ANA LUIZA FONSECA DESTRO

METAIS PESADOS EM PEQUENOS MAMÍFEROS: EFEITOS EM MODELOS MURINOS E EM MORCEGOS FRUGÍVOROS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Animal, para obtenção do título de *Doctor Scientiae*.

Orientador: Mariella Bontempo Duca Freitas

Coorientadores: Reggiani Vilela
Leandro Licursi de Oliveira
Jerusa Maria de Oliveira

VIÇOSA - MINAS GERAIS
2023

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
D477m
2023

Destro, Ana Luiza Fonseca, 1992-
Metais pesados em pequenos mamíferos: efeitos em modelos murinos e em morcegos frugívoros / Ana Luiza Fonseca Destro. – Viçosa, MG, 2023.
1 tese eletrônica (116 f.): il. (algumas color.).

Texto em português e inglês.
Inclui anexos.
Orientador: Mariella Bontempo Duca de Freitas.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Biologia Animal, 2023.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2023.673
Modo de acesso: World Wide Web.

1. Mamíferos - Efeito dos metais pesados. 2. Toxicologia ambiental. 3. Estresse oxidativo. 4. Histologia. I. Freitas, Mariella Bontempo Duca de, 1974-. II. Universidade Federal de Viçosa. Departamento de Biologia Animal. Programa de Pós-Graduação em Biologia Animal. III. Título.

CDD 22. ed. 571.95431

Bibliotecário(a) responsável: Alice Regina Pinto Pires CRB-6/2523

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Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Meteorologia Aplicada, para obtenção do título de *Doctor Scientiae*.

APROVADA: 24 de agosto de 2023

Assentimento:

Documento assinado digitalmente
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ANA LUIZA FONSECA DESTRO
Data: 25/10/2023 18:42:30-0300
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Ana Luiza Fonseca Destro
Autora

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MARIELLA BONTEMPO DUCA DE FREITAS
Data: 27/10/2023 10:48:53-0300
Verifique em https://validar.iti.gov.br

Mariella Bontempo Duca de Freitas
Orientadora

Aos meus pais, irmã e amigos.

AGRADECIMENTOS

Agradeço primeiramente a Deus.

Aos meus pais e irmã, Ione, Luiz e Letícia Destro que confiaram em mim mais do que eu mesmo em todo o processo e por não me deixarem desistir.

Ao meu namorado, Lucas Ribeiro, por permanecer ao meu lado oferecendo suporte e apoio emocional.

À minha orientadora Mariella pelos quase 11 anos de orientação. Aos meus coorientadores, Reggiani Vilela, Jerusa M. Oliveira e Leandro Licursi pelo suporte, auxílio e sugestões durante toda a construção da tese. Principalmente, a Jerusa M. Oliveira, que além de coorientadora, é amiga e professora desde que iniciei os caminhos da pesquisa.

Ao professor e amigo Donald Wlodkowic pelos ensinamentos e conversas sobre políticas, gatos e ciência. Também por ter me proporcionado, com paciência, uma das experiências mais engrandecedoras da minha vida, de estar do outro lado do mundo (Austrália).

A(os) amigas(os) Débora Cardoso, Thaís Silva, Thaís Ribeiro (mini), Kemilli Pio, e Guilherme Garbino, pela amizade, pelas companhias em coletas durante a madrugada, pelas risadas, e pelo apoio emocional durante os surtos.

Aos professores Rômulo Dias e Mariáurea Sarandy pela ajuda com a escrita do primeiro capítulo.

Aos membros do laboratório de Ecofisiologia de Quirópteros pelo auxílio com análises e pelas prosopopeias flácidas.

Aos diversos professores e membros dos diversos laboratórios que me ajudaram durante o processo tirando dúvidas e auxiliando em análises laboratoriais.

Ao departamento de Biologia Animal e todos os pós-graduandos, técnicos, e professores pela presença, auxílio e experiência durante os últimos 7 anos de pós-graduação.

À Universidade Federal de Viçosa, pela oportunidade de realizar a pós-graduação.

Aos membros da banca pela disponibilidade de participar deste momento e contribuírem com valiosas sugestões.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e à Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), pelo apoio e suporte financeiro na aquisição de reagentes e outros gastos relacionados à essa pesquisa.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pela concessão da bolsa de estudos.

“E você aprende que realmente pode suportar... que realmente é forte, e que pode ir muito mais longe depois de pensar que não se pode mais”.

Verônica Shoffstall

RESUMO

DESTRO, Ana Luiza Fonseca, D.Sc., Universidade Federal de Viçosa, agosto de 2023. **Metais Pesados em Pequenos Mamíferos: Efeitos em Modelos Murinos e em Morcegos Frugívoros.** Orientadora: Mariella Bontempo Duca de Freitas. Coorientadores: Reggiani Vilela, Leandro Licursi e Jerusa Maria de Oliveira.

Os metais liberados no ambiente durante a mineração são persistentes no ambiente e contaminam cadeias alimentares. Dentre os animais que são afetados por poluentes, os morcegos estão susceptíveis a contaminação oral ou por contato. Devido ao hábito alimentar variado e a capacidade de voo, esses mamíferos prestam serviços ecológicos e econômicos importantes como dispersão de sementes, polinização, contribuição na fertilidade do solo e indicador ecológico de qualidade ambiental, portanto, a contaminação de populações de morcegos é prejudicial à conservação ambiental. Apesar dos efeitos de diversos metais em modelos murinos ser bem descrito, as formas de prevenção, os efeitos danosos da contaminação em animais selvagens e a avaliação dos danos da liberação de metais pela mineração ainda não estão claros. Portanto, essa tese foi dividida em três capítulos no formato de artigos científicos. No primeiro capítulo foi apresentado uma revisão sistemática dos efeitos de extratos vegetais na toxicidade hepática induzida por chumbo (Pb) em modelos murinos. Esta revisão sistemática teve como objetivo trazer uma atualização e novas perspectivas da ação dos extratos vegetais no metabolismo hepático como prevenção da toxicidade de Pb. No segundo capítulo, foi realizado uma análise experimental e comparativa da toxicidade de baixas doses de quatro metais: Niquel (Ni), Cadmio (Cd), Cromo (Cr) e Chumbo (Pb) em morcegos fugívoros (*Artibeus lituratus*) em cativeiro. O terceiro capítulo foi realizado um estudo *in situ* com o objetivo de avaliar possíveis efeitos da mineração de Ferro (Fe) e Alumínio (Al) em morcegos frugívoros morcegos. uma avaliação dos efeitos fisiológicos da. No trabalho experimental, observou-se que o fígado mostrou maior sensibilidade quando os animais foram tratados com Ni ou Pb. Cr, Cd e Ni foram mais nocivos para os rins. Nos testículos, o Ni dobrou os níveis de dano em comparação aos outros metais. Já os músculos mostraram ser mais sensíveis ao Pb e Cd. O cérebro demonstrou ser mais suscetível a Pb e Ni. Nossos resultados demonstram que doses agudas, mesmo em baixas doses (1,5mg/kg), causaram efeitos prejudiciais em vários órgãos de morcegos frugívoros, o que é uma preocupação para o ecossistema. Assim, baseados em nossos achados, propomos a seguinte ordem de toxicidade dos metais em morcegos frugívoros: Ni> Pb> Cd> Cr. Nós observamos que morcegos frugívoros (*A. lituratus*) coletados em área de mineração de Fe na

Mata Atlântica possuem mais estresse oxidativo no fígado, rins, cérebro e músculo, além de histopatologias no fígado e nos rins em comparação à *A. lituratus* de fragmento de Mata Atlântica sem mineração. Já os morcegos frugívoros (*Sturnira lilium*) coletados em área de mineração de Al, apresentaram alterações em alguns marcadores de balanço redox, indicando estresse oxidativo, quando comparados aos *S. lilium* de fragmento de Mata Atlântica sem mineração. Os morcegos da área de mineração de Al apresentaram histopatologias nos rins e fígado, bem como fibroses no fígado. Os resultados do terceiro capítulo demonstram que morcegos da mineração de Fe na Mata Atlântica estão mais afetados do que os da mineração de Al. Porém ambas as áreas demonstram que os morcegos frugívoros estão sofrendo com os efeitos da mineração. Todos os resultados dos três capítulos da tese, demonstram que metais pesados são um problema para a sobrevivência de pequenos mamíferos e a legislação deve ser atualizada e seguida para diminuir a exposição dos animais selvagens aos metais pesados vindos da ação antrópica.

Palavras-chave: Metais pesado. Ecotoxicologia. Mamíferos. Estresse-oxidativo. Histologia.

ABSTRACT

DESTRO, Ana Luiza Fonseca, D.Sc., Universidade Federal de Viçosa, August, 2023. **Heavy metals in small mammals: effects on murine models and fruit-eating bats**. Advisor: Mariella Bontempo Duca de Freitas. Co-advisors: Reggiani Vilela, Leandro Licursi and Jerusa Maria de Oliveira.

The metals released into the environment during mining activities are persistent and contaminate food chains. Among the animals affected by pollutants, bats are susceptible to oral or contact contamination. Due to their varied dietary habits and flying capabilities, these mammals provide important ecological and economic services, such as seed dispersal, pollination, soil fertility contribution, and serve as ecological indicators of environmental quality. Therefore, the contamination of bat populations is detrimental to environmental conservation. Despite the well-described effects of various metals in murine models, the prevention methods, the harmful effects of contamination in wild animals, and the assessment of damage from metal release through mining are not yet clear. Hence, this thesis has been divided into three chapters in the format of scientific articles. In the first chapter, a systematic review of the effects of plant extracts on lead (Pb)-induced liver toxicity in murine models was presented. This systematic review aimed to provide an update and new perspectives on the action of plant extracts in hepatic metabolism as a means of preventing Pb toxicity. In the second chapter, an experimental and comparative analysis of the toxicity of low doses of four metals—Nickel (Ni), Cadmium (Cd), Chromium (Cr), and Lead (Pb)—was conducted on captive frugivorous bats (*Artibeus lituratus*). The third chapter involved an in situ study to evaluate the possible effects of Iron (Fe) and Aluminum (Al) mining on frugivorous bats and assess their physiological effects. In the experimental work, it was observed that the liver was more sensitive when the animals were treated with Ni or Pb, while Cr, Cd, and Ni were more harmful to the kidneys. Ni doubled the levels of damage in the testicles compared to the other metals, and muscles were more sensitive to Pb and Cd. The brain was found to be more susceptible to Pb and Ni. Our results demonstrate that acute doses, even at low levels (1.5 mg/kg), caused detrimental effects in various organs of frugivorous bats, which is a concern for the ecosystem. Based on our findings, we propose the following order of metal toxicity in frugivorous bats: Ni > Pb > Cd > Cr.

We observed that frugivorous bats (*A. lituratus*) collected in the Iron mining area in the Atlantic Forest showed more oxidative stress in the liver, kidneys, brain, and muscles, as well as histopathologies in the liver and kidneys when compared to *A. lituratus* from a non-mining

Atlantic Forest fragment. Frugivorous bats (*Sturnira lilium*) collected in the Aluminum mining area exhibited changes in some redox balance markers, indicating oxidative stress, compared to S. lilium from a non-mining Atlantic Forest fragment. Bats from the Aluminum mining area also displayed histopathologies in the kidneys and liver, as well as liver fibrosis. The results of the third chapter demonstrate that bats from the Iron mining area in the Atlantic Forest are more affected than those from the Aluminum mining area. However, both areas show that frugivorous bats are suffering from the effects of mining. All the results from the three chapters of the thesis indicate that heavy metals are a problem for the survival of small mammals, and legislation should be updated and followed to reduce the exposure of wild animals to heavy metals from anthropogenic activities.

Keywords: Metals. Ecotoxicology. Bats. Oxidative Stress. Histology.

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1. Introdução

O acelerado crescimento da população humana, o uso extensivo de recursos naturais, industrialização e urbanização durante os últimos anos, principalmente no século XXI, culminou com grandes problemas ambientais como a poluição (ALI; KHAN, 2017). Em função disso, áreas do conhecimento como ecotoxicologia e toxicologia ambiental vêm se tornando muito importante. A ecotoxicologia é uma área científica que investiga os efeitos das substâncias tóxicas no meio ambiente, considerando sua dispersão, deposição, mobilização ou transformação por influência antrópica e não-antrópica. A toxicologia ambiental, por sua vez, está interligada à ecotoxicologia e concentra-se na análise dos efeitos de agentes tóxicos nos organismos e em sua morfofisiologia (TOLLESON, 2018). Dentre os compostos estudados pela toxicologia ambiental estão agrotóxicos, plastificantes e metais potencialmente tóxicos.

O termo "metal pesado" é utilizado como um nome de grupo para metais e metaloides associados com contaminação e potencial toxicidade (ALI; KHAN; ILAHI, 2019). Por isso, nós utilizaremos o termo "metal pesado" para se referir aos metais estudados nessa tese. Alguns autores definem metais pesados (MPs) como elementos metálicos com alta densidade em relação à água (TCHOUNWOU et al., 2012), outros autores consideram como elemento com densidade maior do que 5 $g \cdot cm^{-3}$ (ALI; KHAN, 2018). Devido à alta densidade, os MP's são amplamente encontrados como contaminantes em diversos ambientes, e possuem características preocupantes como não biodegradabilidade, insolubilidade em água e alta persistência (NIGHAT SHAGUFTA; et al., 2018). Dessa forma, uma vez liberados no ambiente - tanto por fontes naturais, como erupções vulcânicas e intemperismo, quanto por fontes antropogênicas, como mineração e atividades industriais– esses elementos contaminam diversos seres vivos, e consequentemente, cadeias alimentares (ALI; KHAN; ILAHI, 2019). Portanto, devido ao seu potencial tóxico, persistência ambiental e capacidade de bioacumulação, os MPs são e devem ser bem estudados por representarem uma ameaça aos organismos vivos (ALI; KHAN; ILAHI, 2019). Em modelos murinos, os efeitos dos MPs nos diversos órgãos têm sido extensivamente documentados e estudados (KARRI; SCHUHMACHER; KUMAR, 2016; MATÉS et al., 2010; SADIGHARA et al., 2023; SANTANA et al., 2023; SILVA et al., 2023).

Embora muitos metais como o Ferro (Fe), Cobalto (Co), Cobre (Cu), Manganês (Mn) Zinco (Zn) e Molibdênio (Mo) sejam cruciais para atividades metabólicas, i.e., são considerados elementos essenciais/micronutrientes, ao exceder os limites fisiológicos, esses

metais podem se tornar tóxicos ao organismo (RAHMAN; SINGH, 2019). Apesar de todos os MP's apresentarem algum risco em altas concentrações, alguns metais são mais preocupantes como o Arsênio (As), Cadmio (Cd), Chumbo (Pb), Cromo (Cr) e Mercúrio (Hg) (RAHMAN; SINGH, 2019). O As e o Pb ocupam a primeira e segunda posição, respectivamente, na Lista de Prioridades de Substâncias de 2022 da Agência de Substâncias Tóxicas e Registro de Doenças (ATSDR), que classifica substâncias com base em sua toxicidade, potencial e frequência de exposição humana (AGENCY FOR TOXIC SUBSTANCES AND DISEASE REGISTRY, 2022).

Em geral, os metais pesados são fonte de preocupação no mundo todo, principalmente devido à mineração (MUSILOVA et al., 2016). No Brasil, especialmente, grandes catástrofes ambientais têm acontecido nos últimos anos, especialmente relacionadas ao setor de mineração, resultando em sérias degradações e contaminações ambientais por MP's (CARMO et al., 2017; VERGILIO et al., 2020). O Brasil ocupa uma posição significativa na mineração, por exemplo, é o terceiro maior produtor de bauxita (minério de alumínio) e o segundo maior produtor de minério de ferro (MARQUES, 2013; TOSTA, 2011). Apesar da expressiva produção mineral, o Brasil enfrenta desafios quanto à sua legislação ambiental, que não acompanhou adequadamente o ritmo de crescimento do setor (FERNANDES et al., 2016). Como resultado, a biodiversidade e a oferta de serviços ecossistêmicos dessas áreas são constantemente ameaçadas. A degradação ambiental resultante das operações de mineração pode ter impactos negativos nos ecossistemas circundantes, levando à perda de habitat e à contaminação do solo e recursos hídricos (BRIDGE, 2004; MUSILOVA et al., 2016).

Com a crescente preocupação em relação aos efeitos tóxicos dos metais pesados na saúde humana, têm surgido esforços para identificar formas de prevenção e tratamento, com destaque para abordagens principalmente naturais (KIM; KIM; KUMAR, 2019; MEHRANDISH; RAHIMIAN; SHAHRIARY, 2019; SEARS, 2013). Além disso, a toxicidade desses metais em animais selvagens tem se tornado uma preocupação significativa tanto para a sociedade em geral quanto para os pesquisadores. Nesse contexto, os morcegos têm sido objeto de estudos em razão de sua importância tanto ambiental quanto a prestação de serviços ecológicos, bem como devido às suas peculiaridades adaptativas, como o amplo hábito alimentar e a capacidade de voo (KASSO; BALAKRISHNAN, 2013). Essas características tornam os morcegos particularmente susceptíveis à exposição aos metais pesados, uma vez que eles podem entrar em contato com essas substâncias por meio da ingestão de alimentos contaminados e por meio do contato com superfícies contaminadas em seus habitats (JONES et al., 2009; KASSO; BALAKRISHNAN, 2013).

Os morcegos, (ordem Chiroptera) possuem grande relevância para o equilíbrio dos ecossistemas. São mamíferos voadores com hábitos alimentares variados e por isso prestam serviços ecológicos e econômicos importantes, como controle de população de insetos, dispersão de sementes e polinização, embora sejam negligenciados (JONES et al., 2009). Esses animais podem ser mais susceptíveis a bioacumulação de metais, principalmente via oral, pois ao contrário de outros pequenos mamíferos e pássaros, esses animais possuem alta taxa de metabolismo e consumo alimentar (RAMOS-H; MEDELLÍN; MORTON-BERMEA, 2020a). O contato com água contaminada é um importante via de exposição, podendo ser de maneira direta ou indireta, quando bebem água ou se alimentam de presas contaminadas (KORINE et al., 2015). A exposição a contaminantes e poluentes ambientais, incluindo metais pesados, vêm sendo apontado como como um dos fatores que contribuem com a queda de populações desses animais (MICKLEBURGH; HUTSON; RACEY, 2002). Alguns estudos já relacionam concentrações de metais pesados no ambiente e em tecidos de morcegos com uma queda brusca em suas populações locais de estudo (HILL et al., 2016; HILL; SCHOEMAN; VOSLOO, 2018; WALKER et al., 2007; ZOCCHE et al., 2010), no entanto ainda não se sabe o que realmente leva a mortalidade dos animais.

Os morcegos frugívoros, que são importantes dispersores de semente, também estão em constante exposição a contaminantes ambientais, tendo a atenção de pesquisas mais voltada a pesticidas (BRINATI et al., 2016; OLIVEIRA et al., 2021, 2017, 2018). Apesar disso, os morcegos com hábito frugívoro também podem se contaminar e bioacumular metais pesados, mas pouco se sabe sobre os efeitos destes contaminantes nos morcegos frugívoros, sendo a maioria dos estudos com morcegos insetívoros (ZUKAL; PIKULA; BANDOUCHOVA, 2015).

Os estudos com dados sobre a contaminação de metais pesados em morcegos ainda são poucos (BENVINDO DE SOUZA et al., 2020). Algumas pesquisas demonstram que esses metais podem bioacumular em diversos tecidos de morcegos (HERNOUT et al., 2016; NIGHAT SHAGUFTA; et al., 2018; RAMOS-H; MEDELLÍN; MORTON-BERMEA, 2020b; WALKER et al., 2007). Porém, nenhum estudo foi feito em morcegos expostos em cativeiro, e, portanto, os atuais estudos disponíveis na literatura não estão livre da influência de outros poluentes sendo difícil afirmar que os danos mensurados foram causados pelos metais analisados. Dentre os metais citados em estudos envolvendo morcegos, o cádmio (Cd), cromo (Cr), chumbo (Pd) e níquel (Ni) são comumente citados pois são encontrados em altas concentrações em tecidos de morcegos em diferentes habitats. (RAMOS-H; MEDELLÍN; MORTON-BERMEA, 2020b; WALKER et al., 2007; ZOCCHE et al., 2010).

À medida que as atividades mineradoras continuam a expandir e os riscos associados à exposição a metais pesados aumentam, é essencial continuar pesquisando e monitorando os efeitos dessas substâncias em animais selvagens, incluindo os morcegos. A proteção dessas espécies e a manutenção da integridade dos ecossistemas requerem ações sustentáveis para minimizar a contaminação ambiental e garantir um ambiente mais saudável tanto para a vida selvagem quanto para as comunidades humanas que compartilham essas regiões. Sendo assim, a presente tese foi dividida em três capítulos onde o primeiro uniu diversos estudos com o intuito de entender se extratos vegetais podem amenizar a intoxicação de metais pesados e como isso ocorre, utilizando o Pb e modelos murinos como base. O segundo capítulo visou avaliar os efeitos de 4 metais pesados em diversos tecidos de morcegos frugívoros a fim de começar a entender, sem influência de outros poluentes, a quão danosa pode ser a contaminação por metais pesados na sobrevivência desses animais utilizando biomarcadores de status redox e histologia de diferentes tecidos. Finalmente, o terceiro capítulo faz uma avaliação completa dos danos de duas diferentes atividades mineradoras (Ferro e Alumínio) em morcegos frugívoros da Mata Atlântica do estado de Minas Gerais utilizando os mesmos biomarcadores.

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2. Capítulo 1:

Impact of plant extracts on hepatic redox metabolism upon lead exposure: A systematic review of preclinical *in vivo* evidence

Ana Luiza Fonseca Destro [a], Patrícia da Silva Mattosinhos [b], Rômulo Dias Novaes [c], Mariáurea Matias Sarandy [b], Reggiani Vilela Gonçalves [b], Mariella Bontempo Freitas [a].

[a] Department of Animal Biology, Federal University of Viçosa, Viçosa, 36570-900, MG, Brazil;

[b] Department of General Biology, Federal University of Viçosa, Viçosa, 36570-900, MG, Brazil;

[c] Institute of Biomedical Sciences, Department of Structural Biology, Federal University of Alfenas, Alfenas, 37130-001, MG, Brazil.

Artigo publicado na revista Environmental Science and Pollution Research

DOI: 10.1007/s11356-023-28620-8

Environmental Science and Pollution Research
https://doi.org/10.1007/s11356-023-28620-8

REVIEW ARTICLE

Impact of plant extracts on hepatic redox metabolism upon lead exposure: a systematic review of preclinical in vivo evidence

Ana Luiza Fonseca Destro[1] · Patrícia da Silva Mattosinhos[2] · Rômulo Dias Novaes[3] · Mariáurea Matias Sarandy[2] · Reggiani Vilela Gonçalves[2] · Mariella Bontempo Freitas[1]

Received: 20 November 2022 / Accepted: 1 July 2023

Abstract

The liver is a central target organ of heavy metals toxicity, and secondary metabolites of several plant species are suggested to attenuate lead (Pb)-induced hepatotoxicity through antioxidant and anti-inflammatory mechanisms. We used a systematic review framework to map the impact of plant extracts and bioactive secondary metabolites on immunological markers and liver redox metabolism in preclinical models of Pb exposure. This is a systematic review performed according to PRISMA guidelines. The structured research of publications was done through PubMed, Scopus, Web of Science, and Embase databases, selecting and analyzing 41 original studies included via the eligibility criteria. Evidence indicates that Pb-exposure increases reactive oxygen/nitrogen species (ROS/RNS) production by δ-aminolevulinic acid auto-oxidation, xanthine dehydrogenase, and xanthine oxidase upregulation. Pb exposure also inhibits antioxidant enzymes, potentiating ROS/NOS levels and reactive cell damage. Plant extracts rich in flavonoids, tannins, alkaloids, anthocyanins, and vitamins exerted hepatoprotective effects by chelating and decreasing Pb bioaccumulation. In addition, plant extracts reinforce exogenous and endogenous antioxidant defenses, attenuating liver oxidative stress and cell death. The lack of blinded evaluators and randomized experimental groups were the main sources of bias identified, which need to be controlled in toxicological studies aimed at identifying natural products applied to the prevention or treatment of Pb poisoning.

Keywords Environmental toxicology · Heavy metals · Hepatotoxicity · Oxidative stress · Natural compounds

Introduction

Lead (Pb) exposure is a growing environmental concern due to its harmful effects on plants, animals, and humans (Kumar et al. 2020). This heavy metal was listed second on the 2022 Substance Priority List of the Agency for Toxic Substances and Disease Registry (ATSDR), which is a prioritization of substances based on a combination of their toxicity, potential for human exposure, and frequency (Agency for Toxic Substances and Disease Registry 2022). Although its use as a fuel additive has been banned in Europe and the US (Dunemann et al. 1999), Pb release from fossil fuel burning still persists in the environment (Larsen et al. 2012). In addition, Pb is still accumulating in the environment from other industrial sources, such as batteries, ammunition, makeup, and paint (Frank et al. 2019). The WHO guideline value for Pb concentration in water established a limit of 10 µg/L in drinking water (World Health Organization 2011). For the European Commission, this limit is also 10 µg/L (Council of the European Union 2020; Jarvis and Fawell 2021). Besides that, soils of recreational areas in the USA represent 11–3604 mg/L of Pb over 20 years (Frank et al. 2019). In China, the soil of rice production showed 26.7 mg/kg of Pb (Mu et al. 2019). For that, Pb pollution is a concern since all ages are highly exposed—for instance, children from Nigeria ingest between 0.09 and 6.82 mg/kg of Pb daily from vegetables and water (Dike et al. 2020).

Responsible Editor: Mohamed M. Abdel-Daim

✉ Ana Luiza Fonseca Destro
nina.destro@gmail.com

[1] Department of Animal Biology, Federal University of Viçosa, Minas Gerais, Viçosa, MG 36570-900, Brazil

[2] Department of General Biology, Federal University of Viçosa, Viçosa, MG 36570-900, Brazil

[3] Department of Structural Biology, Institute of Biomedical Sciences, Federal University of Alfenas, Alfenas, MG 37130-001, Brazil

Published online: 26 July 2023

Pb exposure and absorption often occur through digestive, respiratory, and skin contact (Fattah et al. 2020). Once absorbed, Pb enters the bloodstream and accumulates in erythrocytes (Dewanjee et al. 2015a), is metabolized and conjugated in the liver, and is then taken to the kidney to be excreted in the urine. However, Pb is not completely excreted and accumulates in different tissues (Fattah et al. 2020).

The liver is a direct target organ of Pb-induced toxicity (Zargar et al. 2020), and necropsies indicate the hat liver and kidney are central organs of Pb bioaccumulation (33%) (Fattah et al. 2020). This represents a great concern because the liver has diverse and essential functions such as detoxification, production of digestive enzymes, and protein synthesis (Abbaszadeh et al. 2018). Pb toxicity is correlated with oxidative/nitrosative stress, which is pointed out as the main cause of tissue damage (Patrick 2006). Pb induces reactive oxygen and nitrogen species (ROS/NOS) formation, interrupts antioxidant enzyme activities, inhibits trace minerals absorption, and deactivates antioxidant sulfhydryl pools (Jomova and Valko 2011). Thus, Pb triggers a marked redox imbalance, resulting in severe tissue damage and reactive cell death (Li et al. 2020).

One of the approved ways to treat Pb poisoning is through chelating agents such as meso-2,3-dimercaptosuccinic acid (DMSA) and mono isoamyl DMSA (MiADMSA). These agents form an insoluble complex with Pb and reduce the toxicity of this heavy metal (Flora et al. 2007; Sharma et al. 2010). In some cases, these compounds are polar and have low cell permeability, showing difficulty in crossing the plasma membrane to chelate intracellular Pb (Sharma et al. 2010). In this sense, natural antioxidants can be useful strategies to prevent or treat toxicity induced by heavy metals (Lakshmi et al. 2013). It is recognized that plants used in popular medicine for centuries synthesize several anti-inflammatory and antioxidant secondary metabolites capable of linking to metals such as ascorbic acid, α-tocopherol, phenols, carotenoids, phenolic acids, quinones, flavones, flavonoids, flavanols, tannins, and coumarins (Jomova and Valko 2011; Gurjar et al. 2012). Therefore, plant products can be renewable and accessible natural resources of potentially useful anti-inflammatory and antioxidant bioactive molecules to prevent or treat Pb poisoning. Here, we used a systematic review framework to map the impact of plant extracts and bioactive secondary metabolites on immunological effectors and liver redox metabolism in preclinical models of Pb exposure, in addition to characterizing the plant species and extracts with the best pharmacological effect and their impact on inflammatory, pro-oxidant, antioxidant and cell death effects. We also analyzed the methodological quality of all reviewed studies and the main source of bias associated with current evidence. This review also supports toxicological studies with greater experimental control, providing scientific evidence with greater internal and external validity.

Materials and methods

Guiding questions

Our research protocol was outlined considering the PICO/PECO strategies (Huang et al. 2006). Following the PICO/PECO strategy, we outlined this guiding question: Do plant extracts stimulate antioxidant activity in the liver of Pb-intoxicated animals? What are the mechanisms of action of different plant extracts on the antioxidant activity and stress-responsive pathways in the liver of Pb-intoxicated animals? What are the main hepatic antioxidant metabolic pathways stimulated by plant extracts?

Search strategy

The strategy used in this study to select the reviewed articles was the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) (Liberati et al. 2009). The registration of this systematic review at the International Prospective Register of Systematic Reviews (PROSPERO) (registration number: CRD42022283991) can be accessed at https://www.crd.york.ac.uk/PROSPERO/. A direct search was carried out from three comprehensive electronic databases: PubMed/MEDLINE, Scopus, Web of Science, and Embase. After that, the secondary search was based on the screening of the reference list of all relevant studies identified in the direct search.

For the search, we structured search filters for each database (Table S1). The search filters were initially constructed considering standardized descriptors extracted from PubMed thesaurus MeSH (Medical Subject Headings, http://www.ncbi.nlm.nih.gov/mesh). All descriptors were combined in a complete four-level search strategy based on (i) liver, (ii) lead (iii) antioxidants, and (iv) plant extracts, which were combined by Boolean connectors [AND]. Standardized descriptors were defined by the MeSH algorithm, and non-MeSH descriptors were characterized by the TIAB algorithm which was also used to recover recently published studies. All relevant studies published until September 09, 2021 (updated search date), were recovered and included in the systematic review. All research records recovered in the database search were analyzed, and duplicates were manually removed considering the authors, title, journal, and year of publication. In order to increase coverage of the search strategy, an indirect screening of reference lists from all studies identified in the database search was selected for further studies. The search strategy is detailed in the supplementary materials (Table S1).

Record screening and eligibility

After title and abstract screening, a full-text review of all potentially relevant studies was performed according to specific inclusion and exclusion criteria. We only included the original studies that investigates the relevance of plant extracts on the liver of animals exposed to Pb in preclinical studies in murine models. The exclusion criteria were based on the following: (i) the investigated organ was not the liver, (ii) the intervention was not a plant extract or it was a combination concomitant of more than one extract, (iii) the absence of toxicity inducer of Pb (iv) animal model was not murine, (v) neonatal animals or pregnant and lactating females, (vi) experiments only in vitro, (vii) secondary studies (literature reviews, book chapter, letters to the editor, case studies, comments, and editorials) and (viii) another language besides English, Portuguese and Spanish (ix) studies not retrieved in full. Eligibility was independently analyzed by two researchers (ALFD and MMS), and disagreements were resolved by consensus.

Data extraction

An initial study selection based on the title and abstract (TIAB) was conducted by two independent reviewers (ALFD, MMS). In order to discard subjectivity in the data collection and selection strategy, the information was independently extracted by the three reviewers (ALFD, PSM, MMS) and analyzed separately through the use of a spreadsheet. Inconsistencies were resolved in consultation with two other reviewers (RVG and MBF). The kappa test was done for the selection (kappa = 0.950).

Data were extracted and tabulated in a descriptive way and categorized as follows: (a) publication characteristics: author, year, and country; (b) characteristics of the animal model: strain, sex, age, and weight; (c) treatment characteristics: number of animals per group, control (CTL) group, time of exposure, the timing of exposure (i.e., frequency), Pb compound, administration, a dose of Pb and extract; (d) plant characteristics: species, family, used part, extraction method, dose, and administration route, (e) outcomes: metabolic pathways and effector molecules related to redox metabolism.

Methodological bias

We analyzed the risk of bias using an instrument based on the Cochrane Collaboration RoB Tool: The Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) Risk of Bias (RoB) tool (Hooijmans et al. 2014). The main objective of this methodology was to perform a careful analysis of the studies and avoid discrepancies in the evaluation of their methodological quality in animal experiments. To facilitate our judgment about the studies, we adopted the following questions in order to give us a better understanding and applicability of the studies: sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, intervention, animals information, intervention, ethical approval, referenced methodology, statistical methods, and other bias (extract toxicity test, Pb bioaccumulation analysis). Independent reviewers (ALFD and PSM) examined the risk of bias in the studies; disagreements between reviewers were solved by consensus with three other reviewers (MMS, MBF, and RVG). Two graphs containing summary of risk of bias (SYRCLE) were generated using the Review Manager 5.3 program (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration).

Results

PRISMA guideline

We recovered a total of 2654 records from the databases (PubMed/MEDLINE n= 196, Scopus n=826, Web of Science n=990, and Embase n=642). After that, 680 duplicates were removed, and 1974 records were screened by reading the title and abstract. Then, the 1836 studies were excluded because they do not fit into the focus of this study. Through the eligibility criteria, 130 studies were assessed and 49 were included. Finally, the reference list of the studies was screened and resulted in 1 other record that met the eligibility criteria. Therefore, 53 records were included in this systematic review (Fig. 1).

Publication data

The general characteristics of the publications and experimental animals used in the selected studies and experimental models are shown in Table 1 and Figs. 2 and 3. The studies were published between 2000 and 2023 and were conducted in several countries. Most studies were conducted in India (n=24, 48%), followed by Egypt (n=10, 20%). Interestingly, all countries are from the African or Asian continent.

Experimental animals data

As shown in Table 1, the most used strain was Wistar/Sprague–Dawley (*Rattus norvegicus)* (n=31, 62%). The sex

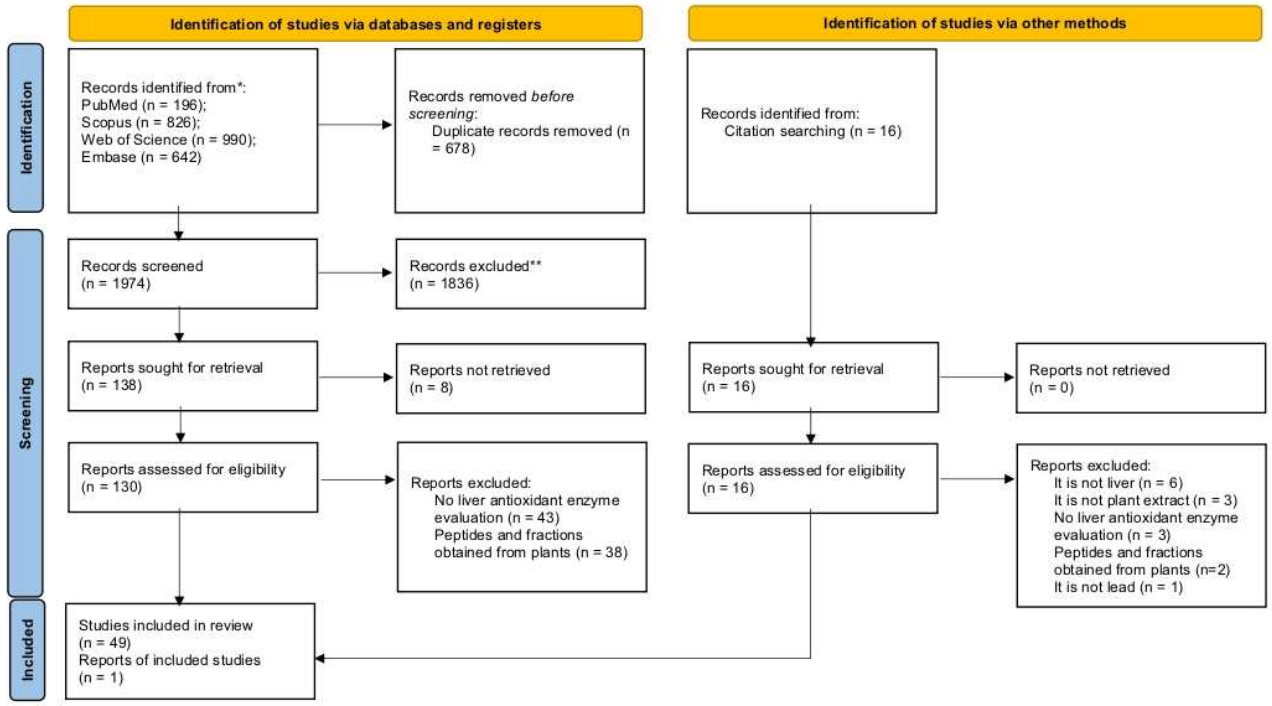


Fig. 1 Flow diagram of the systematic review literature search results based on PRISMA statement (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) (http://www.prisma-statement.org/)

of the animals was 90% male ($n = 45$). In 24% of studies ($n = 12$) adult or young animals were described without a specific age. Body weight data was specified in most studies (94%, $n = 47$) while 6% of studies omitted this information ($n = 3$). And the number of animals most used was 14% 6 animals ($n = 24$). The main experimental animals' data can be seen in Fig. 3

Characteristics of Pb poisoning

As shown in Table 2. Among the Pb compounds used to poison the liver of animals, Pb acetate was the most used (78%, n=39). The route most used to affect intoxication 26% was intraperitoneal injection (n=13). The vehicle of the form of contamination was informed in 62% of the articles (n=31). Also, 40% used distilled water (n=19), and 18% used water (n=9). The main characteristics of Pb poisoning data can be seen in Fig. 4.

About the time of exposure. The time most used by the studies was 6 weeks above (> 38 days) (34%, n=17) followed by the studies that used 2 weeks (8–14 days) (22%, n=11). Exposure frequency was reported in only 60% of articles (n = 30). Twenty-six studies (52%) reported that animals were exposed to Pb daily, while 2 studies (4%) were exposed only once to Pb, and 1 study (2%) reported that exposure occurred in interspersed days or weekly. Regarding the dosage, the studies used mg/kg, ppm, mg/L, and percentages. The most used dosage was 20 mg/kg (n=10, 20%), followed by 5 mg/kg (n=7, 4%).

Characteristics of plant extracts

The characteristics of the extracts used can be seen in Fig. 5 and Table 3. The types of extracts used were aqueous extracts (n=25, 50%), alcoholic extract (n=22, 44%), hydroalcoholic extract (n=2, 4%), and chloroform extract (n=1, 2%). Four percent analyzed aqueous and ethanolic extract in different experimental groups (n=2), and another study analyzed alcoholic and flavonoid-rich extract also in different experimental groups (n=1, 2%).

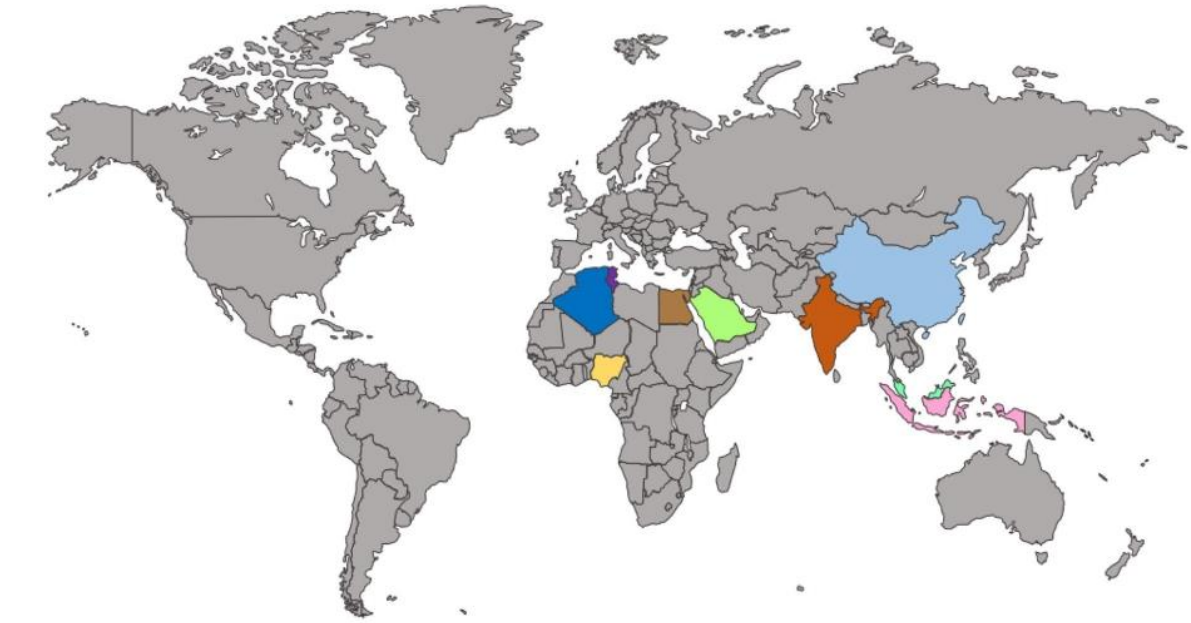
The form of exposure most mentioned form was "orally" (n=25, 50%) without specification. The extract offering vehicle most cited was distilled water (n=19, 38%). Regarding the time of exposure and frequency, the most common time was between 4 and 5 weeks (21–37days) (n=17) and daily (n=21).

Regarding the extract dose, the studies used mg/kg, g/kg, ppm, or ml/g as a unit of measure to inform the concentration of the exposure dose. Some studies used more than one

Table 1 Characteristics of the publications and experimental animals used in all studies that evaluated the effects of plant extracts on the liver of Pb-intoxicated adult murine models

Reference	Country	N° p/group	Sex	Age	Strain	Weight (g)
Adebisi et al. 2022	Nigeria	5	M	10–12 w	Wistar	140 ± 20
Adeyemi et al. 2023	Nigeria	6	F	?	Wistar	120–150
Akilavalli et al. 2011	India	6	F/M	?	Wistar	150–200
Akinlolu et al. 2021	Nigeria	5	F	Adult	Wistar	200
Albasher et al. 2020	Saudi Arabia	8	M	10–11w	Wistar	200 ± 25
Asiwe et al. 2022a	Nigeria	5	M	Adult	Wistar	180–200
Asiwe et al. 2022b	Nigeria	5	M	?	Wistar	150–200
Barkaoui et al. 2020	Tunisia	6	M	10–12w	Wistar	?
Chaurasia et al. 2000	India	7	M	8w	Swiss	30 ± 2
Dewanjee et al. 2013	India	6	?	8–12w	Wistar	200 ± 20
Dewanjee et al. 2015b	India	6	M	?	Swiss	25 ± 5
Diab et al. 2018	Egypt	18	M	Adult	Sprague–Dawley	150–200
Dua et al. 2016	India	6	M	?	Swiss	22 ± 2
El Azab 2021	Saudi Arabia	6	M	Adult	Wistar	130–150
El-Boshy et al. 2019	Egypt	8	M	?	Sprague–Dawley	150±10
El-Hashash et al. 2022	Egypt	8	M	Adult	Sprague–Dawley	150 ± 5
El-Nekeety et al. 2009	Egypt	10	M	12w	Sprague–Dawley	100–120
Fattah et al. 2020	Egypt	6	M	?	Wistar	180–190
Gad et al. 2022	Egypt	8	M	Adult	Sprague–Dawley	150–160
Ghosh et al. 2012	India	6	M	?	Wistar	160–180
Ghosh et al. 2013	India	6	M	?	Wistar	160–180
Haleagrahara et al. 2010	Malaysia	8	M	12w	Sprague–Dawley	80–200
Hamadouche et al. 2014	Algeria	10	M	Adult	Wistar	120–142
Jayan et al. 2017	India	6	F	?	Wistar	150–200
Kansal et al. 2011	India	12	M	8–10w	Swiss	15–30
Koriem 2009	Egypt	6	M	10w	Albino	100±10
Kumar et al. 2014	India	5	M	8–12w	Wistar	100–150
Kumar et al. 2016	India	6	M	8w	Wistar	?
Kumar et al. 2017	India	8	M	?	Wistar	100–120
Laamech et al. 2016	Tunisia	10	M	Adult	Mice	25–30
Lakshmi et al. 2013	India	6	M	Adult	Sprague–Dawley	150 ± 10
Mehana et al. 2012	Saudi Arabia	15	M	?	Sprague–Dawley	170–200
Mohamed et al. 2016	Egypt	6	M	8w	Albino	100–120
Mohammed et al. 2013	India	12/6	M	8–12	Swiss	25-30
Moneim 2016	Egypt	7	M	8w	Wistar	150-180
Mostafa et al. 2018	Egypt	6	M	Adult	*Rattus norvegicus*	130–140
Nishanthi and Anuradha 2012	India	6	M	?	Albino	130–150
Obafemi et al. 2019	Nigeria	5	?	?	Wistar	180 ± 20
Pal et al. 2013	India	6	M	Adult	Swiss	20 and 25
Phatak and Matule 2017	India	6	M	?	Swiss	25–30
Sainath et al. 2011	India	6	M	10–12w	Wistar	?
Sharma and Pandey 2010	India	6	M	?	Swiss	15–30
Sharma et al. 2010	India	12	M	12–16w	Swiss	25–35
Sharma et al. 2009	India	6	M	Adult	Swiss	25–35
Sharma et al. 2011	India	6	M	8–12w	Swiss	25–30
Sharma et al. 2012	India	07/08	M	12–16w	Swiss	25–35
Wang et al. 2020	China	10	M	6w	Kunming	24–29
Xia et al. 2010	China	20/10	M	?	Wistar	80 ± 5
Yuniarti et al. 2021	Indonesia	4	M	10–12w	Swiss	25-30
Zargar et al. 2020	India	7	?	?	Wistar	176.2 ± 13.1

M, male; *F*, female; *w*, weeks



India – 46%

Withania somnifera: **16.7%**
Murraya Koenigii: **16.7%**
Allium sativum: **16.7%**
Corchorus olitorius: **5.6%**
Ipomoea aquatica: **5.6%**
Enhydra fluctuans Lour: **5.6%**
Cissus quadrangular: **5.6%**
Coriandrum sativum: **5.6%**
Abutilon indicum: **5.6%**
Vitis vinifera: **5.6%**
Terminalia arjuna: **5.6%**
Tagetes erecta: **5.6%**
Mangifera indica L.: **5.6%**
Centella asiatica: **5.6%**
Tinospora cordifolia (Willd) Miers: **5.6%**
Asparagus racemosus Wild: **5.6%**
Hippophae rhamnoides: **5.6%**
Ocimum sanctum Linn: **5.6%**

Egito – 20%

Ficus carica L.: **10%**
Thymus vulgaris: **10%**
Aquilegia vulgaris L.: **10%**
Moringa oleifera Lam.: **10%**
Cupressus sempervirens: **10%**
Zingiber officinale L.: **10%**
Indigofera oblongifolia: **10%**
Casimiroa sapota.: **10%**
Hibiscus sabdariffa L.:**10%**
Physalis peruviana: **10%**

Nigeria – 12%

Synsepalum dulcificum: **16.7%**
Morinda lucida: **33.3%**
Annona muricata: **16.7%**
Ginkgo biloba: **16.7%**
Brassica oleracea Linnea*:* **16.7%**
Punica granatum L.*:* **16.7%**

Saudi Arabia – 6%

Malus micromalus Makino: **33;3%**
Smilax glabra Roxb: **33.3%**
Actinidia deliciosa: **33.3%**

China – 4%

Malus micromalus Makino: **50%**
Smilax glabra Roxb: **50%**

Tunisia – 4%

Plantago albicans: **50%**
Berberis vulgaris L.: **50%**

Malaysa – 2%

Etlingera elatior: **100%**

Algeria – 2%

Camellia sinensis: **100%**

Indonesia – 2%

Ocimum sanctum: **100%**

Fig. 2 Geographic distribution where the studies reviewed were conducted (in different colors), and below, the plant extracts used at each location to prevent or treat lead-induced intoxication

dose. The most used dose was 100 mg/kg (n=17, 34%), the dosages from 1 to 100 mg/kg were evaluated in 76% (n=37), from 101 to 200 in 12% (n=6), from 201 to 300 mg/kg in 42% (n=21) and above 301 in 32% (n=16).

Among the families of plants used, there was a wide variation. The Rutaceae and Solanaceae family was the most used, in 8% of articles (n=4, each) followed by Amaryllidaceae, Malvaceae, Lamiaceae, and Rubiaceae (n=3, 6%).

Main outcomes

In all studies analyzed, plant extracts had positive results under oxidative stress. Pb decreases the antioxidant activity of the main enzymes correlated with oxidative stress and produces more reactive species contributing to cell damage. This reduction in antioxidant activity in at least one enzyme was mainly observed in most of the studies with Pb-exposure compared to the CTL group (n=46, 92%), and

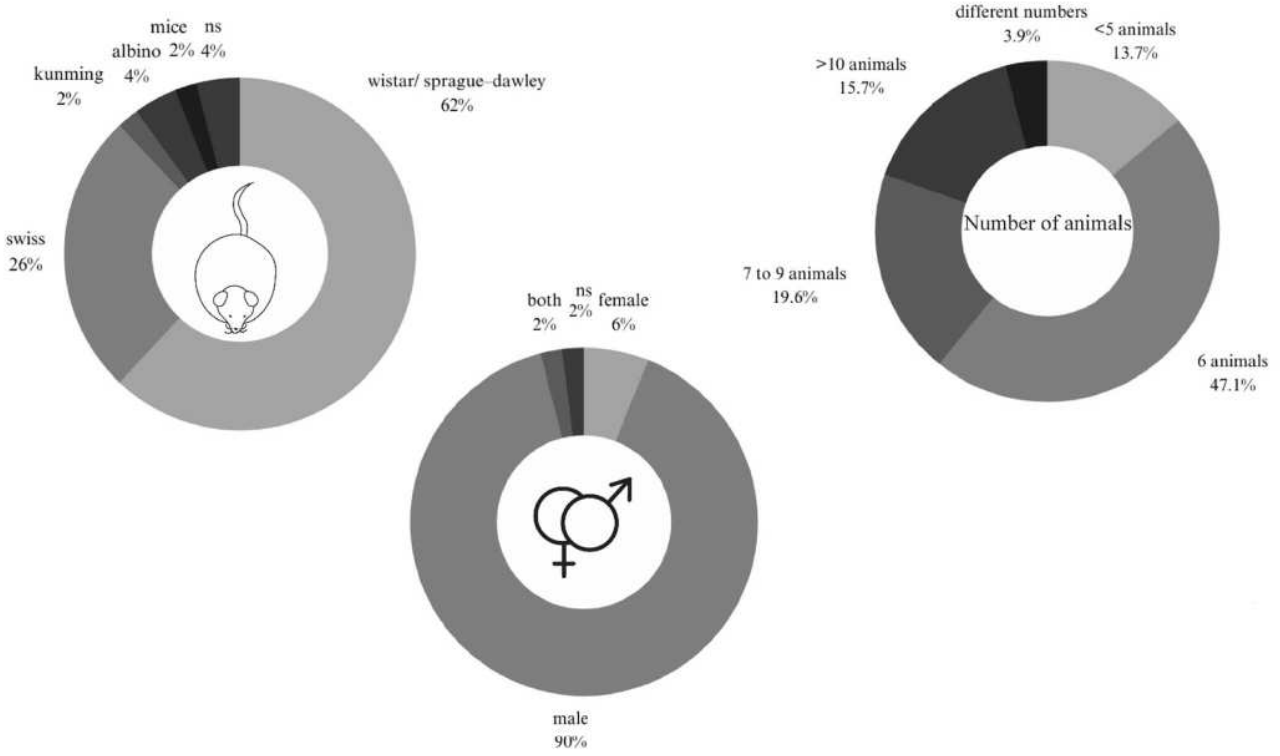


Fig. 3 The main characteristics of the experimental animals in the percentage of studies specified in their publications: the strains, sex, and the number of the murine models used in the analyzed studies. ns, not specified; different numbers, different numbers between groups

all enzymes analyzed (SOD, CAT, and GSH or GST) had their activity decreased at the highest doses (4 to 500 mg/kg) of Pb-exposure (n=43, 82%). In contrast, 6 studies (12%) that used 1 to 15 mg/kg observed an increase in antioxidant activity along with an increase in cell damage biomarkers. In most of the studies (n=43, 86%), plant extracts were able to help increase the antioxidant capacity of enzymes (CAT, SOD, GST, GSH, GPX, or GR) compared to the antioxidant capacity of these enzymes in Pb-exposed animals. The lowest dose of extract evaluated between the studies was 1 mg/kg of *Withania somnifera* and showed no effects in antioxidant biomarkers but decreased the MDA content (Kumar et al. 2014). And the lowest frequency of offer of extracts was 4 times a week, 350mg/kg of *Zingiber officinale* L., and there was no effect in MDA, nor in the antioxidant activity of GSH and GST; only CAT showed an increase (Mohamed et al. 2016). Even in studies where the enzymes did not suffer a decrease in activity; the plant extracts helped bring the values closer to normal (CTL) (n=3, 7.3%). Furthermore, levels of oxidative damage have decreased with plant extracts in almost all studies that Pb had been shown to induce oxidative damage (n=34, 82.9%). However, this change was mostly dose-dependent. Among the doses analyzed, the higher doses of extract had a greater effect on toxic effects.

Pb-exposure increased the expression of Tumor necrosis factor alpha (TNF-α) (n=4, 8%), nuclear factor kappa β (NF-kβ) (n=4, 8%), Caspase 3 (Casp 3) (n=4, 8%), protein X associated with BCL-2 (Bax) (n=4, 8%), interleukin 1 beta (IL-1β) (n=2, 4%), cytochrome c oxidase activity (CYT-C) (n=2, 4%), interleukin (IL-6) (n=1, 2%), xanthine oxidase (XO) (n=1, 2%), xanthine dehydrogenase (XDH) (n=1, 2%), tumor protein (p53) (n=2, 4%), nitric oxide synthases (iNOS) (n=1, 2%), caspase 9 (Casp 9) (n=1, 2%), c-Jun N-terminal kinase (JNK) (n=1, 2%), inhibitor of nuclear factor kappa-B kinase subunit alpha (IKK-α) (n=1, 2%), mitogen-activated protein kinases (p38) (n=1, 2%), heme oxygenase (HO-1) (n=1, 2%), matrix metallopeptidase 9 (MMP-9) (n=1, 2%), NF-kappa-B inhibitor *alpha* (IKB-α) (n=1, 2%), apoptosis (n=1, 2%), calcium (Ca) (n=1, 2%), calreticulim (CRT) (n=1, 2%), protein kinase C (PKC-α) (n=1, 2%) and decreased the expression of BCL-2 (n=4, 8%), hepatic pyruvate dehydrogenase activity (PDH) (n=1, 2%), isocitrate dehydrogenase (ICDH) (n=1, 2%), alpha keto glutarate dehydrogenase (α-KGDH) (n=1, 2%), succinate dehydrogenase (SDH) (n=1, 2%), CYT C (n=1, 2%), NADH cytochrome c oxido-reductase activity (NADH CYT C) (n=1, 2%), interleukin (IL-4) (n=1, 2%), interleukin (IL-10) (n=1, 2%), apoptotic peptidase activating factor 1 (Apaf-1) (n=1, 2%). The extracts were also able to improve all immunological marker expression (Table 4) Fig. 6.

Table 2 Characteristics of the experimental pb-intoxication in the liver of adult murine models

Reference	Pb compound	Exposure form	Vehicle	Time of exposure	Dose (mg/kg)/frequency
Adebisi et al. 2022	Pb acetate	Orally	?	2 w	30/?
Adeyeme et al. 2023	Pb nitrate	Gavage	Distilled water	2 w	30/daily
Akilavalli et al. 2011	Pb acetate	Orally	?	1/2w	14/?
Akinlolu et al. 2021	Pb acetate	Orally	?	2 w before extract, and 5 w with extract	100/?
Albasher et al. 2020	Pb acetate	ip	Distilled water	2w	20/?
Asiwe et al. 2022a	Pb acetate	Orally	Distilled water	4 w	25/?
Asiwe et al. 2022b	Pb acetate	Orally	Distilled water	2 w	25/daily
Barkaoui et al. 2020	Pb acetate	Drinking water	Distilled water + 0.1mL of acetic acid	4 w	100/daily
Chaurasia et al. 2000	Pb acetate	ip	?	2.8 w	4/?
Dewanjee et al. 2013	Pb acetate	Drinking water	Water	5.7 w	5/daily
Dewanjee et al. 2015a	Pb acetate	?	?	5.7 w	5/?
Diab et al. 2018	Pb acetate	Gavage	?	6 w	20/daily
Dua et al. 2016	Pb acetate	Drinking water	Distilled water	5.7 w	5/daily
El Azab 2021	Pb acetate	ip	Distilled water	3w	25/weekly
El-Boshy et al. 2019	Pb acetate	Drinking water	Water	6 w	500/?
El-Hashash et al. 2022	Pb acetate	Gavage	Distilled water	6w	20/daily
El-Nekeety et al. 2009	Pb acetate	Orally	?	4 w	20*/?
Fattah et al. 2020	Pb acetate	Gavage	Distilled water	4 w	100/daily
Gad et al. 2022	Pb acetate	Gavage	Distilled water	4w	10*/?
Ghosh et al. 2012	Pb acetate	ip	Distilled water	1 w	15/daily
Ghosh et al. 2013	Pb acetate	ip	?	1 w	15/?
Haleagrahara et al. 2010	Pb acetate	Drinking water	Water	2 w	500*/daily
Hamadouche et al. 2014	Pb acetate	?	Water	4 w	40,000*/?
Jayan et al. 2017	Pb acetate	Orally	?	1-2 w	25/daily
Kansal et al. 2011	Pb nitrate	Gavage	Distilled water	4.7 w	40/daily
Koriem 2009	Pb acetate	Diet	Deionized water	8 w	500/daily
Kumar et al. 2014	Pb nitrate	ip	?	2 w	40/?
Kumar et al. 2016	Pb acetate	Gavage	?	2 w	1500*/daily
Kumar et al., 2017	Pb acetate	?	Water	2 w	1000*/daily
Laamech et al. 2016	Pb acetate	Orally	Distilled water	5.7 w	5/daily
Lakshmi et al. 2013	Pb nitrate	ip	Distilled water	4 w	5/daily
Mehana et al., 2012	Pb acetate	Drinking water	Distilled water	8 w	4000*/?
Mohamed et al. 2016	Pb acetate	Drinking water	Water	6 w + 2 w without	1*/daily
Mohammed et al. 2013	Pb acetate	ip	Saline solution	2 w	150/once in treatment
Moneim 2016	Pb acetate	ip	Distilled water	24h	20/once in treatment
Mostafa et al. 2018	Pb acetate	ip	Distilled water	1w at end	100/daily
Nishanthi and Anuradha 2012	Pb acetate	Orally	?	3 w	160/?
Obafemi et al. 2019	Pb acetate	?	?	2 w	50/?
Pal et al. 2013	Pb nitrate	Orally	Water	0.9 w	5/daily
Phatak and Matule 2017	Pb acetate	ip	?	1.14 w	15/?
Sainath et al. 2011	Pb acetate	Drinking water	Water	10 w	819*/daily
Sharma and Pandey 2010	Pb nitrate	?	?	4 w	5/daily
Sharma et al. 2010	Pb nitrate	Gavage	Distilled water	5.7 w	50/daily
Sharma et al. 2009	Pb nitrate	ip	?	6.4 w	2/?
Sharma et al. 2011	Pb nitrate	Gavage	Distilled water	6 w	20/daily
Sharma et al. 2011	Pb nitrate	Gavage	Distilled water	6.4 w	20/daily
Wang et al. 2020	Pb chloride	Gavage	?	1.4 w	20/daily

Table 2 (continued)

Reference	Pb compound	Exposure form	Vehicle	Time of exposure	Dose (mg/kg)/frequency
Xia et al. 2010	Pb acetate	ip	?	2.9 w	20/every other day
Yuniart et al. 2021	Pb acetate	?	?	2 w	20/?
Zargar et al. 2020	Pb acetate	Drinking water	Water	6.4 w	250*/daily

Pb, lead; *ip*, intraperitoneal; *w*, weeks, *values adjusted to mg/kg for better understanding

Secondary results

Plant extracts have shown promising effects in decreasing the Pb content accumulated in the tissue. Pb concentrations in exposed murine tissues increased compared to CTL in all studies that analyzed, and among the organs analyzed, the liver was the second organ that accumulated the most Pb, only behind the kidney. However, bioaccumulation values decreased with the use of extracts.

Liver health plasma indices also improved in most studies (n=37, 74%) after offering the extract; these indices include AST (aspartate aminotransferase), ALT (alanine aminotransferase), ALP (alkaline phosphatase), ACP (acid phosphatase), albumin, globulin, bilirubin, calcium, cholesterol, total protein, SGOT (serum glutamic-oxoloacetic transaminase), and SGPT (serum glutamic-pyruvic transaminase). Finally, a high number of studies (n=32, 72%) analyzed pathologies in the liver of animals Pb-exposed. The histological damage caused by Pb in the liver includes mild, moderate, and severe damage. The most cited damage was inflammatory infiltrate and necrosis (n=19, 38%). Vascular congestion (n=15, 30%), cellular degeneration (mainly fat) (n=14, 28%), and nuclear pyknosis (n=8, 16%) were also cited in large quantities. Plant extracts were able to reduce

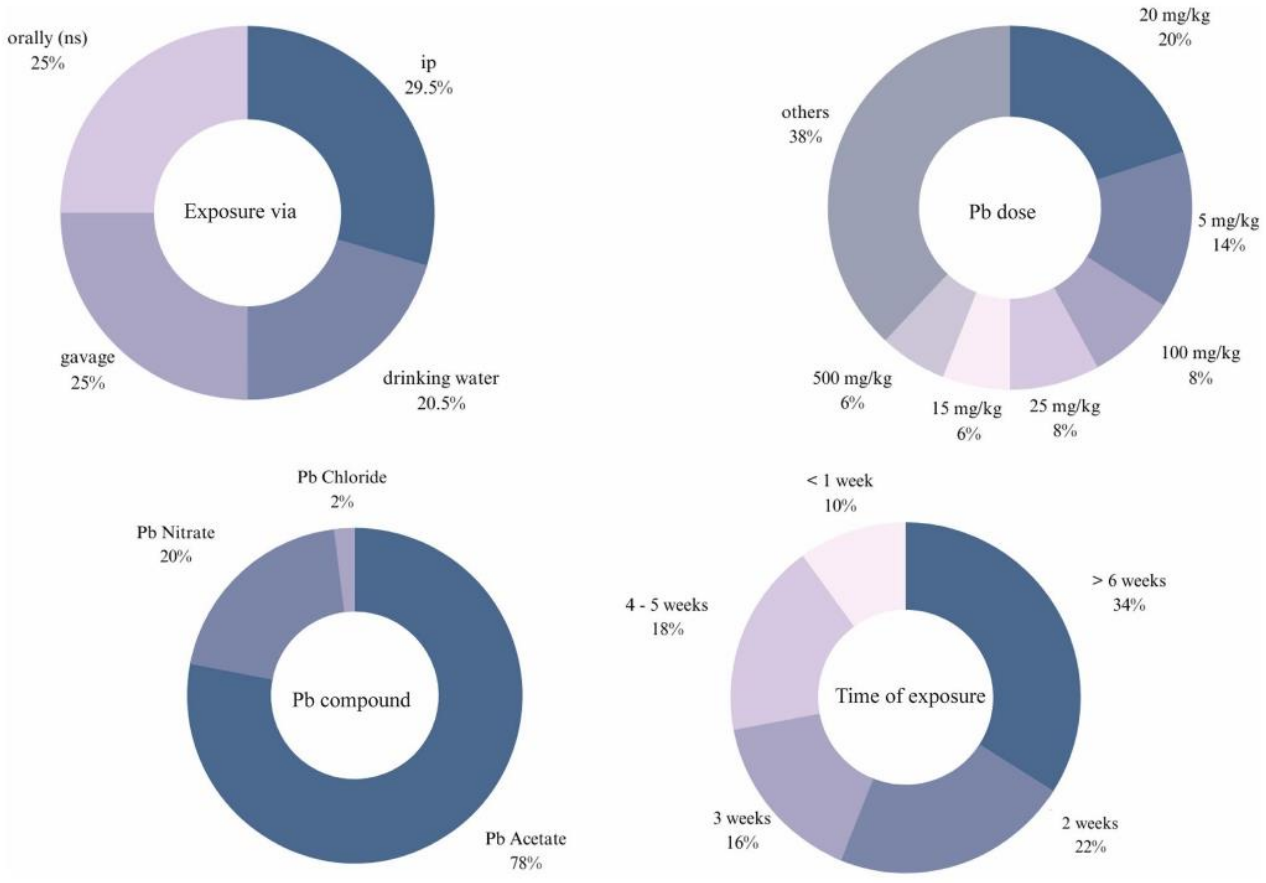


Fig. 4 The main characteristics of Pb poisoning in the percentage of studies specified in their publications: Exposure via, Pb compound, time of exposure, and Pb dose. ns: not specified. > 6 weeks: > 38 days, 2 weeks: 8–14 days, 3 weeks: 15–21 days, 4–5 weeks: 21–37 days, <1 week: <7 days.

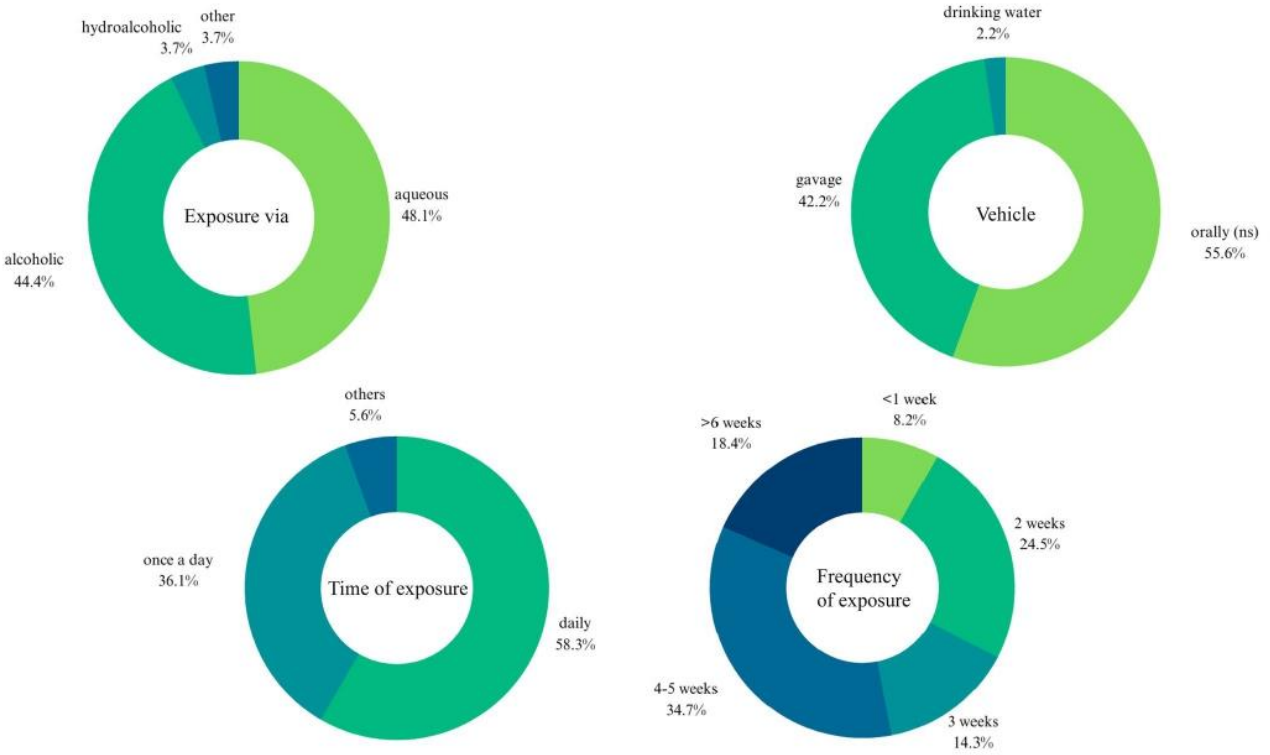


Fig. 5 The main characteristics of the plant extracts in the percentage of studies specified in their publications: Exposure via, vehicle, frequency, and time of exposure. ns: not specified. > 6 weeks: > 38 days, 2 weeks: 8–14 days, 3 weeks: 1521 days, 4–5 weeks: 21–37 days, <1 week: <7 days

Wistar rats followed by Swiss mice were the main animal models investigated, probably due to the easy access and acceptance of the strains in toxicological studies. Although mostly male rats were chosen, other studies suggest that females are more likely to be susceptible to disturbances caused by heavy metals (Menon et al. 2016); therefore, more studies with females are encouraged. Intraperitoneal (ip) was the main route of Pb exposure. This technique is widely used because it is fast and minimally stressful for animals compared to gavage, for example, but its use is often questioned because it avoids the gastrointestinal tract, interfering with the absorption of the administered compound (Al Shoyaib et al. 2020). Notwithstanding this fact, the intraperitoneal route is faster and more efficient compared to the oral and intramuscular route (Al Shoyaib et al. 2020), so it is interesting to use it when the focus of the study is to ensure exposure. The ip route is also more convenient and safer for researchers since they know that the toxicant has been administered and the exact dose. However, when the focus is to ensure the exposure route, it is interesting to keep the most common route (the oral route for plant extracts). In our review, the form of exposure for plant extracts was always oral, which varied from gavage to drinking water. The oral ingestion of extracts is usually used as an attempt to bring studies closer to human consumption of aqueous extracts (Rajgopal 2001). Regarding exposure frequency and duration, there were no notable differences in results. However, some results suggest that the same time of exposure of the extract administered after intoxication, or during intoxication, induced a more efficient effect than administered before (El-Nekeety et al. 2009).

As for the dosages of Pb and extracts used, the results from the studies taken together present shreds of evidence that Pb doses above 20mg/kg decreased the antioxidant activity and doses below that can increase antioxidant activity. The dose of 20mg/kg is almost 22.5 times less than LD50 oral (450mg/kg) (Gad et al. 2022). These results are possibly associated with the enzymatic exhaustion that normally occurs after exposure to higher doses of heavy metals, and on the other hand, the increase in the antioxidant enzymes might be associated with the cell survival gene expression that is usually activated after moderate stress. The highest doses of plant extracts, above 100mg/kg, showed the most satisfactory results regarding the oxidative balance, returning the levels of the antioxidant enzyme activities to the values of the control group and decreasing considerably the important oxidative damage markers, such as malondialdehyde.

Table 3 Characteristics of plant extract exposure in lead (Pb)-intoxicated animals

Reference	Plant species	Extract type	Exposure form of extract	Vehicle	Time/frequency of exposure	Dose (mg/kg)	Family	Plant's part	Main secondary metabolite
Adebisi et al. 2022	*Punica granatum* L.	Alcoholic	Orally	?	2w	100 and 200	Lythraceae	?	
Adeyeme et al. 2023	*Morinda lucida*	Alcoholic	Gavage	Distilled water	2 w after Pb/ daily	100, 250 and 500	Rubiaceae	Stem bark	Alkaloids, flavonoids, tannins, cardiac glycosides, saponins, terpenoids, and anthraquinones
Akilavalli et al. 2011	*Ocimum sanctum* Linn.	Aqueous	Orally	?	3w	100, 200 and 300	Lamiaceae	Whole plant	
Akinlolu et al. 2021	*Morinda lucida and Annona muricata*	Alcoholic	Orally	?	3 w after Pb and 5 w with Pb/?	*M. lucida*: 7.5, 15 *A. muricata*: 7.5, 10	Rubiaceae/ Annonaceae	Leaves	?
Albasher et al. 2020	*Moringa oleifera*	Alcoholic	Orally	Distilled water	2 w/?	250	Moringaceae	Leaves	?
Asiwe et al. 2022a	*Brassica oleracea* Linne	Aqueous	Orally	Distilled water	4w/?	1ml/100g	Brassicaceae	Leaves	Carbohydrates (5.8g), sugars (3.2g), energy (25kcal), dietary fiber (2.5g), protein (1.28g), vitamin C (44%), zinc (2%), potassium (4%), phosphorous (4%), magnesium 12mg (3%), calcium 40mg (4%), vitamin B6 0.124mg (10%), folate (Vit. B5), 53µg (13%)
Asiwe et al. 2022b	*Ginkgo biloba*	?	Orally	Distilled water	2w after Pb/?	50 and 100	Ginkgoaceae	Leaves	24–28% flavonoids, 5–12% terpene lactones (2.8–6.0% ginkgolides A, B, and C, with at least 1.3% ginkgolides B and 2.6–5.6% bilobalide), and less than 5 ppm ginkgolic acids in a standardized form

Table 3 (continued)

Reference	Plant species	Extract type	Exposure form of extract	Vehicle	Time/frequency of exposure	Dose (mg/kg)	Family	Plant's part	Main secondary metabolite
Barkaoui et al. 2020	*Plantago albicans*	Aqueous	Gavage and orally	Distilled water	4 w/daily	100(drinking water), 300 (gavage)	Plantaginaceae	Leaves	Phenolic (gallic acid equivalent), flavonoid (catechin), polyphenols, hydroxycinnamic acids (p-coumaric acid and chlorogenic acids), flavones (apigenin, luteolin, cirsiliol, and luteolin-7-O-rutinoside), and an anthocyanin (cyanidin 3-glucoside).
Chaurasia et al. 2000	*Withania somnifera*	Alcoholic	Gavage	Water+ tween 80 (9:1)	2.8 w/daily	700, 1400*	Solanaceae	Roots	?
Dewanjee et al. 2013	*Corchorus olitorius*	Aqueous	Orally	Distilled water	3 w/daily	25, 50, 100	Malvaceae	Leaves	Flavonoids (rutin, quercetin) and phenolic compounds (gallic acid, chlorogenic acid, p-coumaric acid, ferulic acid, and ellagic acid)
Dewanjee et al. 2015b	*Ipomoea aquatica*	Aqueous	?	Distilled water + tween 80 (1%)	3 w/daily	100	Convolvulaceae	Aerial part	Flavonoids, phenolics, saponins, carbohydrates, ascorbic acid, myricetin, quercetin, and apigenin
Diab et al. 2018	*Ficus carica* L.	Alcoholic	Gavage	?	6 w/daily	200	Moraceae	Leaves	?
Dua et al. 2016	*Enhydra fluctuans* Lour.	Aqueous	Orally	Distilled water +tween 80 (1%)	4 w/daily	100	Asteraceae	Aerial part	Flavonoids, saponins, phenolics, ascorbic acid, carbohydrates, quercetin, myricetin, gallic acid, and chlorogenic acid
El Azab 2021	*Actinidia deliciosa*	Alcoholic	Orally	Distilled water	4w/daily	250 and 500	Actinidiaceae	Fruit	
El-Boshy et al. 2019	*Thymus vulgaris*	Alcoholic	Gavage	?	6 w/daily	500	Lamiaceae	Leaves	Phenol, flavonoids, alkaloids, caffeic acid, and quercetin
El-Hashash et al. 2022	*Hibiscus sabdariffa* L.	Aqueous	Gavage	Distilled water	6w/daily	0.5ml/100g	Malvaceae	Calyx	

Table 3 (continued)

Reference	Plant species	Extract type	Exposure form of extract	Vehicle	Time/frequency of exposure	Dose (mg/kg)	Family	Plant's part	Main secondary metabolite
El-Nekeety et al. 2009	*Aquilegia vulgaris* (L.)	Alcoholic	Orally	?	4 w/daily	100*	Ranunculaceae	Leaves and stems	?
Fattah et al. 2020	*Moringa oleifera* Lam	Aqueous	Gavage	Distilled water	4 w/daily	200	Moringaceae	Leaves	Iron, potassium, phosphorus, calcium, vitamins D and A and essential amino acids, flavonoids, vitamin C, β-carotene, amino acids, alkaloids, sterols, proteins, reducing sugars, flavonoids, tannins, saponins, carbohydrates, gallic acid, protocatechuic acid, p-hydroxybenzoic acid, catechin, gentisic acid, chlorogenic acid, singeo acid, caffeic acid, vanillic acid, ferulic acid, sinapic acid, p-coumaric acid, rutin, rosmarinic acid, apigenin-7-glucoside, cinnamic acid, quercetin, kaempferol, apigenin, chrysina, and andrhamnetina
Gad et al.	*Physalis peruviana*	Aqueous/alcoholic	Gavage	Distilled water	4w after Pb/daily	200, 300 and 500	Solanaceae	Fruit	?
Ghosh et al. 2012	*Murraya Koenigii*	Aqueous	Orally	Distilled water	1 w/daily	12.5, 25, 50, 100	Rutaceae	Leaves	?
Ghosh et al. 2013	*Murraya koenigii* (L.) Spreng	Aqueous	Orally	Distilled water	1 w/daily	50	Rutaceae	Leaves	Alkaloids, tannins, flavonoids, glycosides, terpenoids, and steroids
Haleagrahara et al. 2010	*Etlingera elatior*	Alcoholic	Gavage	Distilled water	3 w/daily	300	Zingiberaceae	Inflorescence	Higher quantities of phenolic compounds, flavonoids, and flavones

Table 3 (continued)

Reference	Plant species	Extract type	Exposure form of extract	Vehicle	Time/frequency of exposure	Dose (mg/kg)	Family	Plant's part	Main secondary metabolite
Hamadouche et al. 2014	*Camellia sinensis*	Aqueous	Orally	Water	4 w/?	66000*	Theaceae	?	?
Jayan et al. 2017	*Cissus quadrangular*	Alcoholic	Orally	Distilled water	2 w/daily	50, 100, 200	Vitaceae	Stalk	?
Kansal et al. 2011	*Coriandrum sativum*	Aqueous/alcoholic	Gavage	Distilled water	3.6 w/daily	250, 300, 500, 600	Umbelliferae	Seeds	?
Koriem 2009	*Cupressus sempervirens*	Alcoholic	Gavage	?	2 w before Pb/daily	80	Cupressaceae		Quercetin, flavonee, rutin, catequina, flavonols, glycoside, tannin, coumarin, unsaturated sterol, leucoanthocyanidins
Kumar et al. 2014	*Withania somnifera*	Aqueous	Orally	Water	2 w/?	1	Solanaceae	Leaves	?
Kumar et al. 2016	*Abutilon indicum*	Aqueous	Gavage	?	2 w/daily	300, 500	Malvaceae	Roots	?
Kumar et al. 2017	*Allium sativum*	Aqueous	Gavage	Distilled water	2 w/daily	250, 500	Amaryllidaceae	Cloves garlic	The main active ingredient was allicin (1.5 %). Other bioactive compounds such as S-allyl cysteine, S-allyl mercaptocysteine, flavonoids, and polyphenols were also present but in less amounts.
Laamech et al. 2016	*Berberis vulgaris* L.	Aqueous	Orally	Distilled water	4 w/daily	25, 50, 100, 150	Euphorbiaceae	Stem bark	?
Lakshmi et al. 2013	*Vitis vinifera*	Hydroalcoholic	Gavage	Saline solution	4 w/daily	400	Vitaceae	Skin, seed, and pulp	?
Mehana et al. 2012	*Camellia sinensis*	Aqueous	Drinking water	Distilled water	8 w/daily	15000*	Theaceae	Leaves	?

Table 3 (continued)

Reference	Plant species	Extract type	Exposure form of extract	Vehicle	Time/frequency of exposure	Dose (mg/kg)	Family	Plant's part	Main secondary metabolite
Phatak and Matule 2017	*Murraya koenigii*	Chloroform	Orally	?	1.1 w/?	50	Rutaceae	Leaves	?
Sainath et al. 2011	*Centella asiatica*	Aqueous	Gavage	Water	10 w/?	200	Umbelliferae	Whole plant	?
Sharma and Pandey 2010	*Tinospora cordifolia* (Willd) Miers	Aqueous	?	?	4 w/daily	400	Menispermacea	Stem and leaves	?
Sharma et al. 2010	*Allium sativum*	Aqueous	Gavage	Distilled water	4 w/daily	100, 250, 500	Amaryllidaceae	Bark	?
Sharma et al. 2009	*Allium sativum*	Aqueous	Orally	Distilled water	3.5w/?	250 and 500	Amaryllidaceae	Cloves garlic	
Sharma et al. 2011	*Withania somnifera*	Hydrometha-nolic	Gavage	Distilled water	6 w/daily	200, 500	Solanaceae	Roots	?
Sharma et al. 2012	*Asparagus racemosus* Willd	Aqueous	Gavage	Distilled water	6.4 w/daily	50, 150	Asparagaceae	?	?
Wang et al. 2020	*Malus micromalus* Makino	Phenolic (alcoholic)	Orally	Deionized water	1.4 w/daily	100	Rosaceae	Pulp	Epicatechin was the most abundant phenolic acid. Quinic acid, chlorogenic acid, vanillic acid, neochlorogenic acid, ellagic acid, 3,5-dihydroxybenzoic acid, and 3,4-dihydroxybenzoic acid
Xia et al. 2010	*Smilax glabra* Roxb.	Alcoholic	Gavage	?	1.4 w/daily	300	Liliaceae	Rhizome	Phenolic compounds and flavonoids
Yuniart et al. 2021	*Ocimum sanctum*	Alcoholic	Orally	Tween-80 (1%)	3.4 w/?	140, 280, 560	Lamiaceae	Leaves	?
Zargar et al. 2020	*Hippophae rhamnoides*	Aqueous	Orally	None	8.5 w/daily	100	Elaeagnaceae	Leaves	?

w, weeks, *values adjusted to mg/kg for better understanding

Table 3 (continued)

Reference	Plant species	Extract type	Exposure form of extract	Vehicle	Time/frequency of exposure	Dose (mg/kg)	Family	Plant's part	Main secondary metabolite
Phatak and Matule 2017	*Murraya koenigii*	Chloroform	Orally	?	1.1 w/?	50	Rutaceae	Leaves	?
Sainath et al. 2011	*Centella asiatica*	Aqueous	Gavage	Water	10 w/?	200	Umbelliferae	Whole plant	?
Sharma and Pandey 2010	*Tinospora cordifolia* (Willd) Miers	Aqueous	?	?	4 w/daily	400	Menispermacea	Stem and leaves	?
Sharma et al. 2010	*Allium sativum*	Aqueous	Gavage	Distilled water	4 w/daily	100, 250, 500	Amaryllidaceae	Bark	?
Sharma et al. 2009	*Allium sativum*	Aqueous	Orally	Distilled water	3.5w/?	250 and 500	Amaryllidaceae	Cloves garlic	
Sharma et al. 2011	*Withania somnifera*	Hydrometha-nolic	Gavage	Distilled water	6 w/daily	200, 500	Solanaceae	Roots	?
Sharma et al. 2012	*Asparagus racemosus* Willd	Aqueous	Gavage	Distilled water	6.4 w/daily	50, 150	Asparagaceae	?	?
Wang et al. 2020	*Malus micromalus* Makino	Phenolic (alcoholic)	Orally	Deionized water	1.4 w/daily	100	Rosaceae	Pulp	Epicatechin was the most abundant phenolic acid. Quinic acid, chlorogenic acid, vanillic acid, neochlorogenic acid, ellagic acid, 3,5-dihydroxybenzoic acid, and 3,4-dihydroxybenzoic acid
Xia et al. 2010	*Smilax glabra* Roxb.	Alcoholic	Gavage	?	1.4 w/daily	300	Liliaceae	Rhizome	Phenolic compounds and flavonoids
Yuniart et al. 2021	*Ocimum sanctum*	Alcoholic	Orally	Tween-80 (1%)	3.4 w/?	140, 280, 560	Lamiaceae	Leaves	?
Zargar et al. 2020	*Hippophae rhamnoides*	Aqueous	Orally	None	8.5 w/daily	100	Elaeagnaceae	Leaves	?

w, weeks, *values adjusted to mg/kg for better understanding

Table 4 Main results obtained from the administration of lead (Pb) with and without treatment with plant extracts on effector molecules related to hepatic redox metabolism

Outcomes Primary								
Reference	ROS/NOS production biomarkers		Antioxidant activity biomarkers		Oxidative stress products biomarkers		Immune biomarkers and others	
	Pb without extract	Pb with extract	Pb without extract	Pb with extract	Pb without extract	Pb with extract	Pb without extract	Pb with extract
Adebisi et al. 2022			↓CAT ↓GSH ↑GST	= CAT = GSH ↓GST	↑MDA	↓MDA		
Adeyeme et al. 2023					↑MDA	↓MDA		
Akilavalli et al 2011			↓SOD ↓GSH	↑SOD ↑GSH	↑MDA	↓MDA		
Akinlolu et al. 2021					MDA =	MDA =	↑ p53	↓ p53
Albasher et al. 2020	↑NO	↓NO	↓CAT ↓SOD ↓GSH ↓GPX ↓GR	↑CAT ↑SOD ↑GSH ↑GPX ↑GR	↑MDA	↓MDA	↑TNF-α ↑ IL-1β, ↑ NF-kβ ↑ iNOs ↑ Bax ↑Casp 3 ↓ Bcl-2/bax ratio ↓ BCL-2	↓TNF-α ↓ IL-1β ↓ NF-kβ ↓ iNOs ↓ Bax ↓Casp 3 ↑ Bc -2/bax ratio ↑ BCL-2
Asiwe et al 2022a			↓SOD ↓CAT ↓GSH	↑SOD ↑CAT ↑GSH	↑MDA	↓MDA		
Asiwe et al 2022b			↓SOD ↑GSH = CAT	↑SOD ↑GSH ↑CAT	↑MDA	↓MDA		
Barkaoui et al. 2020			↓CAT ↓SOD ↓GSH ↓GPX	↑CAT ↑SOD ↑GSH ↑GPX	↑MDA	↓MDA	↑ TNF-α ↑ IL-6 ↑NF-kβ	↓ TNF-α ↓ IL-6 ↓ NF-kβ
Chaurasia et al. 2000			↓CAT ↓SOD	↑CAT ↑SOD	↑MDA	↓MDA	-	-
Dewanjee et al. 2013			↓CAT ↓SOD ↓GST ↓GSH ↓GPX ↓GR ↓TOTAL COENZYME 9 ↓TOTAL COENZYME 10	↑CAT ↑SOD ↑GST ↑GSH ↑GPX ↑GR ↑TOTAL COENZYME 9 TOTAL COENZYME 10 =	↑MDA ↑DNA DAMAGE	↓MDA ↓DNA DAMAGE	-	-
Dewanjee et al. 2015a	↑ROS ↑ATP LEVELS	↓ROS ↓ATP LEVELS	↓CAT ↓SOD ↓GST ↓GSH ↓GPX ↓GR ↓TOTAL COENZYME 9 ↓TOTAL COENZYME 10	↑CAT ↑SOD ↑GST ↑GSH ↑GPX ↑GR ↑TOTAL COENZYME 9 ↑TOTAL COENZYME 10	↑MDA ↑CP ↑DNA DAMAGE	↓MDA ↓CP ↓DNA DAMAGE	-	-
Diab et al. 2018			↓SOD ↓GSH	↑SOD ↑GSH	↑MDA	↓MDA	-	-
Dua et al. 2016	↑ROS ↑ATP LEVELS	↓ROS ↓ATP LEVELS	↓CAT ↓SOD ↓GST ↓GSH ↓GPX ↓GR ↓TOTAL COENZYME 9 ↓TOTAL COENZYME 10	↑CAT ↑SOD ↑GST ↑GSH ↑GPX ↑GR ↑TOTAL COENZYME 9 ↑TOTAL COENZYME 10	↑MDA ↑DNA DAMAGE	↓MDA ↓DNA DAMAGE	-	-
El Azab 2021			↓SOD ↓CAT ↓GPx ↓GR	↑SOD ↑CAT ↑GPx ↑GR	↑MDA	↓MDA		

Table 4 (continued)

Outcomes Primary								
Reference	ROS/NOS production biomarkers		Antioxidant activity biomarkers		Oxidative stress products biomarkers		Immune biomarkers and others	
	Pb without extract	Pb with extract	Pb without extract	Pb with extract	Pb without extract	Pb with extract	Pb without extract	Pb with extract
El-Boshy et al. 2019			↓CAT ↓SOD ↓GSH ↓GPX	↑CAT ↑SOD ↑GSH ↑GPX	↑MDA	↓MDA		
El-Hashash et al. 2022	↓NO ↓TAC	= NO ↑ TAC	↓ALAD	↑ALAD	↑MDA	↓MDA		
El-Nekeety et al. 2009			↓GSH	↑GSH	↑MDA	↓MDA		
Fattah et al. 2020			↓SOD ↓GSH	↑SOD ↑GSH	↑DNA DAMAGE	↓DNA DAMAGE	↑ TNF-α ↑NF-kβ	↓ TNF-α ↓ NF-kβ
Gad et al. 2022			↓SOD ↓CAT ↓GSH	↑SOD ↑CAT ↑GSH	↑MDA	↓MDA		
Ghosh et al. 2012			↑CAT ↑SOD ↑GSH	↓CAT ↓SOD ↓GSH	↑MDA	↓MDA	-	-
Ghosh et al. 2013			↑CAT ↑SOD ↑GSH ↑GPX ↑GR ↑GSSG	↓CAT ↓SOD ↓GSH ↓GPX ↓GR ↓GSSG	↑MDA ↑CP	↓MDA ↓CP	↑XO ↑XDH ↓PDH ↓ICDH ↓α-KGDH ↓SDH ↓CYT C ↓NADH CYT C	↓XO ↓XDH ↑PDH ↑ICDH ↑α-KGDH ↑SDH ↑CYT C ↑NADH CYT C
Haleagrahara et al. 2010			↓ total antioxidant ↓SOD ↓GST ↓GPX	↑ total antioxidant ↑SOD ↑GST ↑GPX	↑MDA ↑CP	↓MDA ↓CP	-	-
Hamadouche et al. 2014			↓SOD ↓GST	↑SOD ↑GST			-	-
Jayan et al. 2017			↓CAT ↓SOD ↓GSH	↑CAT ↑SOD ↑GSH	↑MDA	↓MDA	-	-
Kansal et al. 2011			↓CAT ↓SOD ↓GSH	↑CAT ↑SOD ↑GSH	↑MDA	↓MDA	-	-
Koriem 2009	↑NO	↓NO	↓SOD ↓GPX	↑SOD ↑GPX	↑MDA ↑DNA DAMAGE	↓MDA ↓DNA DAMAGE	-	-
Kumar et al 2014			↑CAT ↓SOD GSH =	↑CAT SOD = GSH =	↑MDA	↓MDA	-	-
Kumar et al. 2016			↓CAT ↓SOD ↓GSH	CAT = ↑SOD ↑GSH	↑MDA	↓MDA	-	-
Kumar et al. 2017	↑ROS	↓ROS			↑MDA ↑CP	↓MDA ↓CP	-	-
Laamech et al. 2016			↓GSH	↑GSH	↑MDA ↑CP	↓MDA ↓CP	-	-
Lakshmi et al. 2013			↑CAT ↑SOD ↑GSH	↓CAT ↓SOD ↓GSH	↑MDA	↓MDA	-	-
Mehana et al. 2012			↓SOD ↓GST	↑SOD ↑GST			-	-
Mohamed et al. 2016			↓CAT SOD = ↑GST ↑GSH GPX =	↑CAT SOD = ↑GST ↑GSH GPX =	↑MDA	↑MDA	-	-

Table 4 (continued)

Outcomes Primary								
Reference	ROS/NOS production biomarkers		Antioxidant activity biomarkers		Oxidative stress products biomarkers		Immune biomarkers and others	
	Pb without extract	Pb with extract	Pb without extract	Pb with extract	Pb without extract	Pb with extract	Pb without extract	Pb with extract
Mohammed et al. 2013			↓CAT ↓SOD ↓GSH ↓GPX	↑CAT ↑SOD ↑GSH ↑GPX	↑MDA	↓MDA	-	-
Moneim 2016	↑NO ↑H_2O_2	↓NO ↑H_2O_2	↓CAT ↓SOD ↓GSH	↑CAT ↑SOD ↑GSH			NRf2 = ↑HO-1 ↓BCL-2 ↑Bax ↑Casp-3 ↑MMP-9	↑ NRf2 ↑ HO-1 ↑ BCL-2 ↓Bax ↓Casp-3 ↓MMP-9
Mostafa et al. 2018			↓CAT ↓SOD ↓GSH ↓GPX	↑CAT ↑SOD ↑GSH ↑GPX	↑MDA	↓MDA	↑TNF-α ↑IL-1β ↓IL-4 ↓IL-10	↓TNF-α ↓IL-1β ↑IL-4 ↑IL-10
Nishanthi and Anuradha 2012			↓CAT ↓SOD ↓GSH ↓GPX ↓Vit E ↓ Vit C	↑CAT ↑SOD ↑GSH ↑GPX ↑Vit E ↑Vit C			-	-
Obafemi et al. 2019			↓SOD ↓GST	↑SOD ↑GST	↑MDA	↓MDA	-	-
Pal et al. 2013	↑ROS	↓ROS	↓CAT ↓SOD ↓GST ↓GSH ↑GSSG ↓GPX ↓GR	↑CAT ↑SOD ↑GST ↑GSH ↓GSSG ↑GPX ↑GR	↑MDA ↑CP ↑DNA DAMAGE	↓MDA ↓CP ↓DNA DAMAGE	↑P38 ↑JNK ↑P53 ↑NF-kβ ↓IKB-α ↑IKK-α ↑BAX ↓BCL-2 ↓Apaf-1 ↑CYT-C ↑CASP-3 ↑CASP-9 ↑APOPTOSIS	↓P38 ↓JNK ↓P53 ↓ NF-kβ ↑IKB-α ↓IKK-α ↓BAX ↑BCL-2 ↑Apaf-1 ↓CYT-C ↓CASP-3 ↓CASP-9 ↓APOPTOSIS
Phatak and Matule 2017			↓ total antioxidant ↓CAT ↓SOD ↓GST ↓GSH	↑ total antioxidant ↑CAT ↑SOD ↑GST ↑GSH	↑MDA	↓MDA	-	-
Sainath et al. 2011			↓CAT ↓SOD	↑CAT ↑SOD	↑MDA	↓MDA	-	-
Sharma and Pandey 2010			↓CAT ↓SOD	↑CAT ↑SOD			-	-
Sharma et al. 2010			↓CAT ↓SOD ↓GSH	↑CAT ↑SOD ↑GSH	↑MDA	↓MDA	-	-
Sharma et al. 2009			↓SOD ↓GSH	↑SOD ↑GSH	↑MDA	↓MDA		
Sharma et al. 2011			↓CAT ↓SOD ↓GST ↓GSH	↑CAT ↑SOD ↑GST ↑GSH	↑MDA	↓MDA		
Sharma et al. 2012			↓CAT ↓SOD ↓GST ↓GSH	↑CAT ↓SOD ↑GST ↑GSH	↑MDA	↓MDA	-	-

Table 4 (continued)

Outcomes Primary								
Reference	ROS/NOS production biomarkers		Antioxidant activity biomarkers		Oxidative stress products biomarkers		Immune biomarkers and others	
	Pb without extract	Pb with extract	Pb without extract	Pb with extract	Pb without extract	Pb with extract	Pb without extract	Pb with extract
Wang et al. 2020			↓SOD ↓GSH	↑SOD↑GSH	↑MDA	↓MDA	↑Ca ↑CRT ↑PKC-α ↑BAX ↑CYT -C ↑CASP-3 ↓BCL-2	↓Ca ↓CRT ↓PKC-α ↓BAX ↓CYT -C ↓CASP-3 ↑BCL-2
Xia et al. 2010			↓CAT ↓GSH ↑GSSG	↑SOD ↑GSH GSSG =	↑MDA	↓MDA	-	-
Yuniart et al. 2021					↑MDA	↓MDA	-	-
Zargar et al. 2020			↓GSH ↑GSSG	↑GSH ↓GSSG	↑MDA	↓MDA	-	-

Pb without extract: statistically different results compared to control group, Pb with extract: statistically different results compared to Pb-exposed group; ↑, increase; ↓, decrease; *NO*, nitric oxide; *CAT*, catalase, *SOD*, superoxide dismutase; *GSH*, reduced glutathione; *GPX*, glutathione peroxidase; *GR*, glutathione reductase; *GST*, glutathione-S-transferase; *GSSG*, oxidized glutathione; *MDA*, malondialdehyde; *CP*, carbonyl protein; *ROS*, reactive species of oxygen; *Vit E*, vitamin E; *Vit C*, vitamin C; *P53*, tumor protein; *TNF-α*, tumor necrosis factor; *IL-1β*, interleukin 1 beta; *IL-6*, interleukin 6; *IL-4*, interleukin 4; *IL-10*, interleukin 10; *NF-kβ*, nuclear factor kappa β; *iNOS*, nitric oxide synthases; *Bax*, protein X associated with BCL-2; *Casp 3*, caspase 3; *Casp 9*, caspase 9; *BCL-2*, B-cell lymphoma 2; *Xo*, xanthine oxidase; *XDH*, xanthine dehydrogenase; *PDH*, hepatic pyruvate dehydrogenase activity; *ICDH*, isocitrate dehydrogenase; *α-KGDH*, alpha keto glutarate dehydrogenase; *SDH*, succinate dehydrogenase; *Cyt C*, cytochrome c oxidase activity; *NADH CYT C*, NADH cytochrome C reductase; *Nrf2*, nuclear factor erythroid 2-related; *HO*, heme oxygenase; *Apaf-1*, apoptotic peptidase activating factor 1; *JNK*, c- Jun N-terminal kinase; *IKB-α*, kappa-B inhibitor alpha; *IKK-α*, inhibitor of nuclear factor kappa-B kinase subunit alpha; *P53*, tumor protein; *p38*, mitogen-activated protein kinases; *Ca*, calcium; *CRT*, calreticulim; *PKC-α*,protein kinase C

histopathological damage; however, in most studies with serious pathologies such as necrosis, the improvement was not enough to maintain CTL standards.

Risk of bias and methodological quality assessments

The detailed results for the analysis of bias using SYRCLE's tool are shown in Figs. 7 and 8 Among the 50 studies included in this review, none of the studies fully met all the set criteria, and most of the studies contained insufficient information. Four of the issues were fully described in all studies (100%), being incomplete outcome data, selecting reporting, validated tools, and statistical analyses. Through careful and comprehensive analysis, when assessing random sequence generation, 52% (n=26) of the studies did not clearly report this information but reported that the experiment was performed randomly and 38% (n=19) of the studies presented a high risk of bias since they did not report any information on how it was performed. Regarding allocation concealment, 32% (n=16) were rated at high risk of bias and 52% (n=26) did not clearly report how the random allocation was done. Only 1 of the studies reported blinding of participants and results, and these two items were rated at high risk of bias (98%; n=49). All articles fully reported the methodology in the results, so the items with incomplete outcome data and selective reporting were assessed at low risk of bias (100%; n=50). Regarding the studies that clearly reported the information, we analyzed that the similarity of intervention characteristics (species, origin, extraction and/or preparation protocol, extract exposure, and vehicle) was clearly reported in 14 studies (28%) and 37 studies did not report all the necessary information (74%), being evaluated with a high risk of bias. Regarding the information about the animal used in the experiment (strain, sex, age, laboratory conditions, and weight), 28 studies reported all the information (n=56%), therefore being evaluated with low risk of bias, and 23 studies did not report all the information, being classified with a high risk of bias (n=46%). When evaluating the studies that show approval from the ethics committee for the use of animals, half of them (50%; n=25) did not clearly report this information, 8% (n=4) have a high risk of bias as they did not report approval and 44% (n=22) of these studies were evaluated with low risk of bias as they reported committee approval and protocol number.

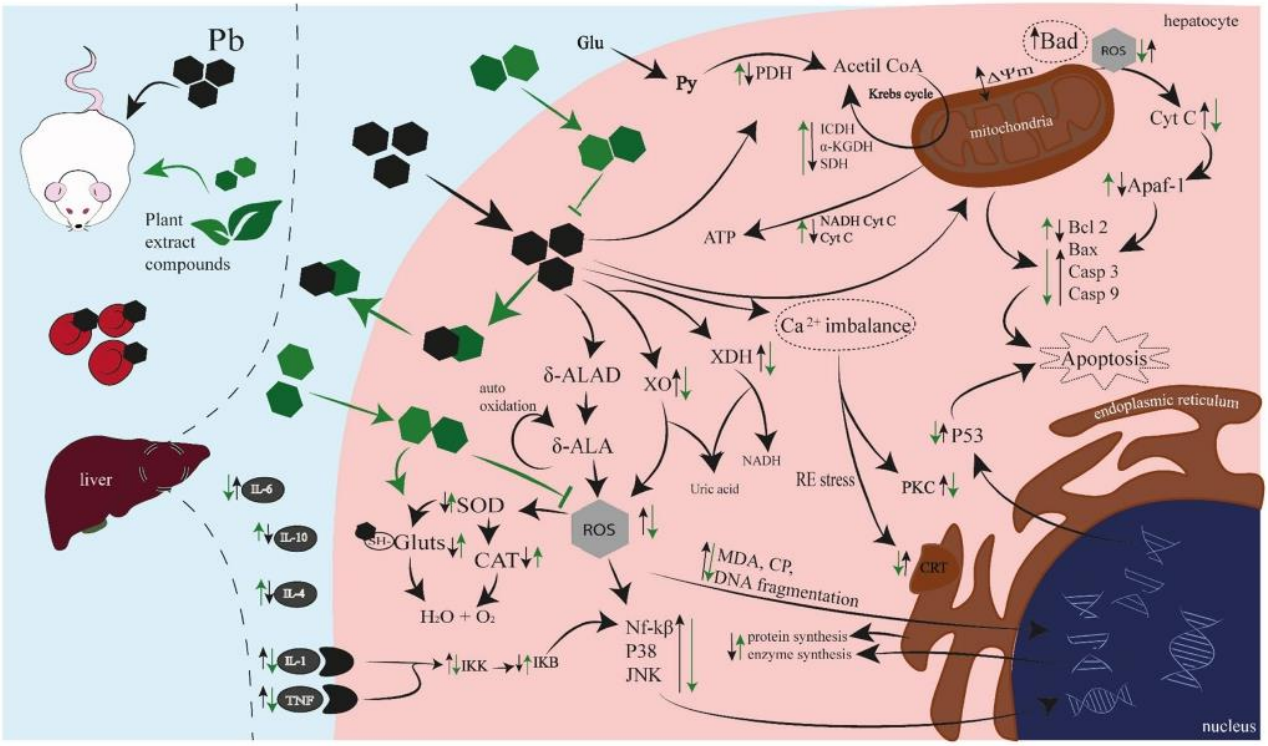


Fig. 6 General effects of plant extracts in the liver from lead (Pb)-intoxicated animals. The effects of Pb can be observed by the black arrow and extracts by the green arrow. Pb, lead; ROS, reactive oxygen species; CAT, catalase, SOD, superoxide dismutase; MDA, malondialdehyde; CP, carbonyl protein; ROS, reactive species of oxygen; Gluts, glutathione; Glu, glucose; Py, pyruvate; ΔΨm, mitochondrial membrane potential; Acetyl Coa, acetylcoenzyme A; SH, sulfhydryl radical; Xo, xanthine oxidase; XDH, xanthine dehydrogenase; PDH, hepatic pyruvate dehydrogenase activity; ICDH, isocitrate dehydrogenase; α-KGDH, alpha keto glutarate dehydrogenase; SDH, succinate dehydrogenase; Cyt C, cytochrome c oxidase activity; Ca, calcium; CRT, calreticulim; PKC-α, protein kinase C; HO, heme oxygenase; TNF-α, tumor necrosis factor; IL-1β, interleukin 1 beta; IL-6; interleukin 6; IL-4, interleukin 4; IL-10, interleukin 10; NF-kβ, nuclear factor kappa β; Bax, protein X associated with BCL-2; Casp 3, caspase 3; BCL-2; B-cell lymphoma 2; NADH CYT C, NADH cytochrome C reductase; MMP-9, matrix metallopeptidase 9

Discussion

Our study reviewed the main hepatic effects of plant extracts in Pb-intoxicated animal models, indicating that disturbances in liver antioxidant defenses and ROS production are key mechanisms of Pb toxicity (Dua et al. 2016). Exposure to Pb is a global concern, and the studies analyzed here demonstrate several shreds of evidence that Pb affects many important redox status pathways leading to cell death. Conversely, although the studies together did not bring results that can be used as a suggestion for use as prevention, plant extracts showed promise in reversing the Pb damages, acting as a metal chelator, providing exogenous antioxidants, and stimulating endogenous antioxidant defense mechanisms in Pb-intoxicated animals.

Interestingly, studies investigating the effect of plant extracts on the hepatic metabolism of Pb-exposed animals are considerably recent, within 2000–2021. The studies identified here were mainly from India, Egypt, and China. China is the largest Pb emitter in Asia, followed by India, through coal combustion (Das et al. 2018). Some of the studies used Ayurveda herbs, which is a traditional medicine system practiced in India (Jain and Mathur 2020; Mukherjee et al. 2021). Surprisingly, Pb was present even in herbal drugs in Asia. Some medications, including Ayurvedic drugs, are manufactured pills. During 2011–2012, six cases of Pb poisoning were associated with the use of oral Ayurvedic medicines produced in India due to the use of Pb in the red dye used to color the pills or the metal itself added to increase the medicine's weight. Pb concentrations in these drugs exceeded 300mg of intake per day if taken according to the labeling instructions. Concentrations as little as 70–80 μg/dL of Pb in blood plasma have been associated with encephalopathies in children, and near to 460 μg/dL seems to be causing the same problem in adults (Agency for Toxic Substances and Disease Registry (ATSDR) 2020).

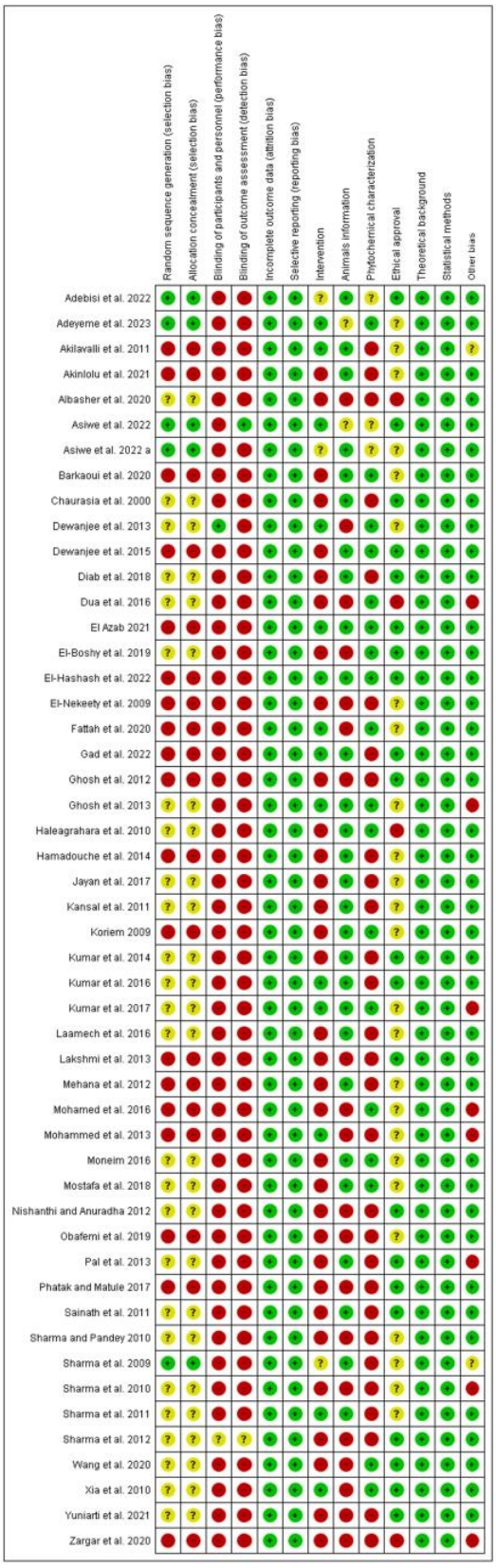


Fig. 7 Results for the risk of bias and methodological quality indicators for all studies included in this systematic review that evaluated the impact of plant extracts on hepatic redox metabolism upon lead exposure. The items in the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) Risk of Bias assessment were scored with "yes" indicating low risk of bias (green), "no" indicating high risk of bias (red), or "unclear" indicating that the item was not reported, resulting in an unknown risk of bias (yellow)

We also highlight that studies that analyzed the plant extract in a Pb-unexposed group demonstrated that the extract concentration tested is not toxic, and animals from this group showed the highest antioxidant activity (higher than the CTL group) since most oxidative markers related to the ROS like H_2O_2 were decreased, and SOD, CAT, and GST expressions were improved (Chaurasia et al. 2000; Haleagrahara et al. 2010; Sainath et al. 2011; Lakshmi et al. 2013; El-Boshy et al. 2019; Fattah et al. 2020).

Many plants evaluated in this study also showed antioxidant protective effects when co-exposed with several other heavy metals, such as *Mangifera indica* L., *Casimiroa sapota*, and *Malus micromalus* Makino, probably due to common intracellular pathway activations (Egwurugwu et al. 2010; Nwokocha et al. 2012; Mishra and Singh Sangwan 2019). That is important since we are daily exposed to many metals, and co-exposition protection can be an interesting factor for choosing the use of a plant to mitigate effects. Only a few studies performed a phytochemical analysis of the extracts. Some of the compounds used in the studies have already been studied for their promising healthy effects. Quercetin (QE), one of the most widely distributed flavonoids, was found in five studies and has anti-inflammatory effects on Pb toxicity, and they showed hydroxyl and carbonyl groups that can be the main metal complexing domains in Pb-chelating processes (Liu et al. 2012). Most studies using extracts with QE showed higher levels of antioxidant activities in the liver than Pb-exposed animals, as well as lower levels of ROS (Dewanjee et al. 2013, 2015b; Dua et al. 2016), lower levels of pro-inflammatory immune biomarkers expression (Fattah et al. 2020), and lower levels of MDA and DNA damage. Rutin (RUT) is a flavine that can protect Ca^{2+} and Na^{+}/K^{+}ATPase activity from Pb effects and has anti-inflammatory effects, which was observed for the authors of one study—lower levels of TNF-α, IL-6, and NF-kβ (Barkaoui et al. 2020; Fattah et al. 2020). Catechins (CA) such allic acid, catechin, epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate were found in 3 studies. CA exhibited an antioxidant activity more potent than vitamin C—20 times higher—related with an ortho-dihydroxyl group in the B-ring and a galloyl moiety at the 3-position (Chen et al. 2003). The studies that observed the presence of CA also found anti-inflammatory effects

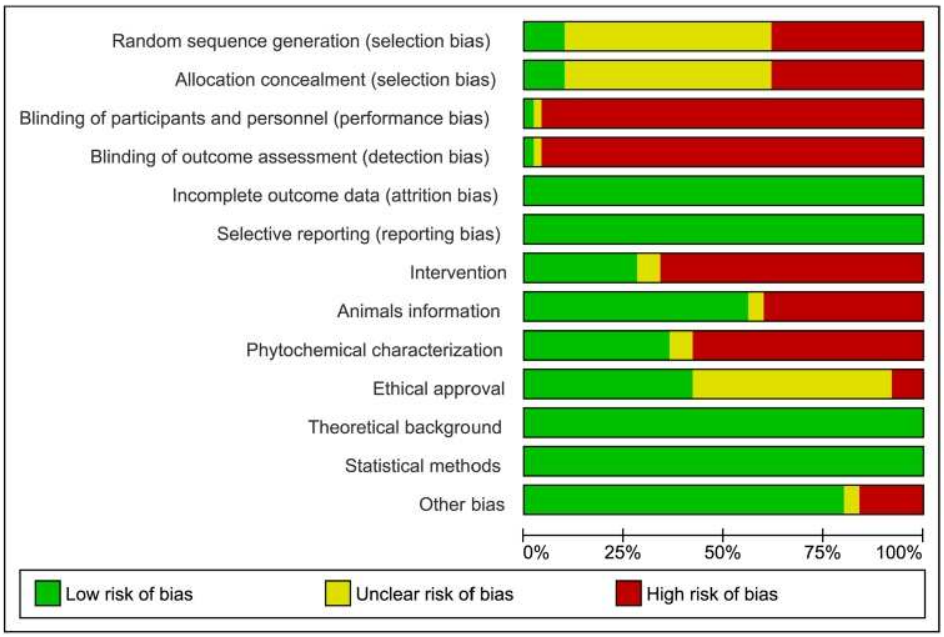


Fig. 8 Risk of bias summary: review authors' judgments about the risk of bias items for each included study. Green, low risk of bias; yellow, unclear risk of bias; red, high risk of bias

(Wang et al. 2020; Barkaoui et al. 2020; Fattah et al. 2020) and calcium homeostasis control—lower levels of Ca and CRT co-exposed animals compared to Pb-exposed animals (Wang et al. 2020). Curcumin (CUR) was observed in one study with phytochemical analyses (Mohamed et al. 2016). This study that found CUR is with *Zingiber officinale*, from the family Zingiberaceae. CUR was proven to be rich in phenol and is found in the rhizome of turmeric plants. This compound has a high ability to bind lead and other toxic elements and is an efficient inhibitor of lipid peroxidation through the inhibition of lipoxygenase and cyclooxygenase pathways (Abu-taweel 2018). However, the study that found CUR in the phytochemical analysis was not able to show lower levels of MDA content after treatment with the plant extract (*Zingiber officinale* L.)(Mohamed et al. 2016). One of the advantages of using plant extracts is that they can contain one or more compounds such as those mentioned here that may act synergistically (Adhikari et al. 2018). For instance, *Moringa oleifera* Lam has QE, RUT, and CA (Fattah et al. 2020).

After oral ingestion, Pb is enterically absorbed through the portal system, transported as Pb-diphosphate in erythrocytes (Dewanjee et al. 2013, 2015a), and then the liver is the first exposed organ (Moneim 2016). Once in the liver, Pb can cause hepatotoxicity through the production of ROS/NOS and the inhibition of δ-aminolevulinic acid dehydrase (δ-ALAD) caused by binding to Pb. This inhibition leads to the accumulation of δ-aminolevulinic acid (δ-ALA). This acid (δ-ALA) undergoes Pb-catalyzed auto-oxidation and thus induces the production of ROS/NOS such as superoxide ion (O_2^+) and hydroxyl radical (OH^+) (Mehana et al. 2012; Kumar et al. 2016; Dua et al. 2016). Another form of ROS production observed in our study was the increase in xanthine oxidase (XO) and xanthine dehydrogenase (XDH), which produce H_2O_2 and O_2 in situations of ATP depletion and Ca^{2+} imbalance. It is possible that Pb-exposure can increase the production of ROS by XO and XDH through its disrupting effect on intracellular Ca^{2+} and consequently damage mitochondrial functions (Ghosh et al. 2013). In addition to the production of ROS, Pb promotes modifications on other important enzymes that use endogenous sources to promote oxidative stress in tissues, including NADPH oxidase and XO (Leto et al. 2009; Schmidt et al. 2019). The increase in this protein activity is responsible to increase the quantity of reactive oxygen species inside the cells and consequently lipid peroxidation, DNA damage, and protein carbonylation (Schmidt et al. 2019). However, some compounds such as flavonoids can cause inhibition of XO, resulting in decreased oxidative injury—and luteolin is one of the most potent inhibitors of XO (Nijveldt et al. 2001) and was found in one study with phytochemistry that did not evaluate XO but evaluated anti-inflammatory markers and observed lower levels of the markers compared to CTL (Barkaoui et al. 2020). In addition, in some studies, Pb decreases the antioxidant activity responsible for neutralizing free radicals and ROS. This decrease in antioxidant activity happens in several ways, including the inhibition of the thiol groups of enzymes. Pb has a strong affinity with amino acid thiol groups, mainly cysteines, affecting important enzymes such as SOD, GST, and GSH (Mehana et al. 2012). Our results showed that almost all concentrations of Pb in the liver induced lower levels of GST and GSH and higher GSSG in exposed animals compared to CTL. This decrease was observed in most articles analyzed. The cellular consequences of excessive

ROS production after Pb exposure are lipid peroxidation, demonstrated by the increase in MDA content (Tsikas 2007), and misfolded proteins associated with increased carbonyl content (Akagawa 2021) and DNA damage (Shokolenko et al. 2009; Srinivas et al. 2019). In addition, the DNA damage culminates in decreased protein (also in enzymatic) synthesis, showing that there is positive feedback among all these molecules (Albasher et al., 2020; El-Boshy et al., 2019; Moneim, 2016). Another process involved with decreased protein synthesis is through disturbances in Ca^{2+} homeostasis in cells. This imbalance causes cell lysis due to changes in the cytoskeleton and induces stress on the endoplasmic reticulum, decreasing protein production (El-Boshy et al., 2019). These findings corroborate the results shown in our review (Haleagrahara et al. 2010; Ghosh et al. 2012; Pal et al. 2013; Dewanjee et al. 2015b; Kumar et al. 2016; Laamech et al. 2017) since the studies describe an increase in DNA fragmentation and consequently a decrease in total protein expression, and an increase in the number of misfolded proteins as a dysfunction, compromising the cell integrity following the Pb exposure.

The studies analyzed in our review showed that some plant extracts can improve the endogenous enzymatic activity by activating enzymes or preventing their oxidation, as is the case with garlic extract (Kumar et al. 2016) and bioactive present in plant extracts such as polyphenols (Laamech et al. 2017) and flavonoids (Xia et al. 2010; El-Boshy et al. 2019). In addition, polyphenols and flavonoids are considered exogenous antioxidants because they can directly neutralize ROS/NOS through the donation of an electron and consequently a hydrogen atom, becoming these molecules in stable compounds (Kurutas 2016; Albasher et al. 2020). Certain flavonoids may act synergistically; therefore, plant extracts—which contain many different flavonoids—may increase the antioxidant effect (Adhikari et al. 2018). Besides the promissory effects of the extracts, some difficulties regarding the systemic bioavailability of the plant metabolites should be considered. Some metabolites such as anthocyanins are unstable and have low bioavailability; the degradation and reaction of the metabolites can interfere with the biological effects (Fleschhut et al. 2006).

The knowledge about redox metabolism and the role of oxidative stress on liver diseases has indicated that there is a direct relationship between redox imbalance and inflammatory processes. During Pb intoxication, there is evidence that inflammatory, oxidative, and nitrosative events are coupled processes potentially mediated by pro-inflammatory cytokines such as TNF-α, IFN-γ, and IL-1 and IL-6, which stimulates the intense production of reactive species in liver tissue (Albasher et al. 2020; Barkaoui et al. 2020). The iNOS also has its expression increased during Pb intoxication and participates in the inflammatory response (Albasher et al. 2020). Our results show us that increased NO was observed in Pb-exposed rat's liver in the studies, and these levels were decreased in animals treated with extracts, especially in studies that used flavonoid and polyphenol-rich extracts (Koriem 2009; Moneim 2016; Albasher et al. 2020). This benefit is likely to occur due to reduced inflammation since iNOS can be induced by immunostimulatory cytokines, monocytes, and macrophages releasing more NO that becomes a NOS, further increasing nitrosative stress (Kurutas 2016). In fact, the inflammatory process itself increases the production of ROS/NOS (El-Benna et al. 2016; Destro et al. 2021), increasing oxidative damage even further. When oxidative damage is too high, pro-apoptotic proteins Casp3 and Bax are activated, modulating changes in the mitochondrial membrane (Dua et al. 2016). The pro-apoptotic event consists of the translocation of the pro-apoptotic Bad protein to the mitochondria, simultaneously with the downregulation of the anti-apoptotic Bcl-2 protein, and then the release of Cyt C to the cytosol. Increasing Cyt C in the cytosol induces apoptosis by cleaving caspases (Dua et al., 2016). In our review, we observed that Pb exposure promoted a decrease in Casp, BAX, and Cyt C and an upregulation of pro-apoptotic genes. On the other hand, the plant extracts analyzed in the studies caused an increase in anti-apoptotic proteins and a downregulation in anti-apoptotic genes like BCL-2, showing that the plant extracts can attenuate apoptotic process caused by Pb exposure through controlling important inflammatory pathways (Pal et al. 2013; Moneim 2016; Albasher et al. 2020; Wang et al. 2020).

Given the beneficial results on antioxidant defenses and the decrease in Pb bioaccumulation, it was clear from our review that metabolites from plant extracts often act together in protecting cells and tissues from metal exposure and, for that, may the different extracts with different metabolites could have better effects together, but we need more studies to understand it better. Some antioxidants can interact with other antioxidants to regenerate their original properties; forming an "antioxidant network" (Kurutas 2016), suggesting another form of action of the antioxidants found in plant extracts. Furthermore, many compounds, such as garlic extract, have a lipophilic nature (Kumar et al. 2016), as opposed to chelators such as meso-2,3-dimercaptosuccinic acid (DMSA) which are lipophobic (Sharma et al. 2010). This lipid affinity facilitates the entry of the extract into the cell, increasing the chances of removing intracellular Pb through their chelating and antioxidant abilities (Kumar et al. 2016). Other extracts also form an insoluble complex, facilitating the removal of intracellular Pb (Patrick 2006), as is the case with green tea (Mehana et al. 2012). Interestingly, after forming the Pb-polyphenols complex, polyphenols still exert an antioxidant capacity sometimes higher than polyphenols alone (Adhikari et al. 2018).

Limitations

In this review, we observed that alternative therapies like plant extracts are very promising as they are involved in the control of redox metabolism and inflammation processes and may represent a rational and useful strategy to treat liver diseases. However, it is important to emphasize that there is a bias in the data found in the literature, where only positive results are published. Thus, the interpretation of the conclusions requires caution. The absence of results reporting side effects or negative effects of plant extracts should not be interpreted as non-existent. Our research did not identify any systematic review that analyzes the use of plant extracts in the modulation of the antioxidant defense in hepatic Pb poisoning. The main strength of this study is reuniting the fragmented literature to map the oxidative and deleterious effects of Pb intoxication in the liver and the possible antioxidant effects of plant extracts against Pb intoxication. This study synthesized the main forms of Pb that act in the liver, and how plant extracts mitigate these negative effects, in addition to providing information on more effective studies in the area. Through bias analysis, we found the absence of fundamental characteristics, such as random sequence generation or random outcome assessment and blinding of participants (caregivers and outcome evaluators). On the other hand, there were no studies with incomplete results, selective reporting, or no description of the statistical method. This demonstrates some reliability in the studies.

The chemical origin of the extracts can vary greatly, and many studies did not show the extract phytochemical analysis, which makes does not shed light on how they act. Also, the families and types of extracts analyzed varied, which makes this review less homogeneous. These main limitations can make it difficult to build a robust conclusion, such as mapping the most effective extracts, possible side effects, and recommended doses. Finally, information regarding the negative effects of plant extracts should be clearer in the studies. We did not find any side effects or information about high dosages, for example. The lack of information in this regard may lead the reader to think that all concentrations and doses are relevant and plant extracts are always beneficial, which may not be true.

Conclusion

Our review reinforces the knowledge about Pb toxicity, demonstrating and synthesizing the main effects of this metal on the liver. Furthermore, it was clear that plant extracts have a great capacity to reduce the toxic effects of Pb poisoning, due to the presence of compounds such as flavonoids, tannins, alkaloids, phenolic essential oils, anthocyanins, and vitamins. Together, these compounds may have an additive or even synergistic protective effect on cells and tissues and act as antioxidants, reducing ROS/NOS, or acting as metal chelators, reducing the metals' concentration in tissues. In summary, the main mode of action of plant extracts are (1) promoting the reduction of oxidative stress acting as metallic chelators and (2) promoting the increase in endogenous antioxidant activity by stimulating anti-inflammatory and anti-apoptotic pathways. We reinforce the importance of studies evaluating the protective effects of plant extracts, with a methodology with the lowest possible risk of bias for robust conclusions in the various fields of toxicology.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-023-28620-8.

Acknowledgements The authors thank the National Council for Scientific and Technological Development (CNPq). A. L. F. Destro received a Ph.D. research fellowships from CAPES (Coordination for the Improvement of Higher Education Personnel).

Author contribution Ana L. F. Destro, conceived/designed the research, analyzed the data, and wrote the manuscript. Patrícia S. Mattosinhos, analyzed the data. Rômulo D. Novaes data curation, validation, writing—review & editing. Mariáurea M. Sarandy analyzed the data and wrote the manuscript. Reggiani V. Gonçalves conceived/designed the research, writing—review & editing. Mariella B. Freitas conceived/designed the research, supervised the study, analyzed the data, and wrote the manuscript. All authors read, corrected, and approved the manuscript.

Data availability The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interest The authors declare no competing interests.

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2. Capítulo 2:

Individual acute exposures to low concentrations of cadmium, chromium, lead, and nickel affect oxidative stress and pathological markers in fruit bats

Ana Luiza Fonseca Destro[a], Thaís Silva Alves[a], Fernanda Ribeiro Dias[b],Reggiani Vilela Gonçalves[a], Jerusa Maria de Oliveira[d], Leandro Licursi de Oliveira[c], Mariella Bontempo Freitas[a]

[a] Department of Animal Biology, Federal University of Viçosa, Viçosa, MG, Brazil.

[b] Department of Veterinary Medicine, Federal Rural University of Pernambuco, Recife, PE, Brazil.

[c] Department of General Biology, Federal University of Viçosa, Viçosa, MG, Brazil.

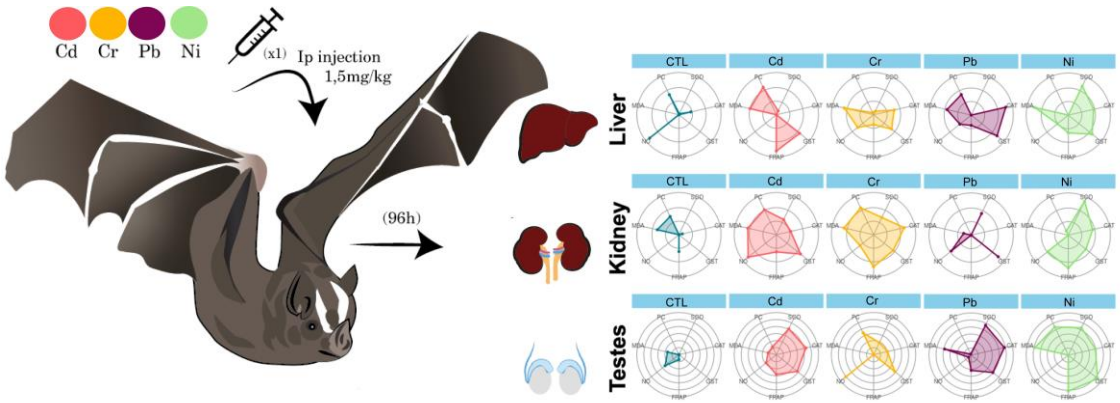
[d]Rede Nordeste de Biotecnologia (RENORBIO), Federal University of Alagoas, Maceió, AL, Brazil.

Corresponding author:

Mariella Freitas, Department of Animal Biology, Federal University of Viçosa, Viçosa Minas Gerais, 36570-900, Brazil.

Email: mfreitas@ufv.br

Artigo a ser submetido à Ecotoxicology and Environmental Safety



HIGHLIGHTS

- Even low concentrations at acute exposure of Ni and Pb can cause severe damage to the kidney and liver.
- Acute exposure to Cd, Cr, Pb and Ni causes oxidative unbalance in bats.
- Integrated biomarker response (IBR) is a good tool for multi-biomarker approaches on bats.
- Based on the IBR indexes this is the order of metals toxicity in fruit-eating bats: Ni> Pb> Cd=Cr.

Abstract

Cadmium (Cd), chromium (Cr), lead (Pb), and nickel (Ni) are heavy metals commonly found in the environment, where they can contaminate animals, including bats. We aimed to investigate the effects of heavy metals in fruit bats. Adult males (*Artibeus lituratus*) were captured and assigned to the following groups, treated with 1.5 mg/kg intraperitoneal (ipi) injection of: CTL sodium chloride (NaCl) 0.9% (n=6); Cd cadmium chloride ($CdCl_2$) (n=6); Cr chromium trioxide IV (CrO_3) (n=6); Pb lead acetate ($Pb(CH_3COO)_2 \cdot 3H_2O$) (Pb, n=6) and Ni) nickel chloride ($NiCl_2.6H_2O$) (n=7). After 96h of exposure, Ni-exposed animals showed higher lipid peroxidation in the liver and higher protein carbonyl in the testes, and in the kidneys, we found increased vascular congestion compared to CTL. Pb-exposed bats showed lower Glutathione S-Transferase (GST) activity in all tested tissues compared to CTL and a decreased percentage of normal cells in the seminiferous tubules in the testes. Bats exposed to Cr showed lower GST activity in the kidneys and testes, and higher leukocyte infiltrate in the liver and higher vacuolization in the testes. Cd-exposed bats showed lower GST activity in all tissues, higher leucocyte infiltrate in the kidneys and a lower percentage of normal cells in the testes. Necrotic and lipidic areas in the liver were also observed in Pb-exposed bats, and necrotic areas for Ni-exposed. In conclusion, we propose the following order of metal toxicity for fruit-eating bats based on the concentrations and exposure time we tested: Ni> Pb> Cr= Cd.

Keywords: Chiroptera, ecotoxicology, heavy metal, bat conservation

1.0. INTRODUCTION

Heavy metals (HM) are often referred in ecotoxicological studies as metals and metalloids with potential toxicity, usually associated with environmental pollution [1]. Anthropogenic activities such as mining and industrialization have been a major concern as they might be involved in increased HM levels in the environment [2]. As metals are not degraded in the environment, they may accumulate in living organisms and within food chains, processes known as bioaccumulation and biomagnification, respectively [2].

Cadmium (Cd) is a non-essential heavy metal [3] and could accumulate in organisms through food chains [4]. In the male mammalian reproductive system, Cd exposure shows one of the main disruptive potentials among metals [5], causing hormone and sperm alterations [5–7]. Low and prolonged Cd concentrations also induced damage to different organs, such as the liver and kidneys (Genchi et al., 2020). Chromium (Cr) is an essential nutrient for animals [9], and it has been heavily used in metallurgical, chemical, and refractory industries [10]. Excessive Cr exposure was found to affect the mammalian reproductive system, inducing oxidative stress due to its strong oxidant capacity [10]. Prolonged exposures also induce genotoxic effects and disturbances to the immune system [9]. Lead (Pb) is a non-essential element [3] widely found in industrial products (batteries, paints, gasoline, pesticides, medicines, cosmetics, etc.) and is considered the most important toxic heavy element in the environment due to its abundant global distribution [11]. Pb induces damage to renal, reproductive, and nervous systems in mammals [11], through excessive reactive oxygen and nitrogen species (ROS/NOS) production and antioxidant enzyme inhibition [12]. Nickel (Ni) has physical and chemical properties that favor its use in stainless steel manufacturing, structural steel alloys, electroplating, and batteries [13]. Although this is an essential nutrient (Genchi et al. 2020a), high Ni levels have been proven to disrupt the Hypothalamus-Pituitary-Gonad (HPG) axis and generate excessive ROS/NOS production in testes [15].

In the environment, variable concentrations of Cd, Cr, Pb, and Ni are often found, depending on the proximity of industrial activities and the natural composition of the soil - for instance, concentrations (mg/kg) up to 11.7 for Cd, 468 for Cr, 510 for Pb, and higher than 583 for Ni in the soil close to gold mining exploration have already been reported [16]. Besides, mining activities are also involved with eventual dam rupture, as recently happened twice in Brazil (in the Atlantic Forest area) and might as well increase the associated metal concentration in the environment [17–19]. These associated metals are often not bioavailable, although they can be released due to anoxic conditions associated with plant and animal

activity [20]. In Brazilian tropical basins, these metals were found in sediments, in concentrations (mg/kg) varying from below the detection limit to 6.6 for Cd, 2.2 to 119.5 for Cr, and from below the detection limit to 92 for Pb [21]. This environmental contamination may impact the local fauna in several ways, and factors like the chemical form of the metal in the environment, availability, concentration, exposure time, interaction with other metals in the environment, and the animal's trophic level may influence the impact extent [22].

Although the effects of HM, as those cited above, have been described in several animal models, its effects on wild species are less understood. Bats are the only true flying mammals, with adaptations that include a low reproduction rate [23], high metabolic rate, high longevity, and a reduced inflammatory response (may be associated with their ability to deal with viral infection with lower impact) (Moreno Santillán et al. 2021). Some of these specific traits could indicate that environmental pollutants may affect bats differently compared to other mammals [25]. Despite this, the risk assessment of pollutants in bats is incipient and requires a specific approach for these animals [26]. Bats also play a vital role in ecosystem services through pollination (549 plants species are pollinated by bats), pest control (752 insect species are consumed by bats, including crop pests and disease vectors), and seed dispersal [27]. The great fruit-eating bat (*Artibeus lituratus*) is an abundant species in the Neotropical region, where it contributes to reforestation, especially in heavily fragmented areas of the Atlantic Rain Forest [28,29]. In these regions, though, fruit bats face several challenges to survive, including heavy metal pollution [30].

Despite a few limitations, bats are considered potential ecological bioindicators, mainly due to their ability to inhabit wide geographic ranges, to be at the top of the food chain, to show ecological and evolutionary interactions with other ecosystem components, such as bat-insect and bat-plant coevolution, and for the key ecosystem services provided [31]. A few *in loco* toxicological studies associate environmental exposure to heavy metals, detected in guano or blood, with damage to the liver, DNA, and cholinergic functions [30]. Although there are not many studies on this, the scientific literature shows that bats can accumulate heavy metals found in the environment. Concentrations (mg/kg) such as 5.8 – 7.32 of Pb, 5.7 – 10.9 of Cr, 3.6 – 4.05 of Cd, and 4.3 – 8.6 of Ni have already been found in insectivorous bats living at coal mining areas (Zocche et al., 2010). However, there is a lack of information on the specific, isolated effect of the main environmentally available heavy metals in bats exposed to each one of them, individually. In a region where mining operations

and forest fragments are closely located, investigations on how HM pollution affects important aspects of local wildlife are needed to understand the extension of these impacts.

Here we aimed at evaluating the toxicological effects of acute exposure to low concentrations of four heavy metals (Cd, Cr, Pb, and Ni) on redox balanceand histopathological parameters in physiologically relevant tissues in the great-fruit-eating bat. Our study was designed based on a previous study with mice, in order to make possible comparisons and assumptions regarding eventual differences between the two animal models [32].

2.0. MATERIAL AND METHODS

2.1. Chemical

The metals $CdCl_2$ (cadmium chloride 99.9%), CrO_3 (chromium VI trioxide ≥99%), Pb $(CH_3COO)^2 \cdot 3H_2O$ (neutral lead acetate P.A. trihydrate) and $Cl_2Ni.6H_2O$ (nickel (II) chloride hexahydrate 99.9%) were obtained from Sigma-Aldrich (St. Louis, Missouri, US) and Merck (Darmstadt, Germany) and diluted in distilled water to obtain the target concentrations (1,5 mg/kg) of Cd, Cr, Pb and Ni, according to other studies [32–34].

2.2. Animals

Adult male great fruit-eating bats (*Artibeus lituratus*, n=31, BW=73.62±5.78 g) were captured using mist nets in a forest area from the Federal University of Viçosa (UFV) (20° 45'S and 42° 52' W), Viçosa, Minas Gerais, Brazil. All animals were under the same condition, gender, and captured in the same location and during the same season, as described in other studies with bats [29,35–37]. Animals were identified according to Díaz et al. (2016), brought to the University, and kept in a half-wall screen-lined bat house located at the Museum of Zoology garden, under trees of the Atlantic Forest. Bats were assigned to individual enclosures (a total of 8 enclosures of 2 m^3 size each), where they could fly freely inside the rooms, and kept under natural cycles of temperature, light, and humidity. All animals were submitted to a 4-day acclimation period before the exposure started. During this time, the animals were offered tropical fruits (*Carica papaya, Musa* sp*, Psidium guajava,* and *Mangifera indica* L.) and water *ad libitum*.

2.3. Experimental design

Following the acclimation period, the animals were treated according to one of the five experimental groups: CTL) control: normal saline solution (NaCl 0.9%) (n=6) and

experimental groups that received 1.5 mg/kg of each metal: Cd) cadmium chloride ($CdCl_2$) (n=6); Cr) chromium trioxide IV (CrO_3) (n=6); Pb) lead acetate ($Pb(CH_3COO)^2 \cdot 3H_2O$) (n=6) and Ni) nickel chloride ($Cl_2Ni.6H_2O$) (n=7). We chose to run the CTL group with the saline solution due to the fact that metals were supplied in the form of a salt in metal-exposed groups, so the saline solution would offer the CTL group similar conditions to the treated ones (Cupertino et al. 2017b; Albasher et al. 2020). Exposure to saline or metals was performed through one intraperitoneal injection (ipi) of 0.7 mL of solution at 8:00 am on day 1 of exposure). The ipi route was chosen as it ensures that the total volume would be completely absorbed by the animal, avoiding consumption bias between metals. Besides, gavage in bats is often complicated and stressful due to the lack of proper tools, specifically designed for their unique anatomy, and ipi absorption is faster and more efficient than oral [40]. Food (fruits) were offered each night at 6:00 pm (150-200 g each) and leftovers were weighted in the morning, according to Oliveira et al. (2021), to make sure that all animals were fed to satisfaction. Water was available *ad libitum*. The metal concentrations we tested were chosen from previous similar experiments with adult male mice [32–34]. Although these doses were already investigated in testes of murine models, this study is the first to use bats as models, and the results will allow a comparison that will advance the understanding on how much the effects observed for murine models can be extrapolated to bats. This study is the first to use bats as models, and therefore the effects of each metal and relevant concentration are unclear. The same concentrations for all metals were chosen to make comparisons among them possible. After 96h of exposure, bats were euthanized through cervical dislocation followed by decapitation. The liver, kidney, and testes were rapidly removed under ice, divided into fragments, weighed, and portions assigned to the redox status determination were flash frozen in liquid nitrogen until storage at -80°C. The other portion of these organs were assigned to histopathological analysis and were fixed for subsequent investigation.

Redox status determinations

2.3.1. Tissue preparation

Samples were homogenized in 0.2 mol/L phosphate buffer and 1 mmol/L ethylenediaminetetraacetic acid (EDTA) (1:1 and 1.5:1, respectively), pH 7.4, using a tissue homogenizer (OMNI) (Kennesaw, USA). The homogenates were centrifuged at 15,000 g for 10 min at 4 °C prior to the analysis.

2.3.2. Assessment of oxidative and nitrosative stress markers

The homogenate supernatant was used for nitric oxide (NO), superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), ferric reducing ability of plasma (FRAP), malondialdehyde (MDA). An additional assay of protein total was done to standardize the results of CAT, SOD, and MDA. The remaining pellets were used for protein carbonyl assays. All samples were randomly assigned to blind analyses without sample identification until the analysis of the results to avoid bias. All samples were run in duplicates using a spectrophotometer (UV-Mini 1240, Shimadzu, Japan) or a microplate reader (Thermo Scientific, Waltham, USA).

NO production was quantified by the standard Griess reaction. Briefly, 50 μL of supernatants described above were incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-(1-Naphthyl) ethylenediamine and 2.5% phosphoric acid) at room temperature for 10 min [41]. The absorbance was measured at 570 nm in a microplate reader. The conversion of absorbance into micromolar concentrations of NO was obtained from a sodium nitrite (0–100 μmol/L) standard curve and expressed as NO concentrations (μmol/L).

SOD activity was determined by the method based on the reduction of the superoxide (O^{-2}) and hydrogen peroxide, thereby decreasing the auto-oxidation of pyrogallol (Dieterich et al. 2000). The reaction mixture contained 99 μL of potassium phosphate buffer (5 mmol/L, pH 8.0) and 30 μL of sample and was started by adding 15 μL of pyrogallol (100 μmol/L). The final reaction was measured by absorbance at 570 nm. SOD activity was calculated as units per milligram of protein, with one U of SOD defined as the amount that inhibited the rate of pyrogallol autoxidation by 50%. Duplicates of standards and blank samples for SOD activity were prepared with and without pyrogallol, respectively.

CAT activity was determined by adapting the (Hadwan and Abed 2016) method. Briefly, 5 μL samples were incubated with 100 μL hydrogen peroxide (20 mmol/L), and 100 μL of sodium and potassium phosphate pH buffer (50 mmol/L, pH 7.0). After 3 min, the reaction was stopped with 150 μL ammonium molybdate (32.4 mmol/L). A control test without hydrogen peroxide was used to exclude the interference of amino acids and proteins. The reading, at 374 nm, was performed in a spectrophotometer. To calculate CAT activity, a standard curve was built with serial dilutions of hydrogen peroxide. CAT activity was expressed in CAT KU/milligrams of protein.

GST activity was measured using the method of (Habig et al. 1974). Briefly, 1 mmol/L of glutathione-conjugated 1-chloro-2,4-dinitrochlorobenzene (CDNB) was added to the buffer containing 1 mmol/L of GSH and to an aliquot (10 μL) of the homogenate

supernatant. Upon the addition of CDNB, the alteration was monitored through absorbance at 340 nm for 60 s. The molar extinction coefficient used for CDNB was ε340=9.6 mmol/L × cm. One unit of GST activity was defined as the amount of enzyme that catalyzed the formation of one μmol of product/min/mL. GST activity was expressed in μmol/min/g.

The total antioxidant capacity was estimated according to the ferric reducing antioxidant power (FRAP), a method described by Benzie and Strain (1996) using TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) as a substrate. The method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}-TPTZ). Samples (10 μL) were added as FRAP solution (190 μL) of 25mL of acetate buffer (300 mmol/L, pH3.6), 2.5mL of TPTZ reagent (10 mmol/L), and 2.5 $FeCl_3.6H_2O$ solution (20 mmol/L) and the increase in absorbance at 593 nm was measured. The reducing Fe^{3+}-TPTZ reagent by antioxidants was determined by using the standard curve of serial dilutions of $FeSO_4 \cdot 7H_2O$ starting with 1 mmol/L. The results were expressed as FRAP value.

Malondialdehyde (MDA) is the major product of lipid peroxidation. MDA was measured according to Buege and Aust (1978). Briefly, 0.2 mL of the tissue supernatant was homogenized in a solution (0.4 mL) of trichloroacetic acid (15 %)/thiobarbituric acid (0.375 %)/hydrochloric acid (0.6 %). The total reaction mixture was kept in a boiling water bath for 40 min. After cooling on ice, butyl alcohol (0.6 mL) was added, then the solution was vortexed for 2 min and centrifuged for 10 min at 9,000 g. The supernatant was used to measure the absorbance at 535 nm. The concentration of MDA was determined by using the standard curve of known concentrations of 1,1,3,3-tetramethoxypropane (TMPO). The results were expressed as μmol/L per mg protein.

Protein carbonyl (PC) content was measured using the 2,4-dinitrophenylhydrazine (DNPH), according to Levine et al. (1994). The homogenate pellet was added to 0.5 mL of DNPH solution (10 mmol/L) diluted in hydrochloric acid (7 %), vortexed, and kept at room temperature in the dark, shaking periodically for 30 min. Then, 0.5 mL of ice-cold 10% trichloroacetic acid (TCA) was added to each tube, which was centrifuged (5,000 g for 10 min at 4 °C) and the supernatant was discarded. The precipitate was washed three times with 1 mL of ethyl acetate and ethanol (1:1 v/v). Finally, 1 mL of sodium dodecyl sulfate (SDS) 6% was added, the tubes were vortexed and the supernatant was measured through absorbance at 370 nm. The results were expressed as nmol/ mg of protein based on the molar extinction coefficient of ε370=22 mmol/L × cm.

Total protein was determined according to Lowry et al. (1951) using bovine serum albumin (BSA) as a standard. Total protein concentrations were used to standardize CAT, SOD, MDA, and PC results.

2.3.3. Integrated biomarker response (IBR)

To integrate the results from different biomarkers and understand the global response, we calculated the integrated biomarker response (IBR), following the method developed by Devin et al. (2014), using the program CALIBRI (Calculate IBR Interface). The mean (m) and standard deviation (s) of a given biomarker were measured, and the group mean (X) is the mean value for the biomarker for a group. After that, we calculated a standardization for each group to obtain Y:

$Y = (X - m)/s$

Then, instead of transforming each biphasic biomarker into two variables with positive and negative values relating to biomarker inhibition/activation, we used the square form control Y score:

$Z = (Y_{Control} - Y)^2$

The score results (S) are:

$S = Z + |Min|$, where $S \geqslant 0$ and $|Min|$ is the lowest absolute value of Z.

Star plots were then used to display and calculate the integrated biomarker response (IBR), where the IBR is the star plot's total area. As the results for each organ are a set of biomarkers, a ray coordinate of the star chart represents the score of a given biomarker in a given organ. To avoid the strong dependencies on the biomarker arrangement along the star plot, the program uses a permutation procedure leading to $(k-1)!/2$ possible values, where k is the number of biomarkers. The final IBR score is a mean of all IBR values corresponding to every possible order of biomarkers along the star plot.

2.4. Histological analyzes

The left testes, left kidney, and a portion of the liver were fixed in paraformaldehyde 4% for 24 h and transferred to 70% ethanol. Tissue fragments were dehydrated in a growing series of ethanol and embedded in glycol-methacrylate (Historesin®, Leica, Germany). Semi-serial sections (3µm) (12/animal) were made using a rotary microtome (RM 2255, Leica, Germany), with a minimum of 40µm between sections, and stained with toluidine blue/sodium borate (testes) or hematoxylin/eosin (HE) (liver). Morphometry and stereology were performed using 10 digital images per animal, captured with a light microscope

(Olympus BX-60®, Tokyo, Japan) connected to a digital camera (Olympus QColor-3®, Tokyo, Japan).

Liver images were morphometrically analyzed using grids with 266 intersections (Image Pro Plus 4.5® Software, Media Cybernetics, Silver Spring, USA). For stereological analysis, a test system of 266 points was used in a standard test area. In sections stained with HE, points were recorded in liver components (cytoplasm and nucleus of hepatocytes, blood vessels), inflammatory infiltrate, and congestion, with a total of 2660 points/animals.

In the kidneys, images obtained were analyzed morphometrically by counting the intersection of points on glomeruli, renal tubules, and blood vessels, totaling 5320 points per animal. In addition, the radius and glomerular area and the number of glomeruli present in each image were measured. For histopathological analysis, counts of leukocyte infiltration, vascular congestion, and leukocyte marginalization were performed, totaling 2660 points per animal. Analyzes were performed by grids with 266 intersections of the Image Pro Plus 4.5® Software (Media Cybernetics, Silver Spring, USA).

In the testis, the mean tubular diameter was obtained after measuring 30 random circular seminiferous tubule cross sections from each animal, regardless of the tubular stage (200x magnification). The seminiferous epithelium height was measured in the same tubular sections in which the tubule diameter was obtained (as the mean of two diametrically opposed measurements). The diameter of the seminiferous tube was obtained using the formula: Diameter of the lumen = tubular diameter - 2 (height of the epithelium). Analyzes were performed using Image J software (National Institutes of Health). Histopathological evaluation scores were used adapted from (Johnsen 1970) to classify degenerative damage into Normal - intact seminiferous tubules with germ cells in their normal places and few vacuoles; Mild - vacuoles at the base or apex of the epithelium; Moderate - vacuoles at the base and apex; or Severe - tubules with only basal cells or only Sertoli cells.

2.5. Statistical analysis

Data distribution was determined by the Shapiro–Wilk test using the program GraphPad Prism 6.0 (San Diego, CA, USA). All data were submitted to unifactorial one-way analysis of variance (ANOVA), followed by the Tukey post-hoc test for multiple comparisons. When the distribution was not considered normal, the data were submitted to Kruskal–Wallis test followed by Dunn's test. Results are expressed as the mean and standard error of the mean (mean ± SEM). Statistical significance was established at $p<0.05$.

3.0. RESULTS

3.1. Redox status

In the liver, SOD activity increased (χ^2=16.99; p=0.002) in Pb-exposed groups compared to CTL. GST activity decreased ($F_{(4,24)=}$5.63; p=0.002) in Cd (p=0.008), Pb (p=0.003), and Ni (p=0.005) exposed groups compared to CTL. MDA concentration increased ($F_{(4,25)=}$ 2.913; p=0.042) only in Ni-exposed groups (p=0.036). NO, CAT, FRAP, and PC did not differ among the groups (Table 1).

Table 1. Levels of nitric oxide production (NO) (µmol/L), activity of superoxide dismutase (SOD) (U/mg protein), catalase (CAT) (KU/mg protein), glutathione S-transferase (GST) (µmol/min/g), total antioxidant capacity (FRAP) (µmol/L), malondialdehyde (MDA) (µmol/mg protein) and carbonylated protein (PC) (nmol/mg protein) in tissues from *A. lituratus* following 96h of exposure to heavy metals.

		NO	SOD	CAT	GST	FRAP	MDA	PC
Liver	CTL	47.33 ± 8.60	0.92 ± 0.12	235.00 ± 25.68	24.42 ± 2.90	675.40 ± 81.12	0.17 ± 0.03	2.16 ± 0.50
	Cd	28.42 ± 10.500	1.02 ± 0.07	267.60 ± 40.73	**11.19 ± 3.37***	492.70 ± 15.79	0.29 ± 0.05	2.47 ± 0.38
	Cr	38.42 ± 4.41	1.01 ± 0.09	211.4 ± 19.98	14.18 ± 1.01	622.40 ± 58.46	0.31 ± 0.02	1.42 ± 0.05
	Pb	35.76 ± 2.61	0.89 ± 0.07	173.20 ± 35.9	**9.72 ± 1.99***	620.70 ± 58.46	0.28 ± 0.03	2.18 ± 0.72
	Ni	35.47 ± 4.07	**2.40 ± 0.37***	198.40 ± 29.71	**10.89 ± 1.99***	580.60 ± 28.09	**0.33 ± 0.03***	1.34 ± 0.27
Kidneys	CTL	4.39 ± 0.93	2.45 ± 0.30	3.02 ± 0.27	6.81 ± 0.27	432.40 ± 46.88	0.61 ± 0.05	21.82 ± 2.67
	Cd	**12.68 ± 1.23***	2.19 ± 0.15	2.65 ± 0.25	**8.78 ± 0.21***	473.40 ± 43.22	0.64 ± 0.04	25.79 ± 3.17
	Cr	8.37 ± 0.77	2.16 ± 0.16	2.09 ± 0.22	**8.41 ± 0.35***	324.10 ± 37.98	0.64 ± 0.06	26.93 ± 4.22
	Pb	**10.22 ± 0.74***	2.08 ± 0.05	3.13 ± 0.21	**9.01 ± 0.34***	521.30 ± 33.07	0.56 ± 0.04	11.08 ± 1.18
	Ni	**10.46 ± 1.47***	1.87 ± 0.11	2.30 ± 0.16	**8.25 ± 0.36***	318.90 ± 37.83	0.54 ± 0.04	13.52 ± 0.76
Testis	CTL	9.55 ± 1.04	4.85 ± 0.21	315.20 ± 18.41	2.78 ± 0.27	325.40 ± 20.06	2.34 ± 0.48	13.11 ± 1.10

Cd	8.70 ± 1.09	7.54 ± 0.77	428.80 ± 50.20	**1.67 ± 0.25***	252.90 ± 27.36	2.16 ± 0.73	16.44 ± 3.86
Cr	12.86 ± 1.96	6.40 ± 0.90	368.60 ± 55.45	**1.64 ± 0.14***	346.10 ± 19.79	1.69 ± 0.62	22.32 ± 3.86
Pb	6.90 ± 1.06	7.87 ± 0.24	442.70 ± 66.48	**1.62 ± 0.14***	286.40 ± 41.25	3.22 ± 1.19	13.54 ± 1.24
Ni	6.28 ± 1.03	7.66 ± 0.84	419.30 ± 94.21	**1.22 ± 0.14***	207.80 ± 47.54	3.61 ± 1.48	**24.96 ± 2.93***

CTL: control, Cd: Cadmium, Cr: Chromium, Pb: Lead, Ni: Nickel. * *Asterisk* means statistical differences among groups ($P \leq 0.05$). Data are shown as mean ± SEM.

In the kidney, NO content values increased ($F_{(4,24)}$=6.841; p=0.0008) in the Cd (p=0.0004), Pb (p=0.0144), and Ni (p=0.0076) exposed groups compared to CTL. In GST activity all exposed groups Cd (p=0.0032), Cr (p=0.0140), Pb (p=0.0010), and Ni (p=0.0317) increased ($F_{(4,22)}$=6.668; p=0.0011) compared to CTL. SOD, CAT, FRAP, and PC did not differ among the groups.

In the testes, GST activity decreased ($F_{(4,24)}$=7.959; p<0.001) in Cd (p=0.007), Cr (p=0.006), Pb (p=0.007) and Ni (p<0.001) exposed groups compared to CTL. PC concentration increased ($F_{(4,25)}$=3.928; p=0.013) in Ni (p=0.029) exposed group and the other parameters did not differ among groups.

3.2. Integrated biomarker response (IBR)

The IBR star plots of the liver, kidney, and testes were shown (Figure S1).

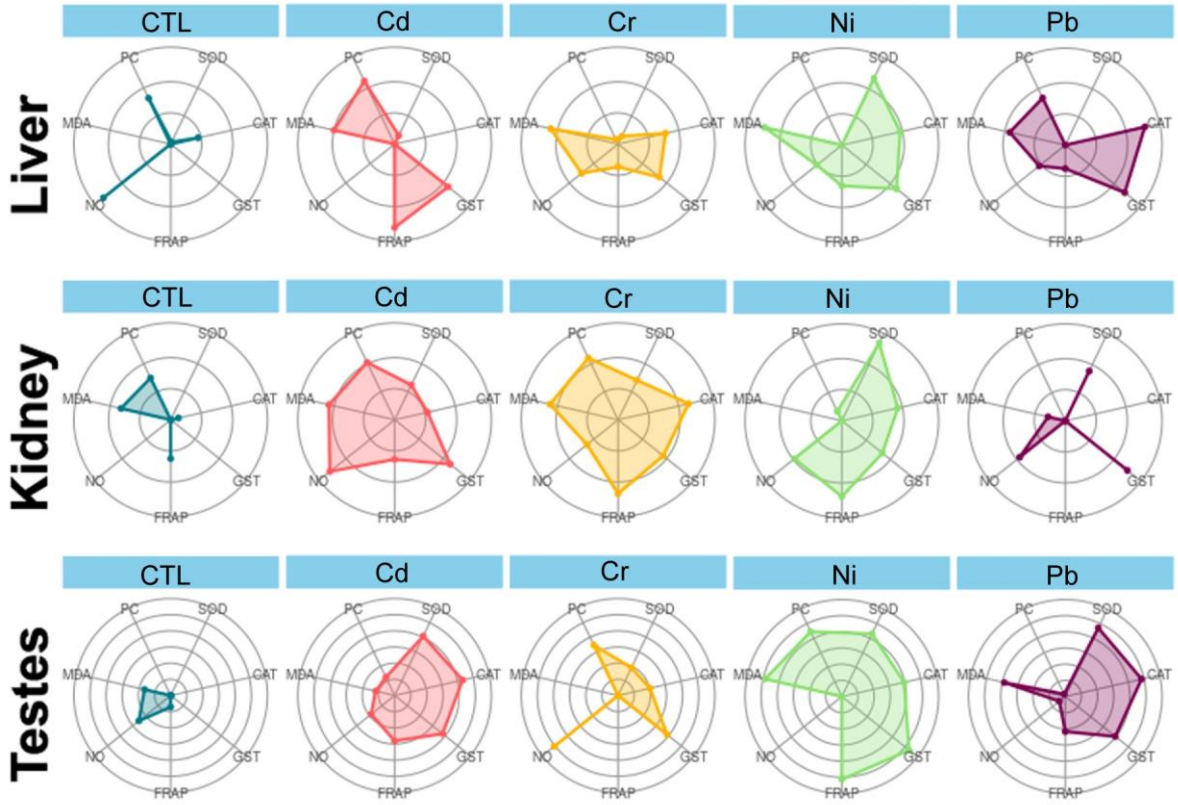


Figure S1. Star plot of tissues exposed to heavy metals. The total area of star plots represents the value of the IBR index.

All groups exposed to HMs in the liver and testes had a rise in global damage. In the kidney, the groups exposed to Cd, Cr, and Ni had global damage increased when compared to the CTL group.

3.3. Histological analyses

Liver histological analyses did not show any differences in the percentage of the liver nucleus, cytoplasm, blood vessels, and vascular congestion compared to the control. However, we found an increase ($F_{(4.17)}$=3.635, p=0.026) in leukocyte infiltrates in Cr (p=0.018), fatty foci were observed in Pb, and areas of necrosis were observed in Pb and Ni exposed groups compared to control (Table 2, Figure 1).

Table 2. Liver morphometric histological parameters from *A. lituratus* following 96h of exposure to heavy metals.

	Treatments				
	CTL	Cd	Cr	Pb	Ni
Nucleus (%)	8.16 ± 0.82	9.62 ± 0.55	9.94 ± 0.80	9.01 ± 3.81	9.96 ± 0.90

Cytoplasm (%)	75.05 ± 1.34	73.01 ± 2.11	69.59 ± 1.58	76.90 ± 2.28	67.63 ± 2.37
Blood vessels (%)	15.53 ± 0.18	18.76 ± 2.47	14.32 ± 0.04	13.61 ± 1.30	20.29 ± 1.07
Vascular congestion (%)	8.47 ± 1.21	5.94 ± 1.16	8.61 ± 0.73	12.35 ± 1.21	7.91 ± 0.75
Leukocyte infiltrate (%)	0.00 ± 0.00	0.45 ± 0.18	**1.38 ± 0.48***	0.92 ± 0.30	0.54 ± 0.17

CTL: control, Cd: Cadmium, Cr: Chromium, Pb: Lead, Ni: Nickel. * *Asterisk* means statistical differences among groups ($P \leq 0.05$). Data are shown as mean ± SEM.

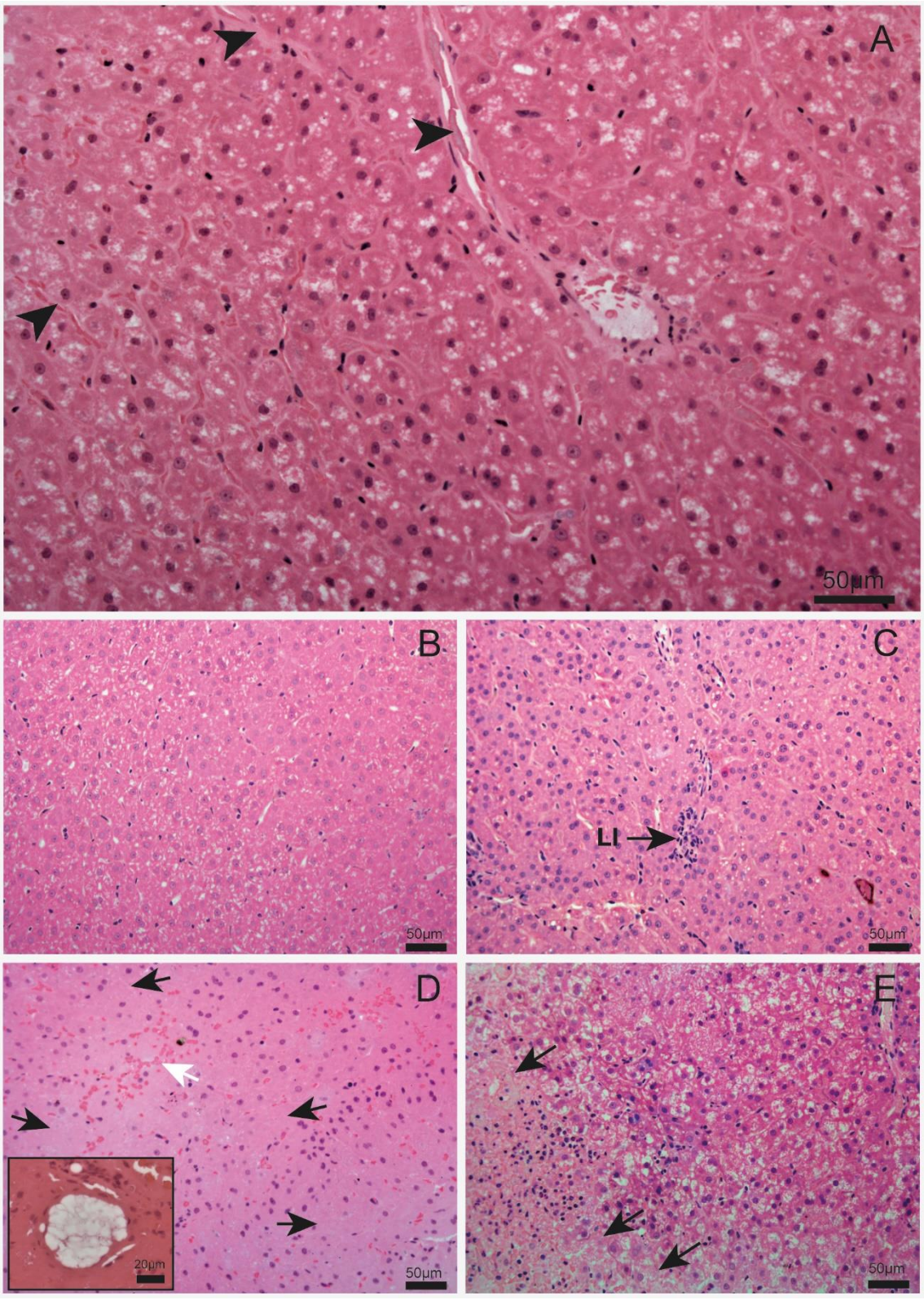


Figure 1. (A-E) Liver sections from *A. lituratus* from the following treatment groups: A) CTL: Control; B) Cd: Cadmium; C) Cr: Chromium; D) Pb: Lead; E) Ni: Nickel (HE staining, 20× objective lens). The highlighted image in D indicates a fatty focci (40× objective lens). Black arrowhead: blood vessels; LI: Leukocyte infiltrate; Black arrow: Necrotic area; White arrow: hemorrhage.

Histomorphometry parameters showed a decrease in capsular space (X^2=17.95, p=0.0013) in Cd (p=0.0073) and Ni (p=0.0019), an increase in the tubular epithelium ($F_{(4.24)}$=5.688, p=0.0023) in Pb (p=0.0171), an increase in blood vessels ($F_{(4.24)}$=15.17, p<0.0001) in Cd (p=0.0005), Cr (p<0.0001) and Pb (p<0.0001) and glomerulus radius ($F_{(4.25)}$=10.15, p<0.0001) in all groups (Cd: p=0.0076, Cr: p=0.0261, Pb: p=0.0004, Ni: p<0.0001), also in glomerulus area ($F_{(4.24)}$=6.181, p=0.0014) in Pb (p=0.0294) and Ni (p=0.0008). In the histopathological parameters, there was an increase ($F_{(4.25)}$=5.398, p=0.0028) in vascular congestion in Ni (p=0.0019) and an increase ($F_{(4.24)}$=3.466, p=0.0227) in leukocyte infiltrate in Cd (p=0.0106) (Table 3; Figure 2).

Table 3. Kidneys histological parameters from *A. lituratus* following 96h of exposure to heavy metals.

	Treatments				
	CTL	Cd	Cr	Pb	Ni
Glomerulus (%)	2.95 ± 0.17	3.62 ± 0.11	4.45 ± 0.39	4.01 ± 0.52	4.31 ± 0.50
Capsular space (%)	0.95 ± 0.20	**0.16 ± 0.05***	0.35 ± 0.05	0.28 ± 0.08	**0.13 ± 0.03***
Tubular epithelium (%)	84.74 ± 0.29	86.24 ± 0.21	85.37 ± 0.92	**87.72 ± 0.56***	84.51 ± 0.36
Tubular lumen (%)	3.83 ± 0.36	4.09 ± 0.15	3.75 ± 0.15	3.53 ± 0.26	4.37 ± 0.17
Blood vessels (%)	7.81 ± 0.47	**5.89 ± 0.12***	**5.43 ± 0.20***	**5.10 ± 0.12***	6.71 ± 0.30
Glomerulus radius (μm)	0.28 ± 0.01	**0.35 ± 0.01***	**0.34 ± 0.02***	**0.37 ± 0.01***	**0.39 ± 0.01***
Glomerulus área (μm²)	27.48 ± 1.37	33.76 ± 1.89	30.99 ± 1.53	**35.26 ± 1.51***	**38.9 ± 2.24***
Vascular congestion (%)	5.93E-03 ± 5.51E-04	1.07E-02 ± 1.15E-03	1.48E-02 ± 2.58E-03	9.82E-03 ± 2.28E-03	**1.96E-02 ± 3.41E-03***
Leukocyte infiltrate (%)	4.90E-03 ± 1.40E-03	**1.36E-02 ± 1.75E-03***	7.66E-03 ± 1.66E-03	9.58E-03 ± 2.03E-03	9.18E-03 ± 1.64E-03

CTL: control, Cd: Cadmium, Cr: Chromium, Pb: Lead, Ni: Nickel. * *Asterisk* means statistical differences among groups ($P \leq 0.05$). Data are shown as mean ± SEM.

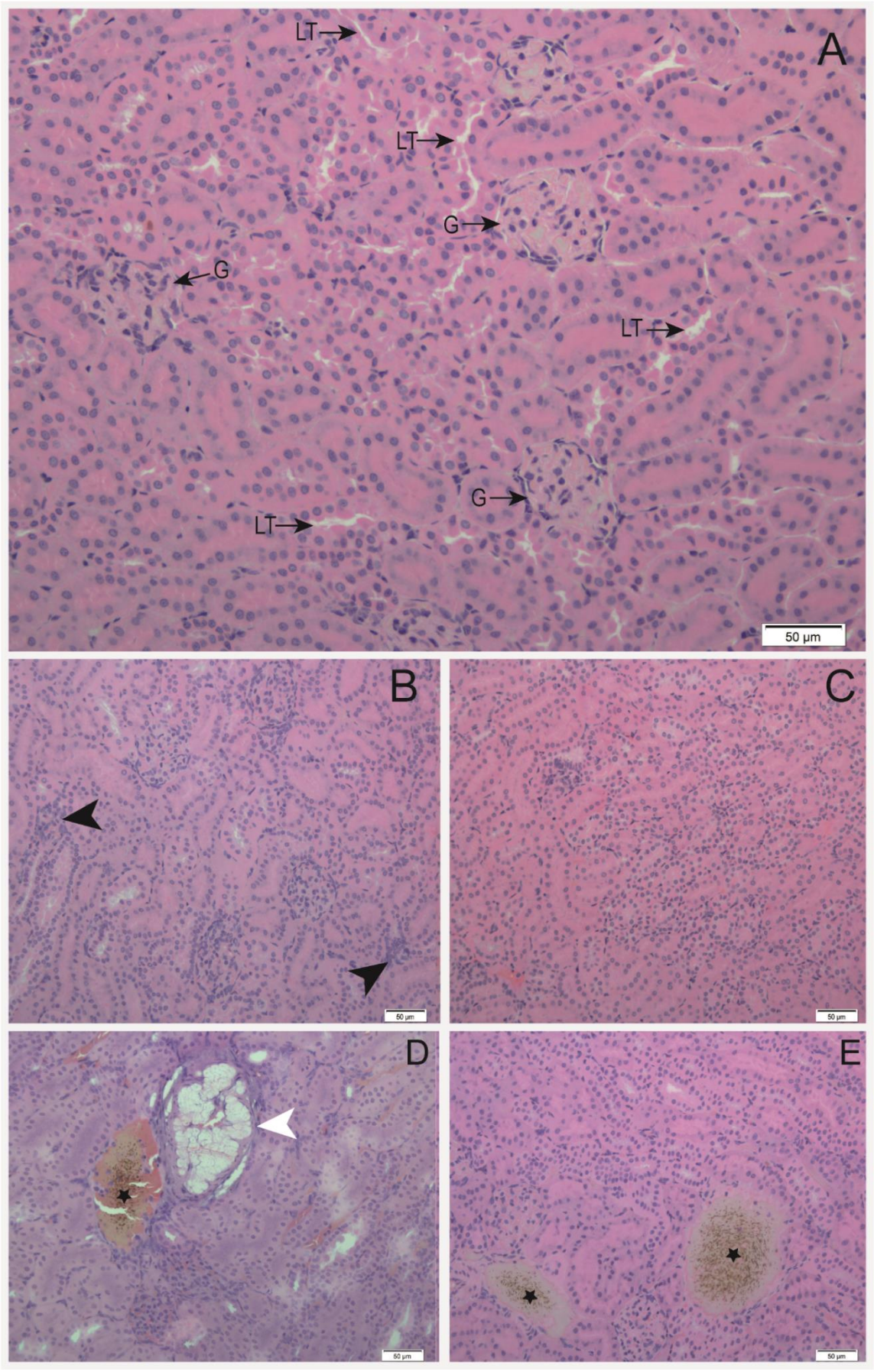

A
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Figure. 2. Kidney sections, the proportion of histopathological components in the kidney of *A. lituratus* from groups after 96h to (A) CTL: Control, (B) Cd: Cadmium (C) Cr: Chromium (D) Pb: Lead (E) Ni: Nickel. G: Glomerulus LT: Tubular lumen Black arrowhead: Leukocyte infiltrates White arrowhead: fatty focci Star: Vascular congestion (Hematoxylin and Eosin staining, 20× objective lens).

In the testes, we found an increase in luminal diameter ($F_{(4.18)}$=4.388, p=0.0119) in Cr-exposed bats (p=0.0171) compared to the control. We also found a decrease ($F_{(4.20)}$=12.16, p<0.0001) in normal cells in all groups and an increase in vacuoles at the base ($F_{(4.19)}$=7.646, p=0.0008) and at the apex and base ($F_{(4.20)}$=3.675, p=0.0212) in Cr-exposed bats compared to control (p=0011 and p=0.0150, respectively). As for the sum of mild histopathology, there was an increase ($F_{(4.19)}$=4.556, p=0.0095) in Cr (p=0.0057) and Ni (p=0.0448) exposed groups bats and an increase in moderate histopathologies in Cr animals (p=0.0147) compared to control (Table 4, Figure 3).

Table 4. Testes histological parameters from *A. lituratus* following 96h of exposure to heavy metals.

	Treatments				
	CTL	Cd	Cr	Pb	Ni
Tubular diameter (μm)	119.40 ± 6.90	147.90 ± 4.36	139.40 ± 15.67	118.90 ± 8.72	120.30 ± 0.81
Luminal diameter (μm)	20.46 ± 2.90	22.42 ± 3.39	**35.35 ± 4.08***	22.00 ± 1.39	29.58 ± 3.10
Epithelium height (μm)	49.5 ± 2.35	59.68 ± 4.27	52.00 ± 6.17	49.16 ± 0.85	44.66 ± 3.09
Normal cells (%)	90.06 ± 1.27	**48.40 ± 7.42***	**32.20 ± 5.21***	**29.40 ± 11.19***	**36.10 ± 6.95***
Vacuole at the apex (%)	9.74 ± 1.22	25.10 ± 6.24	24.30 ± 6.46	29.00 ± 6.72	28.80 ± 8.33
Vacuole at the base (%)	0.00 ± 0.00	6.30 ± 1.94	**24.30 ± 6.46***	2.30 ± 1.28	10.50 ± 2.80
Vacuole at the apex and base (%)	0.00 ± 0.00	15.30 ± 5.47	**31.80 ± 11.01***	7.80 ± 2.19	20.30 ± 6.61
Epithelial desquamation (%)	0.00 ± 0.00	1.20 ± 0.87	0.00 ± 0.00	1.63 ± 0.94	0.60 ± 0.37
Seminiferous tubules with only basal cells (%)	0.00 ± 0.00	3.70 ± 2.24	2.40 ± 0.89	18.60 ±11.12	3.10 ± 2.04
Seminiferous tubules with only Sertoli cells (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	6.80 ± 4.45	0.60 ± 0.40

CTL: control. Cd: Cadmium. Cr: Chromium. Pb: Lead. Ni: Nickel. *Asterisk means statistical differences relative to CTL groups ($P \leq 0.05$). Data are shown as mean ± SEM.

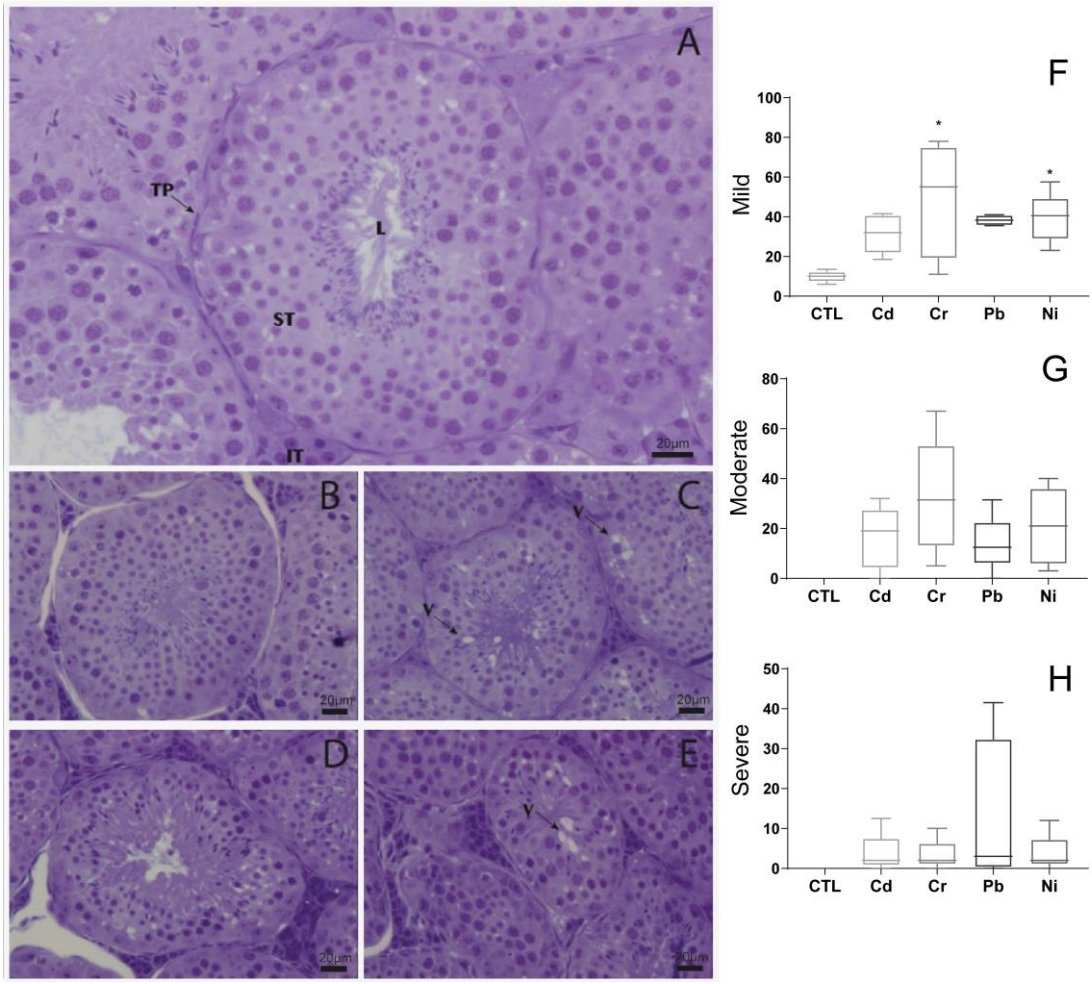

Figure 3. (A-E) Testis sections from *A. lituratus* from the following treatment groups: A) CTL: Control, B) Cd: Cadmium C) Cr: Chromium D) Pb: Lead E) Ni: Nickel (Toluidine Blue staining, 40× objective lens). TP: Tunica propria; L: Lumen; ST: Seminiferous tubules; IT: Intertubule; V: Vacuole regions. (F-H) Johnsen's (1970) histopathological evaluation score for degenerative damage in *A. lituratus*. *Asterisk means statistical differences relative to CTL groups ($P \leq 0.05$). The data shown represent the median and interquartile range.

4.0. DISCUSSION

Although some metals are essential to physiological processes in animals, excessive environmental concentrations through anthropic activities may potentially impair reproduction and other functions that could lead to decreases in wildlife populations in the long term (Karri et al. 2016). The concentrations analyzed in this study are lower than the concentrations found in insectivorous bats living in coal mining areas [52] and lower than those found in tropical basin sediments in Brazil, so they are considered low concentrations [21]. This is the first study evaluating the isolated effect of heavy metals on wild bats.

Liver alterations

The analysis of IBR in the liver demonstrates that exposure to all metals harms the organ even in low concentrations. Environmental studies showed that insectivorous bats living in coal mining areas can bioaccumulate higher metal concentrations in the liver than those analyzed in our study, such as 5.8 – 7.32 of Pb, 5.7 – 10.9 of Cr, 3.6 – 4.05 of Cd and 4.3 – 8.6 of Ni (mg/kg) (Zocche et al., 2010). In the liver, antioxidant enzymes were altered by Cd, Ni, and Pb, which affected SOD and GST differently (Ni increased SOD activity, while Cd, Pb, and Ni decreased GST activity). Ni is considered an essential element in plants and bacteria, and possibly in animals, because its deficiency seems to affect the metabolic processes, increasing total lipids in the liver and decreasing phospholipids [53]. Here, Ni exposure increased SOD activity, which may be associated with hydrogen peroxide (H_2O_2) overproduction [54]. Excessive H_2O_2 formation would further activate CAT and Glutathione peroxidase (GPx). In our study, there was no increase in CAT activity following Ni exposure. GST activity decreased, and that may indicate this enzyme could no longer protect the cells from lipid peroxidation [55], corroborating the increased MDA observed in this group. Decreasing GST activity following Cd, Ni, and Pb exposure might be explained by this metal's ability to bind to thiol (-SH) groups on proteins and make them inactive, thus decreasing the activity of thiol-containing antioxidants, such as reduced glutathione (GSH) [56], we suggest that this decreased activity of GSH also results in GST activity decreases since GST catalyzes the conjugation of GSH to toxics electrophiles [57]. Corroborating this, a decrease in GSH activity and sulfhydryl content was observed in lungs from *Artibeus lituratus* collected in a mining area [58]. Another factor could be the alteration in the Nrf2 pathway that initiates the transcription of other target genes that encode proteins responsible for ROS detoxification. Some metals can affect this pathway, such as Pb, Ni and Cr [59]

Although morphometrical analysis showed alteration, we observed increased leukocyte infiltrate in the liver of Cr-exposed bats, indicating inflammation. In murine models, exposure to higher Cr concentrations for 1-7 days induced pro-inflammatory cytokines and chemokines the release, which may result in the recruitment of inflammatory cells [60]. In our study, histological hepatic images showed necrotic areas and hemorrhage in Ni-exposed animals, and fat deposits and necrosis in the Pb-exposed group. Lipid degeneration in Pb-exposed rats has been previously documented, when animals were treated through oral ingestion at a concentration of 20 mg/kg for 4 weeks, higher than tested in this study [61].

Kidney alterations

The global analysis of the kidney showed an unexpected result the amount of Pb used in the study was not harmful to the kidney. However, the exposure to other metals (Cr, Cd, and Ni) was toxic. The antioxidant enzymes were influenced by Cd, Cr, Ni, and Pb, in different ways. NO increased in all exposed groups. In fact, low levels of Pb in chronic exposure led to an increase in blood pressure, and eNOS/iNOS in the kidney, also, a reduction in urinary NO excretion [62]. NO has several functions in the kidneys as regulation of renal hemodynamics and tubuloglomerular feedback [63]. NO produced by the macula densa in the kidneys inhibits tubular sodium reuptake, resulting in increased urinary excretion and water and solutes [62]. This can contribute to the excretion of toxic compounds. NO reacts with superoxide radical (O^{2-}) and produces peroxynitrite ($ONOOH^{-}$) which can produce hydroxyl radicals ($HO^{\bullet}$) in the presence of hydrogen ions [37], therefore, an increase in NO without a sufficient increase in antioxidant enzymes can lead to cellular oxidative stress. Besides that, levels of the CAT and SOD activity were not observed, only in GST for all groups. In the kidney, GST activity increased, unlike in other tissues where GST activity decreased which may indicate an effort by the kidney to detoxify heavy metals, a defense mechanism, since GST is a non-enzymatic detoxification defense[64]. In fact, Sprague Dawley rats that received 114 mg of lead acetate per kilogram of body weight (i.p.) exhibited a time-dependent decrease in liver GST and an increase in kidney GST [65].

In kidney histology, we found damage although does not indicate damage to MDA and PC. This is worrisome as the kidney is the main organ of detox [66]. The narrowing of the capsular space of the kidneys caused by Ni has already been observed in chronic and subchronic exposure at dosages higher than those tested in our studies, from 7.5 to 30mg/kg. One possible reason for the decrease in Capsular space is a response to a decrease in blood pressure indicated by increased vascular congestion. As for the increase in the tubular epithelium observed for the groups exposed to Pb, a possible explanation would be the unbalance of the Ca^{2+} homeostasis, which can cause cell damage and produce ROS. A study with primary cultures of rat proximal tubular cells (rPT) suggested that Pb increases the cytosolic and mitochondrial concentration of Ca^{2+} and depletes the endoplasmic reticulum (ER) [67] which may affect the tubular epithelial cells. The narrowing of blood vessels found in all groups except Ni may indicate an attempt to avoid contamination of the kidneys by metals. The glomerulus enlargement in the same groups may represent a response to this blood deprivation. The increase in leukocyte infiltrate observed in the group exposed to Cd demonstrates that this metal can induce a faster response to inflammation in the kidneys. The

kidneys of bats collected in an area of pollution showed an increase in infiltrates and necrosis, compared to the control area [68]. We did not find tissue necrosis, but some bats from the Pb group had large foci of fat as we found in liver histology, which is a severe pathology.

Testes alterations

The IBR index for testes showed that all HMs presented a global increase in damage levels, highlighting the Ni that doubled compared to the other metals. Regarding the antioxidant defense, only GST was affected by HM exposure in the testes, decreasing in all exposed groups. GSTs are a family of antioxidant isoenzymes that participate in the cellular detoxification of several xenobiotics, and the inhibitory effects of metals in GST activity may be harmful to the cell [69]. Several studies showed decreased GST activities following HM exposure in testes of mammals, such as decreased GST after 20mg/kg of Pb (ipi) for 5 days in rats (Ahmed E. Abdel Moniem and Al-Quraishy 2010), after 0.025mg/kg of Pb and Cd (ipi) for 15 days in rats [71] and after a single dose of 3.58 mg/kg of Cd and 59.5 mg/kg of Ni (ipi) in rats [72]. Differently from our study, chronic exposure to the same concentrations and the same metals did not show alterations in GST in testes, but Cr and Pb had alterations in SOD [32]. Also, this same study found alterations in PC for Ni-exposure as ours [32], reinforcing our results that Ni is one of the most harmful metals, principally to the testis. Protein carbonylation is an irreversible post-translational modification caused by excessive ROS formation, considered a major hallmark of oxidative stress disorders [73]. Ni exposure also showed higher mild histopathologies. Cr exposure induced moderate histopathologies in the testes, increasing lumen diameter, indicative of tubular damage. Furthermore, all metals induced a decrease in normal tubular cells. That can compromise the detoxification ability of bats, as well as their excretion capacity. Other authors suggested Ni> Cd> Cr > Pb for the male reproductive toxicity order in mice [32]. Here, for bats, we are proposing Ni> Pb> Cd> Cr male reproductive toxicity. Studies in rats showed that the other metals (Ni, Pb, and Cd) also induced reproductive toxicity at higher concentrations and/or exposure times [7,15,74].

5.0. CONCLUSION

Taken together, our results draw attention to oxidative and tissue damage from heavy metals exposure in fruit bats, even under a short exposure time. Our results indicate that bats may be susceptible to the effects of heavy metals, and the liver seems to be a more sensitive tissue, due to the severe histological damage, including necrosis and lipidic area, found in this tissue. In addition, we propose the following order of metal toxicity, based on the degree of oxidative and histological damage: Ni> Pb> Cr= Cd. Ni-exposed animals showed the highest

IBR in the liver and testes, where they showed oxidative damage, also showed histological damage in the kidneys and necrosis area in the liver. Considering that, the most worrisome metal was Ni and must be a priority in pollutant mitigation plans, such as bioremediation. Also, findings of higher levels of protein carbonyl and vacuolization in testes demonstrate that Cr, constantly released into the environment, may affect the reproductive capacity of bats and therefore their ecological contribution. More studies with different concentrations and exposure times are needed to better assess other toxicological effects. Assessing and monitoring bat populations in highly contaminated areas are critical to better understanding the damage caused by environmental pollution on key ecological species.

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3. Capítulo 3:

Iron and aluminum ore mining pollution induce oxidative and tissue damage on fruit-eating bats from the Atlantic Forest

Ana Luiza Fonseca Destro[1], Déborah Cardoso Gonçalves[1], Thaís Silva Alves[1], Kemilli Pio Gregório[1], Vinicius Melo da Silva[2], Victor Rodrigues Santos[3], Olagide Wagner de Castro[4], Hernando Baggio Filho[5], Guilherme Siniciato Terra Garbino[1], Reggiani Vilela Gonçalves[1], Jerusa Maria de Oliveira[6], Mariella Bontempo Freitas[1]

[1] Department of Animal Biology, Federal University of Viçosa, Viçosa, MG, Brazil

[2] Department of General Biology, Federal University of Viçosa, Viçosa, MG, Brazil

[3] Department of Morphology, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil

[4] Department of Physiology, Federal University of Alagoas, Maceió, AL, Brazil

[5]Department of Geography, Federal University of the Jequitinhonha and Mucuri Valleys, MG, Brazil

[6]Rede Nordeste de Biotecnologia (RENORBIO), Institute of Chemistry and Biotechnology, Federal University of Alagoas, Maceió, AL, Brazil

Correspondence:

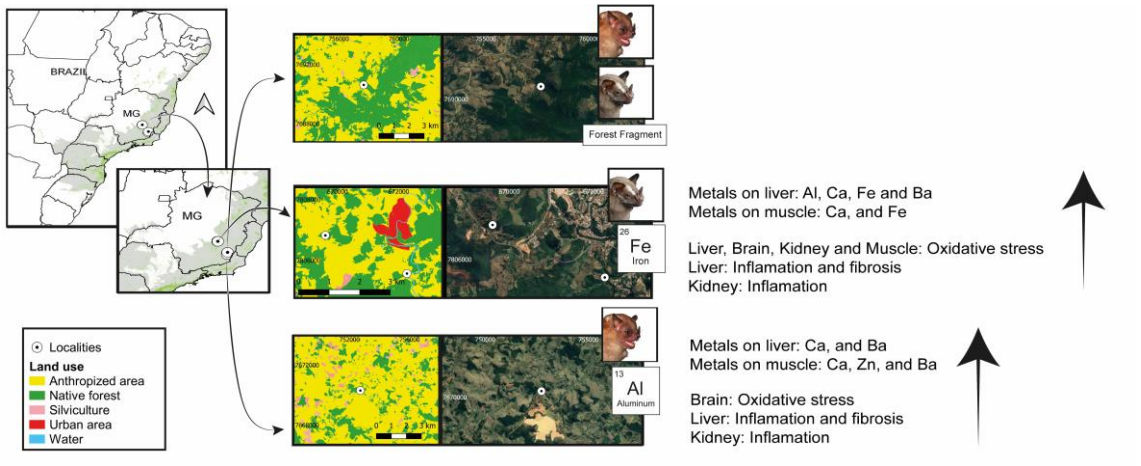
Ana Luiza Destro, Departament of Animal Biology, Federal University of Viçosa, Viçosa Minas Gerais, 36570-900, Brazil.

Email: nina.destro@gmail.com

Abstract

Neotropical fruit-eating bats are important seed dispersers and pollinators for a variety of tropical plants and are crucial for forest ecosystem restoration. A major threat to bat populations is habitat loss, and metal contamination due to mining activities is thought to be involved. We evaluated the effect of mineral exploration areas in two key species (*Artibeus lituratus* and *Sturnira lilium*) collected from an iron ore area (FeOA) or an aluminum ore area (AlOA), respectively, and compared to individuals from a preserved Atlantic Forest fragment (FFA). Bats from FEOA showed higher Aluminum (Al), Calcium (Ca), Iron (Fe) and Barium (Ba) liver accumulation, Ca and Fe muscle accumulation. These animals also showed higher liver, brain, muscle, and kidneys oxidative damage, liver fibrosis and kidneys inflammation. Bats from ALOA showed higher Ca and Ba liver accumulation and Ca, Zinc (Zn), and Ba muscle accumulation, along with higher brain oxidative stress, liver fibrosis, and kidneys inflammation. Our findings indicate that iron ore mining activities along fragments of the Atlantic Forest had a higher impact on fruit-eating bats, impairing bats' redox balance and inducing extensive tissue damage, which may be a potential threat to these crucial bat populations, considering their long-term exposure in the field.

Keywords: oxidative stress, metal accumulation, ecotoxicology, histopathology



HIGHLIGHTS

Artibeus lituratus from an iron ore mining area showed liver oxidative stress and fibrosis.

Sturnira lilium from an aluminum ore mining area showed liver fibrosis and kidneys inflammation.

Fruit-eating bats from mining areas along the Atlantic Forest showed metal bioaccumulation on the liver and muscle.

Iron mining areas had a higher impact on fruit-eating bats than aluminum mining ones.

1.0. INTRODUCTION

Bats are important mammals with a critical role in the ecosystem [1]. Bat colonies reach millions of individuals, and they provide various ecological and financial services due to their diverse eating habits [2]. As insectivorous species are crucial in controlling invertebrate pests in agriculture [3,4], fruit-eating bats are important seed dispersers and pollinators, playing a relevant role in the reforestation of degraded areas [5,6]. Fruit-eating bats are one of the most abundant seed dispersers in Neotropical forests [7]. The great fruit-eating bat (*Artibeus lituratus*) and the little yellow-shouldered bat (*Sturnira lilium)* are commonly found in the Atlantic Forest, the second-largest rainforest in South America [8]. This ecosystem is considered a hotspot for biodiversity conservation due to its elevated species richness, endemism, and a high degree of fragmentation [9,10], and in spite of that, the Atlantic Forest is one of the most threatened ecosystems on the planet, and most devastated as well [11]. In forest environments, the smaller species, *S. lilium*, consumes pioneer plant species and has a large foraging area, facilitating natural forest regeneration [12]. The larger species, *A. lituratus*, also has an important role in the maintenance and recovery of forest fragments, as they also disperse pioneer plant seeds [7,13]. In fact, *A. lituratus* colonizes restored areas in the initial stage of restoration, unlike other seed dispersal animals with limited colonization capacity [7]. This species is also showing promissory results through the use of fruit essential oils to attract more specimens and increase seed dispersal in degraded areas, reinforcing this as a key species associated with reforestation roles [14].

Longevity, diverse eating habits, and large home ranges are among the factors that make bats more susceptive to environmental pollutants such as metals and metalloids [15]. Several studies have reported metal accumulation in bats, particularly in insectivorous species [16–18]. Although important, these studies do not focus on the potentially adverse ecotoxicological effects of these contaminants in bats, as most studies are restricted to quantifying the metal's bioaccumulation. Investigating the physiological effects of their accumulation in tissues is essential, since many metal-binding proteins such as metallothioneins and glutathiones may not reflect the actual metals harmful effects [19]. In addition, published studies have focused on insectivorous bats, while frugivorous species have received less attention [20]. Routes of exposure for fruit-eating bats contamination include the oral, respiratory, and skin contact pathways since metals occurs naturally in all environments. In the last few decades, metals concentrations in the air, water, and soil have

increased due to anthropogenic sources such as combustion, extraction, and industrial activities [21,22], and consequently, wildlife has been exposed to higher concentrations. Insectivorous bats collected in metals-polluted areas showed metal bioaccumulation [23], DNA damage [24], oxidative stress in blood [25], lungs, and emphysema [26]. The few studies investigating metal toxicity in fruit-eating bats were focused on flying foxes (family Pteropodidae) [27–29], showing that flying foxes can accumulate metals and can cause even pulmonary, liver, and renal histopathologies. We aimed at investigating metals tissue concentration, oxidative stress, and histopathological damage on fruit bats from iron ore and aluminum ore mining areas. This is the first study to investigate two bat species crucial to the Atlantic Forest restoration as models on studies of iron and aluminum mining areas´ impacts.

2.0. MATERIAL AND METHODS

2.1. Study site

For this study, we chose two areas neighboring an iron ore (Fe) (19º49'38" S, 43º22'50" W) and an Aluminum (Al) ore mining site (21º06’06”S 42º27’39”W), and compared to a third, a well-preserved Atlantic Forest fragment area (21°02'02.4"S, 42°35'05.4"W), without any mining activity. The iron ore area (FEOA) is 149km from the Forest Fragment (FFA), and the Aluminum ore area (ALOA) is 23km from the FFA. The areas FEOA and ALOA was in particular propriety inside the mining industry area and circled by anthropized area such as agriculture and pasture. Near the areas, also had silviculture, *i.e*. eucalyptus plantation. We collected water samples from the three sites for metal analysis. All areas are in southeastern Brazil, in the state of Minas Gerais (Figure 1).

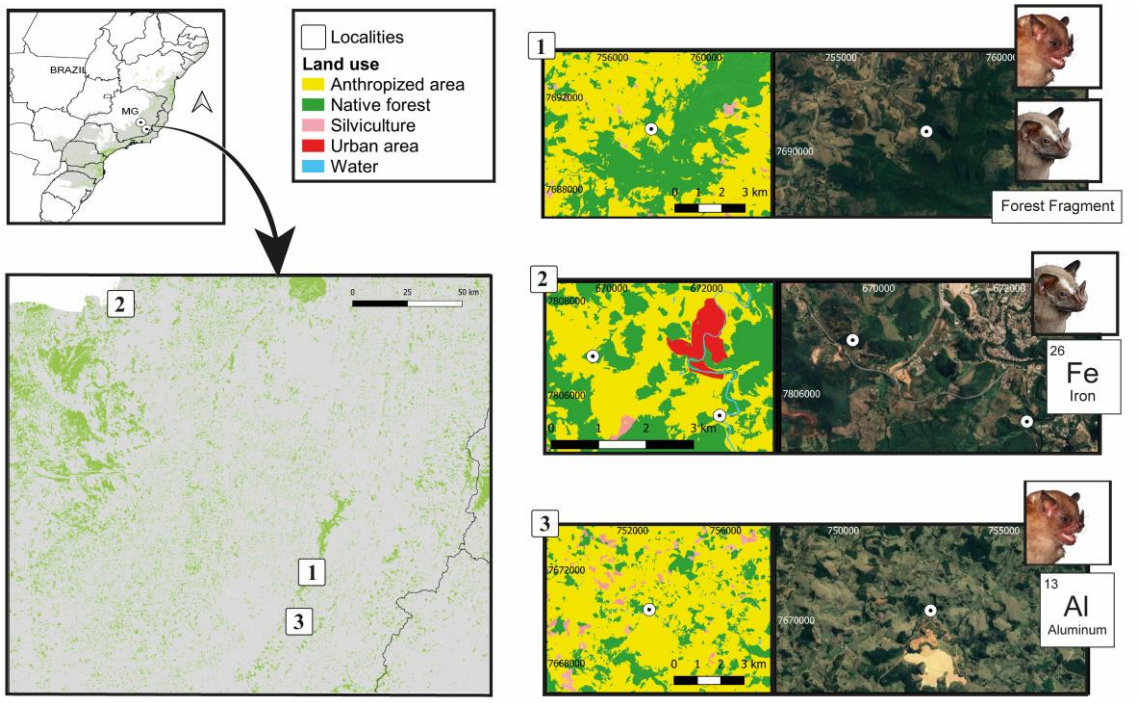


Fig 1. Locations in the state of Minas Gerais (MG) - Brazil that was chosen for collection and the use of the area of the respective locations. Fe: Fe ore area where the FEOA group was collected. Al: Aluminum area where the ALOA group was collected. Control: Atlantic Forest area without mining where the FFA group was collected.

2.2. Animals

A total of 32 adult female and male bats were captured in all three areas. Bats were captured with mist nets set at ground level. At the iron ore area (FEOA, n=8) we collected bats from the species *Artibeus lituratus,* and at the aluminum ore area (ALOA, n=8) we collected bats from the species *Sturnira lilium*. The species selection was based on the abundance of them in each experimental site. In the area free of mining activities, the Atlantic Forest area (FFA, n=16), we collected bats from both species (N=8 each) since they were equally abundantly available. Individuals were identified according to an identification key of South American bats [30]. The bats were euthanized through cervical dislocation and the organs were removed. The liver, breast muscle and brain were removed and divided into two parts. One portion of these organs and one entire kidneys were immediately stored in liquid nitrogen and then taken to the laboratory where they were stored in an ultra-freezer at -80°C for oxidative stress analysis and metals quantification. The other part of the organs and the other kidneys were fixed, as described below, for subsequent histological analyses. Only *A. lituratus* kidneys were analyzed for the kidneys' redox status. The rest of the analyses and organs were entirely performed for the two species and areas collected.

Permits to capture and handle the animals were provided by the Chico Mendes Institute for Environment Conservation (SISBIO 77322-1). Procedures to capture and collect

the specimens followed the latest guidelines for the use of wild mammal species in research [31] and were approved by the Animal Ethics Committee of the Federal University of Viçosa (CEUA-UFV 10/2021).

2.3. Metal Analyses

Liver and muscle samples (~1g) were dried in an oven until a constant mass was obtained. The dry mass was mineralized in 1.5 mL of a nitric-perchloric acid mixture (2: 1) until the complete removal of organic matter and the extract was resuspended in 10 mL of distilled water and used to determine the concentrations of Mg, Al, Ca, Cr, Fe, Co, Cu, Cd, Ti, Mn, Ni, Zn, Ba, and Pb by Inductively Coupled Plasma Optical Emission Spectrometry (Spectro Analytical Instruments). We also collected water samples from the small lakes and streams in the studied area (where the mist nets were set up). The water was filtered and subjected to the same analyses.

2.4. Oxidative biomarkers and antioxidants enzymes

2.4.1. Tissue preparation

Samples of the pectoral muscle, liver, kidneys (0,1g each), and brain (0,15g) were homogenized in 0.2 mol/L pH 7.4 phosphate buffer (1mL), 1 mmol/L ethylenediaminetetraacetic acid (EDTA) using a homogenizer (OMNI). The homogenates were centrifuged at 15,000 g for 10 min at 4 °C and the supernatants were used for the analysis of the enzymatic activity of superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) and ferric reducing ability of plasma (FRAP) and content of malondialdehyde (MDA), nitric oxide (NO) and total protein (TP) assays. The resulting pellets were used for analyses of carbonyl protein (CP) content.

2.4.2. Superoxide dismutase activity

The activity of SOD was determined according to Dieterich et al, 2000 [32]. Briefly, 99 µL of potassium phosphate buffer (5 mmole/L, pH 8.0) was pipetted with 30 µL of sample and 15 µL of pyrogallol (100 µmole/L). The reaction mixture was measured by absorbance at 570 nm. SOD activity was calculated as units per milligram of protein, with one U of SOD defined as the amount that inhibited the rate of pyrogallol autoxidation by 50%. Duplicates of white and standards for SOD activity were prepared without and with pyrogallol, respectively.

2.4.3. Catalase activity

Catalase activity (CAT) was determined by adapting Hadwan's method[33] using hydrogen peroxide (H_2O_2) as substrate. Briefly, 100 µL hydrogen peroxide (20 mmol/L) was pipetted into 5 µL samples. After 3 min 150 µL ammonium molybdate (32.4 mmol/L) was

added to stop the reaction. The white samples were made by replacing hydrogen peroxide with sodium and potassium phosphate pH buffer (50 mmol/L, pH 7.0). The reading was at 374 nm in the spectrophotometer. A standard curve was made to calculate the CAT value with the serial dilution of H_2O_2. The test values were subtracted from the control test values to eliminate interference from other serum compounds that may react with the mol ammonium molybdate and used to obtain the concentration from the curve. CAT activity was expressed in CAT KU/per milligram of protein.

2.4.4. Glutathione S-transferase activity

GST activity was measured using the method of Habig et al, 1974 [34]. Briefly, 10 µL of glutathione-conjugated 2,4-dinitrochlorobenzene (CDNB) (1 mmol/L) was added to the 970 µL buffer (50 mmol/L, pH 7.0) with 10 µL of GSH (1 mmol/L) and the aliquot 10 µL of the sample to be tested. Upon the addition of CDNB, the change was monitored with absorbance at 340 nm for 60 s. The molar extinction coefficient used for CDNB was ε340=9.6 mmol/L × cm. One U of GST activity was defined as the amount of enzyme that catalyzed the formation of one µmole of product/min/mL. GST activity was expressed in µmol/min/g.

2.4.5. Total antioxidant capacity determination

The total antioxidant capacity was estimated according to the ferric-reducing ability of plasma (FRAP) with the method described by Benzie et al, 1996 [35] using TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) as a substrate. The method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}-TPTZ). Samples (10 µL) were added as FRAP solution (190 µL) of 25mL of acetate buffer (300 mmol/L, pH3.6), 2.5mL of TPTZ reagent (10 mmol/l), and 2.5 $FeCl_3.6H_2O$ solution (20 mmol/l) and the increase in absorbance at 593 nm was measured. The reducing Fe^{3+}-TPTZ reagent by antioxidants was determined by using the standard curve of serial dilution of $FeSO_4 \cdot 7H_2O$ starting with 1 mmol/L. The results were expressed as FRAP value.

2.4.6. Nitric oxide production

NO production was quantified by the standard Griess reaction according to Tsikas, 2007 [36]. Briefly, 50 µL of samples were incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-(1-Naphthyl) ethylenediamine, and 2.5% phosphoric acid) at room temperature, for 10 min. The absorbance was measured at 570 nm in a microplate reader. The micromolar concentrations of NO (µmol/L) were obtained from a sodium nitrite (0–100 µmol/L) standard curve.

2.4.7. Malondialdehyde determination

The lipid peroxidation was measured according to Buege et al, 1978[37], through the quantification of total malondialdehyde (MDA) which is a product of lipid peroxidation. Briefly, 0.2 mL of the supernatant of tissues was homogenized in 0.4 mL of a solution of trichloroacetic acid (15 %)/thiobarbituric acid (0.375 %)/hydrochloric acid (0.6 %). The reactions were left for 40 minutes in a water bath (90ºC). After cooling on the ice, we added 0.6 mL of butyl alcohol. After that, the solution was vortexed for 2 min and centrifuged for 10 min at 9,000 g. The supernatant was used to measure the MDA using the absorbance at 540 nm in a microplate scanning spectrophotometer (Multiskan GO). A standard curve of known concentrations of 1, 1, 3, 3-tetramethoxypropane (TMPO) was used to determine the concentration of MDA. The results were expressed as μmol/L per mg protein.

2.4.8. Protein carbonyl determination

Protein carbonyl (PC) content was measured using the 2, 4-dinitrophenylhydrazine (DNPH) procedure [38], based on the carbonyl groups' reaction with DNPH. The pellets resulting from previous extraction homogenates were added to 0.5 mL of solution DNPH 10 mmol/L diluted in hydrochloric acid (7 %). Then, the solution was vortexed and kept at room temperature in the dark, shaking periodically for 30 min. After that, we added 0.5 mL of ice-cold 10% trichloroacetic acid (TCA) to each tube, that was centrifuged (5,000 g for 10 min at 4 °C). After centrifugation, the supernatant was discarded, and the precipitate was washed three times with 1 mL of ethyl acetate and ethanol (1:1 v/v). Finally, 1 mL of sodium dodecyl sulfate (SDS) 6% was added, the tubes were vortexed to dissolve the pellet, and the supernatant was measured by absorbance at 370 nm. The results were expressed as nmol/ mg of protein based on the molar extinction coefficient of $\varepsilon 370=22$ mmol/L × cm.

2.4.9. Total protein

Total protein was determined according to Lowry et al, 1951 [39] using bovine serum albumin (BSA) as a standard. Total protein concentrations were used to standardize CAT, SOD, MDA, and PC results.

2.5. Histological analysis

The left kidneys and half of the liver were fixed in paraformaldehyde 4% for 24 h, then transferred to 70% ethanol. Part of the brain was fixed on paraformaldehyde 4% for 24h and kept on sucrose (30%) until the fragment exceeds the density of the solution. Tissue fragments of the liver and kidneys were dehydrated in a growing series of ethanol and embedded in glycol-methacrylate (Historesin®, Leica). Semi-serial sections (3μm) were made using a rotary microtome (RM 2255, Leica), with a minimum of 40μm between sections, and

stained with hematoxylin/eosin (HE). Morphometry and stereology were performed using 10 digital images/per animal captured with the light microscope (Olympus BX-60®, Tokyo, Japan) connected to a digital camera (Olympus QColor-3®, Tokyo, Japan).

For the liver, images of the organ were morphometrically analyzed by points (intersections) count on the nucleus and cytoplasm of hepatocytes and blood vessels, totaling 2660 points/animals. For pathological analysis in the liver, points over leukocyte infiltrate, and vascular congestion were counted, totaling 2660 points/animals. We also measured the diameter of 150 hepatocytes and nuclei per animal.

For the kidneys, images obtained were analyzed morphometrically by counting the intersection of points on glomeruli, renal tubules, and blood vessels, totaling 5320 points per animal. In addition, the radius and glomerular area and the number of glomeruli present in each image were measured. For histopathological analysis, counts of leukocyte infiltration, vascular congestion, and leukocyte marginalization were performed, totaling 2660 points per animal. Analyzes were performed by grids with 266 intersections of the Image Pro Plus 4.5® Software (Media Cybernetics, Silver Spring, USA).

Also, brain and liver fragments were dehydrated in crescent ethanol series, cleared in xylene, and embedded in paraffin. Semi-serial sections (3μm) were made using a rotary microtome (RM 2255, Leica), with a minimum of 40μm between sections. The liver sections were stained with Sirius (Sirius red F3B, Mobay Chemical Co., Union, NJ, USA) for collagen fiber differentiation under polarizing microscopy to evaluate liver fibrosis. Slides were visualized and images were captured using a light and polarizing microscope (Olympus BX-60, Tokyo, Japan) connected to a digital camera (Olympus QColor-3, Tokyo, Japan). The brain sections containing the hippocampus were incubated in a 0.06% potassium permanganate solution, washed in water, and incubated in a 0.0001% Fluoro-Jade C solution (in acetic acid 0.1%) for 30 min at 25 °C [40]. The slides were washed in water and dried at 37 °C. Images were captured using a light and polarizing microscope (Olympus BX-35, Tokyo, Japan) connected to a digital camera (Olympus DP73, Tokyo, Japan). To validate the technique, the brain of epileptic rats was used as a positive control.

2.6. Statistical analysis

Data distribution was determined by the Shapiro–Wilk test using the program GraphPad Prism (version 6.0, Graph Pad Software Inc., San Diego, CA, USA). Pairwise comparisons among groups were made using Student's t-test. For data not normally distributed, we used Mann-Whitney tests. Individuals from the species *Artibeus lituratus*

collected in the forest fragment area (FFA) were compared to those collected in the Iron ore area (FEOA) and individuals from the species *Sturnira lilium* collected in the forest fragment area (FFA) were compared to those collected in aluminum ore area (ALOA). Results are expressed as means and standard error of the mean (mean ± SEM). Statistical significance was established at $p<0.05$. The Pearson test was used to verify the existence of a correlation between metal bioaccumulation and oxidative stress on muscle and liver.

3.0. RESULTS

3.1. Bioaccumulation of metal analysis

3.1.1. *Iron ore mining area*

Regarding the animals used for the iron ore impact evaluation, in the liver, higher concentrations were observed for Al ($p=0.0414$), Ca ($p<0.0001$), Fe ($p=0.0021$) and Ba ($p=0.0270$) from the FEOA group compared to FFA. In muscles, higher concentrations of Ca ($p=0.0041$) and Fe ($p=0.058$) from FEOA group were obtained.

3.1.2. Aluminum ore mining area

Among animals used for the aluminum impact evaluation, we found higher concentrations of Ca ($p=0.0344$) and Ba ($p=0.0498$) in the liver of ALOA and a higher concentration of Ca ($p=0.0189$), Zn ($p=0.0465$) and Ba ($p=0.0070$), and lower Ti ($p=0.0463$), in muscles from ALOA animals compared to FFA. The other metals tested did not show any differences among groups (Table 1).

Table 1. Levels of metals (µg/g) on liver and muscle from Artibeus lituratus of Fe ore area (FEOA) and Forest Fragment Area (FFA) and Sturnira lilium of Al ore area (ALOA) and Forest Fragment Area (FFA). *Asterisk means statistical differences with control groups ($P \leq 0.05$). Limits established (µg/mL) for ** drinking water regulations by the European Parliament and of the Council.[#] drinking water regulations from United States Environmental Protection Agency – US EPA.*** freshwater by Resolution 357 from the National Council for the Environment - CONAMA.

			Mg	**Al**	**Ca**	**Cr**	**Fe**	**Co**	**Cu**	**Cd**	**Ti**	**Mn**	**Ni**	**Zn**	**Ba**	**Pb**
Reference Value (European Parliament 2020/2184***) [41]			-	0.2	-	0.025	0.2	-	2	0.005		0.05	0.02	-	-	0.005
Reference Value (US EPA[#]) [42]			-	0.2	-	0.1	0.3	-	1.0	0.005	-	0.05	0.1	5	2	0.015
Reference Value (CONAMA 357**) [43]			-	0.1	-	0.05	0.3	0.05	0.009	0.001	-	0.1	0.025	0.18	0.7	0.01
Artibeus lituratus	Liver	FFA	25.66 ± 0.16	5.64 ± 0.80	29.54 ± 0.83	nd	22.29 ± 2.69	nd	nd	nd	0.11 ± 0.04	nd	nd	1.95 ± 0.23	0.06 ± 0.03	nd
		FEOA	28.43 ± 1.42	**7.85 ± 0.49 ***	**48.01 ± 0.69***	nd	**51.92 ± 7.74***	nd	0.18 ± 0.15	nd	0.17 ± 0.03	0.09 ± 0.06	nd	2.01 ± 0.27	**0.23 ± 0.06***	nd
	Muscle	FFA	25.84 ± 1.52	6.58 ± 1.11	25.31 ± 2.83	nd	11.53 ± 1.15	nd	nd	nd	0.07 ± 0.03	nd	nd	0.49 ± 0.11	0.04 ± 0.03	nd
		FEOA	29.07 ± 1.53	6.96 ± 0.74	**40.69 ± 3.31 ***	nd	**25.68 ± 3.87***	nd	0.03 ± 0.02	nd	0.16 ± 0.07	3.54 ± 1.90	nd	0.67 ± 0.16	0.09 ± 0.05	nd
Sturnira lilium	Liver	FFA	30.26 ± 1.15	7.28 ± 0.85	31.94 ± 2.91	nd	32.69 ± 2.84	nd	0.03 ± 0.03	nd	0.27 ±0.04	0.28 ± 0.46	nd	2.27 ± 0.47	0.05 ± 0.03	nd
		ALOA	30.28 ± 2.38	7.92 ± 0.83	**44.94 ± 4.56***	nd	30.42 ± 5.37	nd	0.90 ± 0.76	nd	0.16 ± 0.043	0.07 ± 0.12	nd	2.80 ± 0.13	**0.18 ± 0.05***	nd
	Muscle	FFA	29.01 ± 1.16	7.10 ± 1.33	28.74 ± 3.08	nd	25.19 ± 4.67	nd	nd	nd	0.18 ± 0.033	2.21 ± 1.43	nd	0.69 ± 0.12	nd	nd
		ALOA	30.63 ± 0.53	7.45 ± 0.91	**41.31 ± 3.04 ***	nd	19.48 ± 2.55	nd	nd	nd	**0.08 ± 0.03 ***	1.76 ± 1.57	nd	**1.15 ± 0.12 ***	**0.05 ± 0.02***	nd

* *Asterisk* means statistical differences among groups ($P \leq 0.05$)). nd mean not determined. Data are shown as mean ± SEM.

Water samples from the iron mining area showed Mg, Ca and Mn and in the aluminum mining area, we found Mg, Al, Ca, and Ba. FFA area showed Mg, Al and Ca (Table S1).

Table S1. Levels of metals (µg/mL) on water collected from the Fe mining area (FEOA), Al mining area (ALOA), and Forest Fragment Area (FFA). Limits established (µg/mL) for ** the quality of water intended for human consumption by the European Parliament and of the Council.[#] drinking water regulations from United States Environmental Protection Agency – US EPA.*** freshwater by Resolution 357 from the National Council for the Environment - CONAMA, Brazil.

		Mg	**Al**	**Ca**	**Cr**	**Fe**	**Co**	**Cu**	**Cd**	**Ti**	**Mn**	**Ni**	**Zn**	**Ba**	**Pb**
Reference Value (European Parliament 2020/2184**) [41]		-	0.2	-	0.025	0.2	-	2	0.005	-	0.05	0.02	-	-	0.005
Reference Value (US EPA[#]) [42]		-	0.2	-	0.1	0.3	-	1.0	0.005	-	0.05	0.1	5	2	0.015
Reference Value (CONAMA 357***) [43]		-	0.1	-	0.05	0.3	0.05	0.009	0.001	-	0.1	0.025	0.18	0.7	0.01
Water	FEOA	2.08	nd	3.07	nd	nd	nd	nd	nd	nd	0.47	nd	nd	nd	nd
Water	ALOA	0.07	0.22	0.26	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.05	nd
Water	FFA	0.09	0.03	0.53	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

* *Asterisk* means statistical differences among groups ($P \leq 0.05$). nd mean not determined. Data are shown as mean ± SEM.

3.2. Oxidative stress

3.2.1. *Iron ore mining area*

In the liver of *Artibeus lituratus* (FEOA compared to FFA group), a higher activity of CAT (p=0.0337), GST (p=0.0025), and MDA (p=0.0362) was observed in the FEOA group compared to FFA. Also, a lower concentration was observed for total proteins (p=0.0166), NO (p=0.0456), and FRAP (p=0.0118) in the FEOA group compared to FFA (Fig. 2). We found a negative correlation between Ca bioaccumulation and NO (p=0.0114; r=-0.6751) and positive between Ca bioaccumulation and GST (p=0.0044; r=0.7330). Also, we found a positive correlation between Fe bioaccumulation and SOD (p=0.0083; r=0.6736), CAT (p=0.0214; r=0.5870), and MDA (p=0.0153; r=0.6321) and negative between Fe bioaccumulation and FRAP (p=0.0027; r=-0.7365) on FEOA animals.

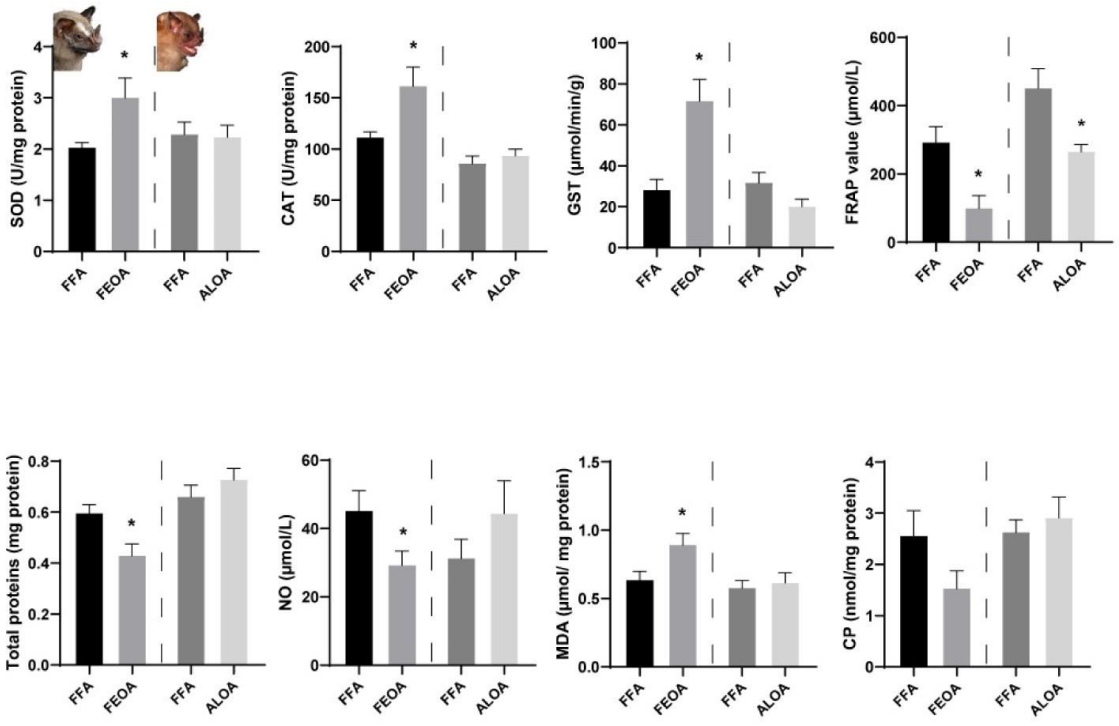


Fig 2. Differences in liver redox balance of *Artibeus lituratus* collected in the Fe ore area (FEOA) and in Forest Fragment Area (FFA) and *Sturnira lilium* collected in the Al ore area (ALOA) and in Forest Fragment Area (FFA). SOD: Superoxide dismutase, CAT: Catalase, GST: Glutathione-S-transferase, FRAP: total antioxidant capacity, NO: Nitric oxide, MDA: Malondialdehyde, CP: Carbonyl protein. * *Asterisk* means statistical differences with control groups ($P \leq 0.05$). Data are shown as mean ± SEM.

In the brain, were found a higher activity of SOD (p=0.0030) and GST (p=0.0002) and a lower level of total proteins (p=0.0051). MDA (p=0.0083) level was higher in FEOA compared to FFA (Figure 3).

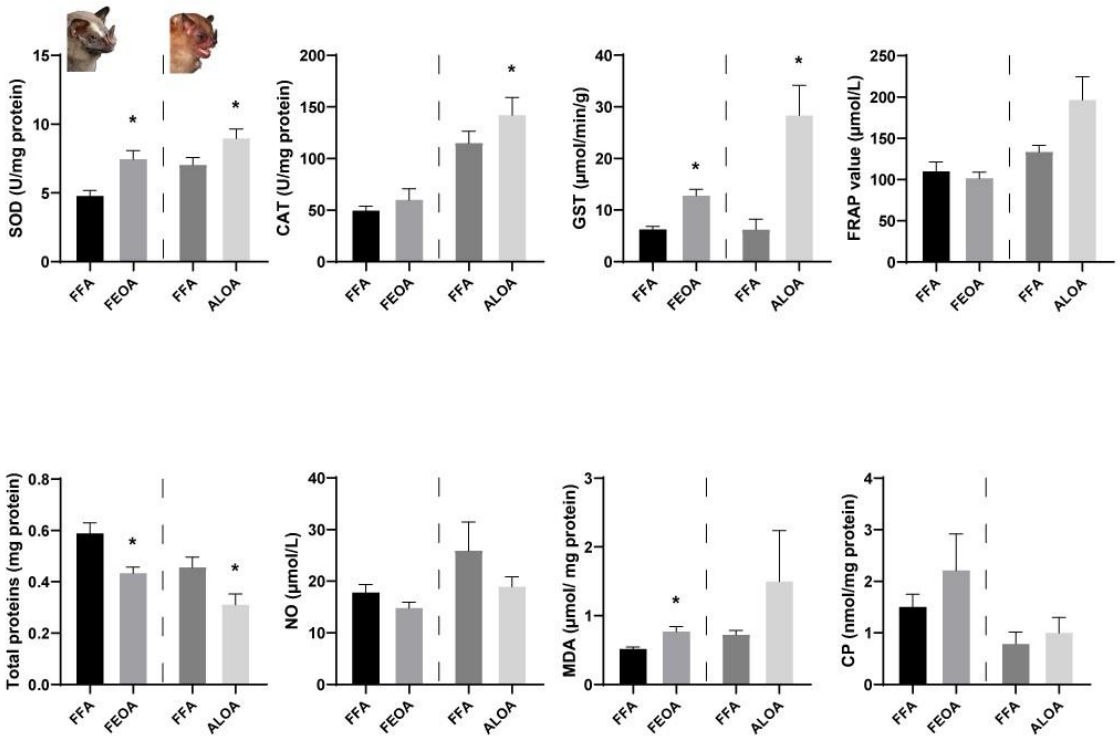


Fig 3. Differences in brain redox balance of *Artibeus lituratus*. collected in Fe ore area (FEOA) and in Forest Fragment Area (FFA) and *Sturnira lilium* collected in the Al ore area (ALOA) and in the Forest Fragment Area (FFA). SOD: Superoxide dismutase, CAT: Catalase, GST: GlutathioneS-transferase, FRAP: total antioxidant capacity, NO: Nitric oxide, MDA: Malondialdehyde, CP: Carbonyl protein. * *Asterisk* means statistical differences among groups ($P \leq 0.05$). Data are shown as mean ± SEM.

In the kidneys, a lower concentration was observed for NO (p=0.0435) and a higher concentration for MDA (p=0.0484) and CP (p=0.0307) in the FEOA group compared to FFA. The other parameters did not differ among groups (Fig. 4).

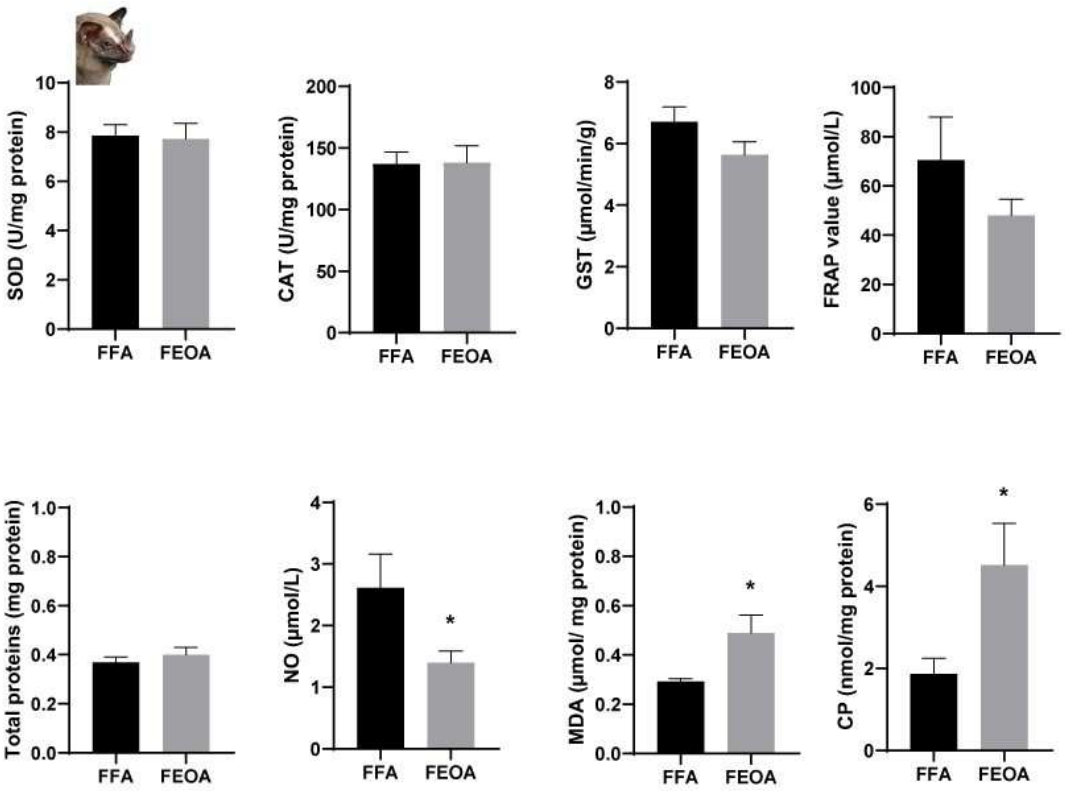


Fig 4. Differences in kidney redox balance of *Artibeus lituratus* collected in Fe ore area (FEOA) and in Forest Fragment Area (FFA). SOD: Superoxide dismutase, CAT: Catalase, GST: Glutathione-S-transferase, FRAP: total antioxidant capacity, NO: Nitric oxide, MDA: Malondialdehyde, CP: Carbonyl protein. * *Asterisk* means statistical differences among groups ($P \leq 0.05$). Data are shown as mean ± SEM.

In the muscle, a higher concentration was observed for total proteins (p=0.0166) and CP (p=0.0368), and a lower activity of SOD (p=0.0074) and GST (=0.0202) in the FEOA group compared to FFA (Fig. 5). We found a negative correlation between Ca bioaccumulation and GST (p=0.02829; r=-0.3223) in FEOA animals.

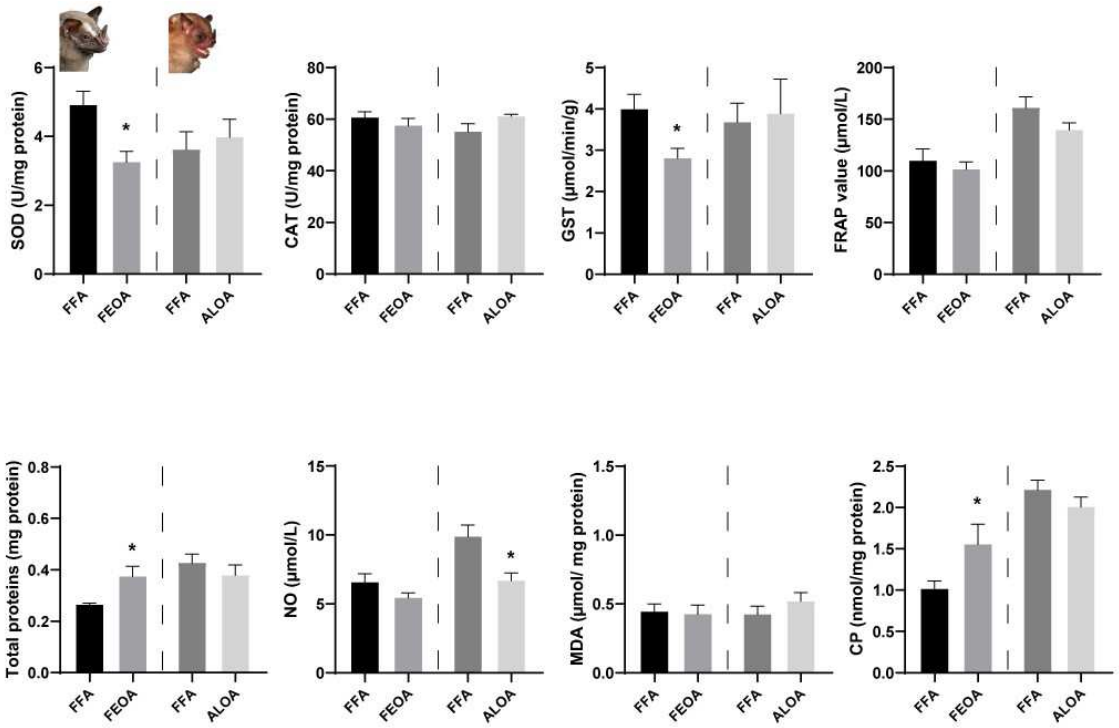


Fig 5. Differences in muscle redox balance of *Artibeus lituratus*. collected in Fe ore area (FEOA) and in Forest Fragment Area (FFA) and *Sturnira lilium* collected in Al ore area (ALOA) and in Forest Fragment Area (FFA). SOD: Super oxide dismutase, CAT: Catalase, GST: Glutathione-S-transferase, FRAP: total antioxidant capacity, NO: Nitric oxide, MDA: Malondialdehyde, CP: Carbonyl protein. * *Asterisk* mean statistical differences control groups ($P \leq 0.05$). Data are shown as mean ± SEM.

3.2.2. Aluminum ore mining area

In the liver of *Sturnira lilium* (ALOA compared to FFA groups) were observed lower concentration of FRAP (p=0.0050). The other parameters did not differ among groups. For ALOA animals, we found a negative correlation between Ba and FRAP (p=0.0366; r=-0.6331) (Fig. 2).

In the brain, were found a higher activity of SOD (p=0.0482) and GST (p=0.0030) and a lower concentration of total proteins (p=0.0274). CAT (p=0.493) activity was higher in ALOA compared to FFA. The other parameters did not differ among groups (Fig. 3).

In the muscle, a lower concentration was observed for NO (p=0.0089) in ALOA compared to FFA (Fig. 5). We found a negative correlation between Ca and FRAP (p=0.0033; r=-0.7707).

3.3. Histological analyzes

3.3.1. *Iron ore mining area*

In the liver of *Artibeus lituratus* (FEOA compared to FFA groups), points over the cytoplasm (p=0.0111) were lower. Also, points over leukocyte infiltrates (p=0.0332) were higher. Bat's liver of FEOA (p=0.0035) presented a fibrosis process (Fig. 6).

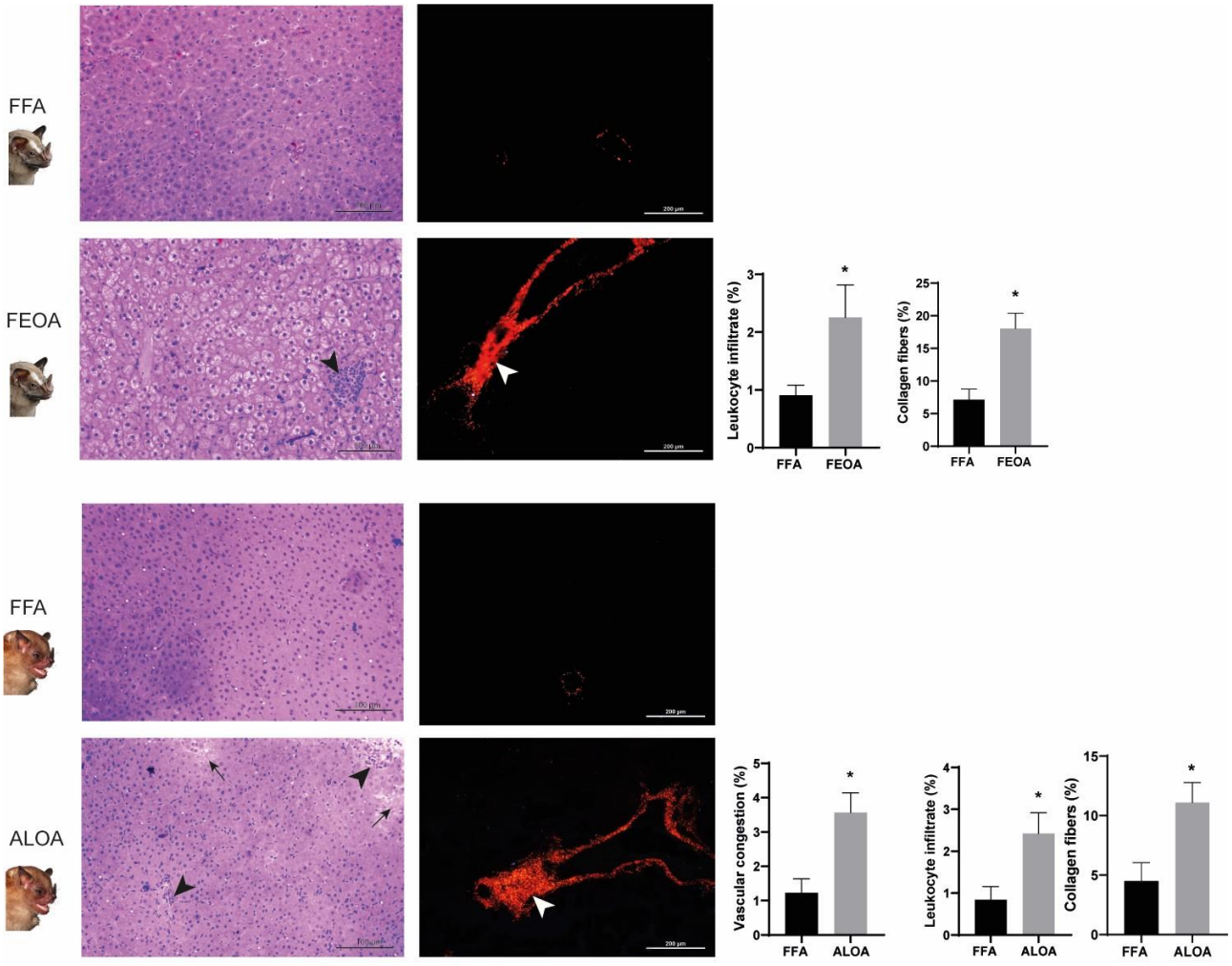


Fig 6. Effects of pollution on liver morphology and stereology of *Artibeus lituratus* collected in Fe ore area (FEOA) and in Forest Fragment Area (FFA) and *Sturnira lilium* collected in Al ore area (ALOA) and in Forest Fragment Area (FFA). Left: Leukocyte infiltrate (blackhead arrow), necrosis (black arrow). HE staining. Scale bars represent 100 µm. Right: Collagen content (white head arrow). Sirius red with polarizing light. Scale bars represent 200 µm. * *Asterisk* means statistical differences with control groups ($P \leq 0.05$). Data are shown as mean ± SEM.

In the kidneys, points over the glomerulus on FEOA (p=0.0115). Also, points over Capsular Space (p=0.0317), Vascular congestion (p=0.0159) and Leukocyte infiltrate (p=0.0195) on FEOA were higher than FFA groups (Fig. 7).

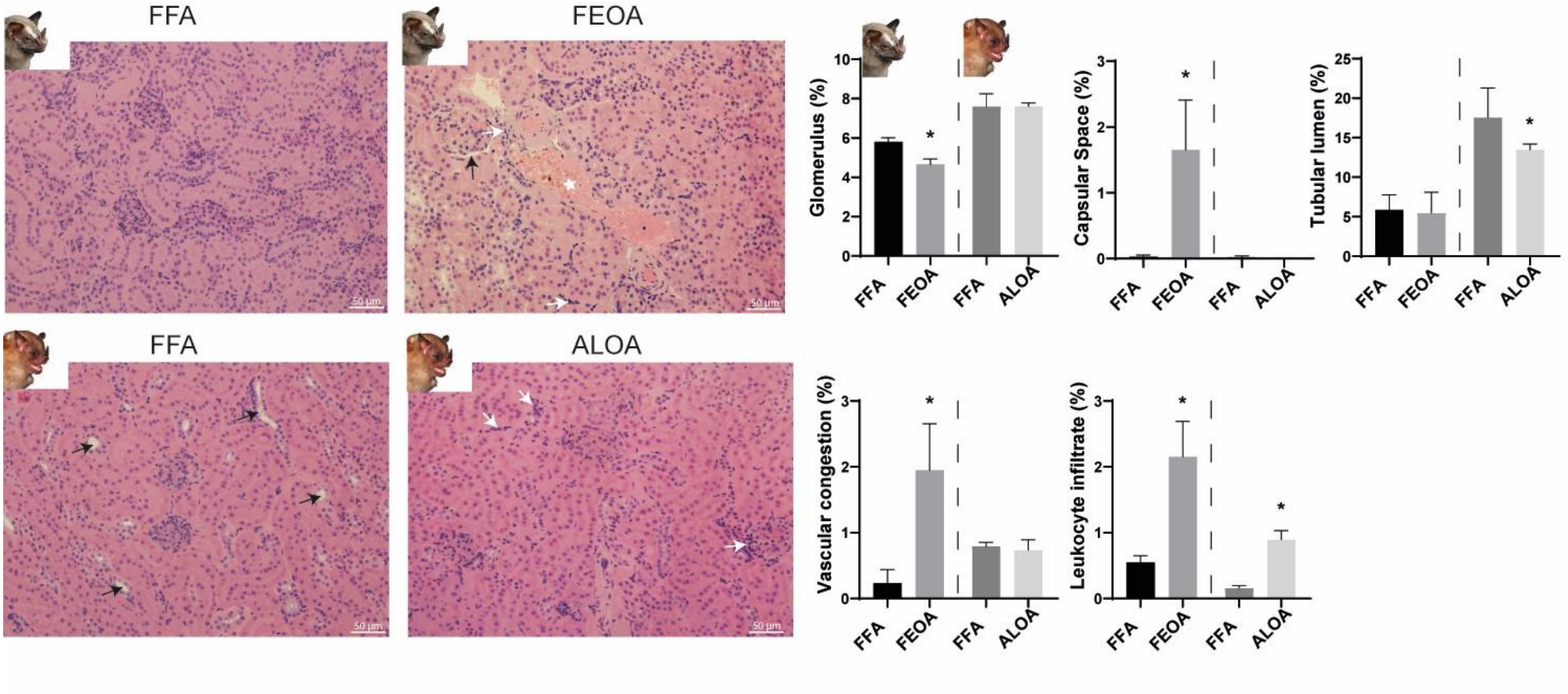


Fig 7. Effects of pollution on kidney morphology of *Artibeus lituratus* collected in Fe ore area (FEOA) and in Forest Fragment Area (FFA) and *Sturnira lilium* collected in Al ore area (ALOA) and in Forest Fragment Area (FFA). FEOA: Capsular space (black arrow), Leukocyte infiltrate (white arrow), Vascular congestion (star). FFA: Tubular lumen (black arrow). ALOA: Leukocyte infiltrate (white arrow). Scale bars represent 50 µm. * *Asterisk* means statistical differences with control groups ($P \leq 0.05$). Data are shown as mean ± SEM.

No changes were found in Fluorojade labeling in the brains of the analyzed bats (Figure 2S).

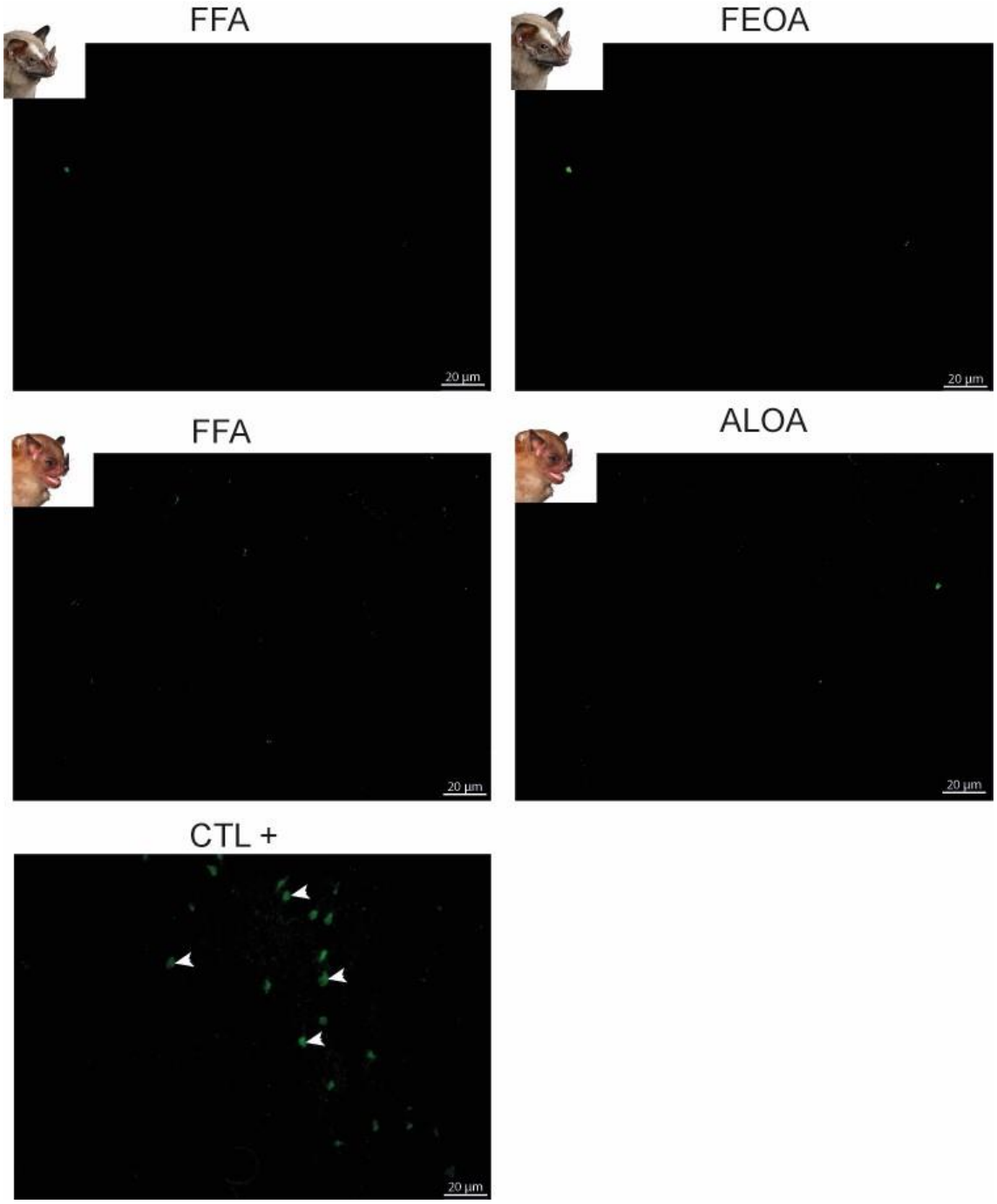


Fig 2S. Neuronal death in selective hippocampal areas of *Artibeus lituratus*. collected in Fe ore area (FEOA) and in Forest Fragment Area (FFA) and *Sturnira lilium* collected in Al ore area (ALOA) and in Forest Fragment Area (FFA). The neurodegeneration-positive process was evaluated using the hippocampal area of rats with epilepsy (CTL+). We did not find neuronal death in the animals collected. Fluorojade staining. Scale bars represent 20 µm.

3.3.2. Aluminum ore mining area

In the liver of *Sturnira lilium* (ALOA compared to FFA groups) points over the nucleus (p=0.0143) and cytoplasm (p=0.0160) were lower. Also, points over vascular congestion (p=0.0145), and leukocyte infiltrates (p=0.0416) were higher. Bat's liver of ALOA (p=0.0205) presented a fibrosis process (Fig. 4).

In the kidneys, the Tubular lumen (p=0.0254) on ALOA was lower. Also, Leukocyte Infiltrate (p=0.0005) on ALOA was higher than FFA groups (Fig. 5).

No changes were found in Fluorojade labeling in the brains of the analyzed bats (Figure 2S).

4. DISCUSSION

The results of this study, the first to assess how iron and aluminum ore mining affects fruit-eating bats, demonstrate how these activities negatively impact a variety of tissues from two key bat species, crucial to the Atlantic Forest restoration. Between the two impacted areas, bats captured in the Iron ore mining area (FEOA) turned out to be the ones with the highest number of altered parameters, compared to the preserved area. We found a correlation between Fe bioaccumulation and increased SOD, CAT, FRAP and MDA in the liver of bats captured in the iron mining area. The organ more severely affected was the liver, which showed leukocyte infiltrate and fibrosis in bats captured in the iron mining area.

4.1. Iron ore mining area

Taken together, bats collected in the iron ore mining area showed bioaccumulation of Al, Ca, Fe, and Ba in the liver and Ca and Fe in the muscles. In fact, some metals tend to accumulate in different organs - some metals like Cd, Se, As, Cu, Mn and Co are more likely to accumulate more in the liver than in the brain, but that may vary according to the species tested [16]. We found a higher concentration of Fe in the liver and muscle of bats captured in the iron mining area compared to the FFA.

Regarding the redox balance biomarkers, bats from the iron mining area showed higher oxidative damage to the liver than to other tissues. Some of these altered oxidative stress biomarkers (higher SOD and CA, lower FRAP and higher MDA levels) were correlated to liver Fe bioaccumulation, indicating that this metal is detrimental to bats occurring in this area. Increased antioxidant enzyme activities may be an attempt to cope with high ROS production. The activity of these enzymes exhibits bidirectional behavior associated with the intensity of cellular metabolic demand, increasing during moderate and

intensive demands, especially in response to oxidative stress [44]. ROS effectors play important roles in several cell signaling pathways, modulating energy metabolism, cell proliferation, and survival [45]. Therefore, higher antioxidant enzyme activities are associated with a protective response of the cells against ROS attacks whenever exogenous or endogenous sources are involved. Calcium bioaccumulation, together with arsenic and lead, for instance, was found to be correlated with higher SOD activities in free-living insectivorous bats [25]. In our study, we found higher Ca bioaccumulation in animals captured in the iron mining area. On the other hand, FRAP determinations can quantify non-enzymatic antioxidants like uric acid, ascorbic acid, and α-tocopherol [46,47]. The lower FRAP levels found in the iron mining area bats, combined with the lower protein content in the liver can be due to the lack of quality food that provides nutrients. In fact, the effects of mining activities on wild animals are also involved with forest fragmentation, habitat loss and changes in the animal's food habits [48]. The potential loss in exogenous antioxidant sources might be a concern since fruit-eating bats diets are usually packed with exogenous antioxidant elements, which help them to enhance their defenses [49,50]. Our results indicate that increased oxidative stress was confirmed by higher liver lipid peroxidation in bats from this area. Damage to lipids can lead to disruption of cell membrane, DNA damage and ultimately result in cell death [51]. Therefore, our results indicate that bats occurring in iron mining areas are showing signs of degeneration processes that may alter cellular homeostasis in the liver. As a consequence, histopathological alterations were also observed in this tissue, such as inflammatory infiltrate, degenerative processes and cellular necrosis, evidenced by excessive collagen fibers, indicating fibrosis, a known response to a chronic stimulus that may lead to cell death [48,49].

In a similar way, in the brain, bats from this area showed lower total protein and higher antioxidant activities, as well as higher lipid peroxidation, than in the protected area. In kidneys, which are essential organs due to its xenobiotic’s excretion role in the body, metals bioaccumulation might be an issue since they may be reabsorbed and accumulated in this organ [54]. Here we did not find any differences in antioxidant defenses in bats from the iron mining area, although two tissue damage markers [55–58] were increased, indicating excessive lipid peroxidation and carbonyl proteins levels in the kidneys. [55–58] Corroborating with that, our histological findings showed higher leukocyte infiltrate and vascular congestion in this tissue in animals from this area. It has been shown that, during metal contamination due to mining activities, acute inflammatory responses might be

trigged, when neutrophils and macrophages are recruited, which increase oxygen uptake [59]. This process is usually associated with excessive ROS generation, which can cause tissue damage and increase lipidic peroxidation and protein carbonylation [59]. Corroborating these findings, animals from iron areas also showed higher capsular space and lower glomerulus area, which can indicate glomeruli atrophy.

In muscles, we also found more signs of unbalanced redox, with lower antioxidant enzyme levels compared to bats from the preserved forest fragment. Several factors may induce this kind of alteration in free-living animals and one possible cause of the drop-in enzymatic activity is the enzyme exhaustion in response to intense ROS formation[60]. Another factor could be the alteration in the Nrf2 pathway that initiates the transcription of other target genes that encode proteins responsible for ROS detoxification. Some metals can affect this pathway, such as Pb, Ni and Cr [61]. Muscles are intensively used by bats in foraging, and the oxygen demand is high due to the flight capacity [62,63]. Therefore, lower SOD and GST activities may be a reflection of a higher antioxidant use, as a counterregulatory mechanism to oxidative stress [64,65]. This theory is supported by the higher carbonylated proteins observed in muscles from bats in this area, which also indicate damage to this tissue, essential to their key role in seed dispersal and reforestation.

4.2. Aluminum ore mining area

The animals from the aluminum mining area showed bioaccumulation of calcium and barium in the liver, and calcium, zinc and barium in the muscle. Bauxite, used to produce Al, is composed of various minerals and oxides, for instance, iron, titanium, silica and aluminum oxides [66,67]. Despite this, we did not find higher bioaccumulation of these metals on bats from the aluminum mining area compared to the forest fragment.

Regarding the liver redox balance biomarkers, the animals from the aluminum mining area showed lower content of FRAP, although they showed the same liver pathologies as animals from the iron mining area - leukocyte infiltrate and collagen fibers, indicating the presence of inflammatory process and necrosis, followed by fibrosis [52,53].

In the brain, we found lower total protein and higher SOD and GST activities, a similar pattern showed in both areas. In the aluminum mining area, though, the bats also showed higher CAT activity, indicating a higher redox unbalance in this tissue, usually one of the last organs to be contaminated with metals [16]. The blood-brain barrier and choroid plexus separate the brain from the systemic circulation, protecting the brain from some toxic metals [68]. Insectivorous bats foraging at wastewater treatment sites showed less arsenic

accumulation in the brain (0.413 ± 0.553 $\mu g \cdot g^{-1}$ dry weight) than in fur (1.514 ± 0.657 $\mu g \cdot g^{-1}$ dry weight), corroborating this idea [46].

In the kidneys, the bats from the aluminum mining area showed higher leukocyte infiltrate, which indicates an initial phase of the inflammatory process [69]. These animals also showed lower tubular lumen area, suggesting tubular atrophy, which is a concern due to the importance of renal tubules in reabsorbing water and useful molecules accidentally filtered by glomeruli, during excretion [70]. Corroborating our results, straw-colored fruit bats captured in a metal-polluted site in Nigeria also showed kidneys histopathologies (necrosis and infiltration) which might be associated with reactive oxygen generation and inflammatory induction by metals [27].

Regarding the muscles from bats captured in the aluminum mining area, in contrast to the bats from the iron mining area, this tissue was not severely damaged. The bats only showed lower NO levels, which, alone, is not enough to indicate oxidative stress.

Taken together, all results indicate that animals from the aluminum area were not as affected by the environmental alterations as those from the iron mining area. The metals released into the environment in these two different areas are one of the factors that might be involved with the tissue oxidative stress and histological damage observed in our study, although other factors such as other sources of pollution and habitat alterations (habitat loss and food availability) might be involved.

5. CONCLUSION

Our study brings a complete and new set of data showing that frugivorous bats occurring in mining areas can have detrimental effects on important physiological processes due to redox balance and histological damage in crucial tissues, and might interfere with their ability to mobilize energy, control and perform their normal activities like the flight. We showed that frugivorous bats occurring in mining areas accumulate metals in several tissues, and bats from iron ore mining sites bioaccumulate more metals and showed more oxidative and histological damage than bats from the aluminum areas. Although our study advances knowledge in this field, other studies are necessary to clarify other aspects involving the effects of mining areas on Neotropical wild bats, which are key to the Atlantic Forest regeneration.

ACKNOWLEDGMENTS

The authors thank the Museu de Zoologia João Moojen da Universidade Federal de Viçosa. We also wish to thank the National Council for Scientific and Technological Development (CNPq). A. L. F. Destro received a doctoral fellowship from the Higher Education Personnel Improvement Coordination, Brazil (CAPES).

AUTHOR CONTRIBUTIONS

Ana Destro: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft, Project administration **Déborah Gonçalves**: Investigation, **Thaís Alves**: Investigation, **Kemilli Gregório**: Investigation, **Vinicius Silva**: Investigation **Victor Santos**: Investigation, Resources **Olagide Casto**: Methodology, Writing - Review & Editing **Hernando Baggio**: Investigation, Resources **Guilherme Garbino**: Methodology, Writing - Review & Editing, Visualization **Reggiani Gonçalves**: Resources, Writing - Review & Editing **Jerusa Oliveira**: Formal analysis, Visualization, Writing - Review & Editing **Mariella Freitas**: Conceptualization, Writing - Review & Editing, Project administration, Supervision.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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5. Conclusões Gerais

Os resultados desta tese demonstram baseado em estudos que compuseram a revisão sistemática de que em modelos murinos, o chumbo afeta diversas vias de sinalização celular, ocasiona estresse oxidativo, genotoxicidade, e apoptose. Porém extratos vegetais podem amenizar essa intoxicação, principalmente se ingeridos após a exposição. Alguns extratos vegetais possuem a capacidade de quelar o chumbo, tornando-o menos tóxico e facilitando sua retirada na célula. Além disso, muitos extratos vegetais são ricos em compostos antioxidantes como flavonoides, taninos, catequinas, quercetinas e ácidos gálicos. Esses compostos além de aumentar a defesa antioxidante exógena da célula, também fortalece a defesa endógena enzimática. Sendo assim, os extratos vegetais demonstram-se como promissores em tratamentos e prevenção de exposição a baixas doses de chumbo.

Em morcegos, nosso estudo indica que esses animais estão mais susceptíveis a danos causados pelo níquel, que demonstrou afetar severamente o fígado e os rins, além de causar estresse oxidativo e danos leves nos testículos. Em segundo lugar, o chumbo demonstrou ser preocupante pois causou estresse oxidativo e histopatologias severas no rim e no fígado, indicando acúmulo lipídico. Cromo está em terceiro junto do cádmio. O cromo teve um efeito expressivo nos testículos e rins, e o cádmio ocasionou menos danos, porém também afetou os rins. Todos os danos encontrados refletem a toxicidade induzida pelos metais mesmo em baixas doses, e, portanto, uma preocupação para a sobrevivência de morcegos de vida livre.

Confirmando que morcegos de vida livre estão em ameaça pela contaminação de metais pesados, e sobretudo, os efeitos da mineração; nosso estudo demonstrou que morcegos frugívoros coletados em área de mineração de ferro e alumínio na Mata Atlântica apresentam inúmeros indícios de danos que podem afetar sua sobrevivência. Morcegos coletados nas áreas de mineração demonstraram severas patologias nos tecidos renais e hepáticos, como fibrose no fígado e infiltrado inflamatório em ambos. Além disso, os morcegos da mineração de ferro demonstraram maiores danos oxidativos e bioacúmulo de metais como ferro e alumínio, indicando que a mineração de ferro é ainda mais danosa para morcegos da Mata Atlântica do que a de alumínio.

ANEXO A – Autorizações do Comitê de Ética em Uso Animal

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 26/2020, intitulado **"Toxicidade comparada de metais em morcegos experimentalmente expostos em cativeiro",** coordenado pela professora Mariella Bontempo Duca de Freitas do Departamento de Biologia Animal, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTI, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTI, portanto sendo aprovado por esta Comissão em 02/02/2021, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 26/2020, named **"Comparative toxicity of metals in bats experimentally exposed in captivity"**, is in agreement with the a actual Brazilian legislation (Lei Nº 11.794, 2008, Normative Resolutions edited by CONCEA/MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI therefore being approved by the Committee on February 02, 2021 valid for 12months.

Prof. ª Mariella Bontempo Duca de Freitas
Coordenadora
Comissão de Ética no Uso de Animais – CEUA/UFV

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 10/2021, intitulado **"Toxicidade de metais pesados em morcegos silvestres da Mata Atlântica coletados em locais de mineração"**, coordenado pela professora Mariella Bontempo Duca de Freitas do Departamento de Biologia Animal, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTIC, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTIC, portanto sendo aprovado por esta Comissão em 07/04/2021, com validade de 2 anos.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 10/2021, named **"Heavy metal toxicity in wild bats from the Atlantic Forest collected in mining sites"**, is in agreement with the a actual Brazilian legislation (Lei Nº 11.794, 2008, Normative Resolutions edited by CONCEA/MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI therefore being approved by the Committee on April 02, 2021 valid for 2 years.

Prof. ª Mariella Bontempo Duca de Freitas
Coordenadora
Comissão de Ética no Uso de Animais – CEUA/UFV