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ELIANA JARDIM FERNANDES

AVALIAÇÃO DA TOXICIDADE E EFEITO PROTETOR DE CURCUMINA NANOENCAPSULADA EM *Drosophila melanogaster*

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Tese de doutorado apresentada ao programa de Pós-Graduação em Bioquímica (PPGBioq), da Universidade Federal do Pampa (UNIPAMPA), como requisito para obtenção do título de Doutora em Bioquímica.
Orientador: Prof. Dr. Gustavo Petri Guerra
Coorientadora: Profa. Dra. Marina Prigol

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A comissão examinadora, abaixo assinada, aprova a tese de Doutorado

AVALIAÇÃO DA TOXICIDADE E EFEITO PROTETOR DE CURCUMINA NANOENCAPSULADA EM *Drosophila melanogaster*

Elaborado por Eliana Jardim Fernandes

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Uruguaiana-RS, Brasil
2024

DEDICATÓRIA

Dedico esta tese a minha mãe Sônia Margarete e ao meu esposo Rui, por todo amor e força, que me incentivaram para chegar até aqui

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RESUMO

O estresse oxidativo e os processos inflamatórios são induzidos por algumas doenças e agentes tóxicos. Assim, o conhecimento de compostos que têm efeito sobre esses marcadores pode ser uma alternativa para tratar doenças já estabelecidas e não tratadas. Nesse sentido, a curcumina merece atenção, um composto bioativo, com grande presença de substâncias polifenólicas que lhe conferem propriedades antioxidantes. Apesar das propriedades, a curcumina livre pode apresentar efeitos tóxicos. Nesse contexto, as nanopartículas são consideradas uma estratégia promissora para diminuir toxicidade. O estudo visa investigar um possível efeito tóxico da exposição crônica de *Drosophila melanogaster* à nanocápsulas carregadas com curcumina e avaliar seu efeito protetor sobre a toxicidade induzida por lipopolissacarídeo (LPS). Para verificar se a nanoencapsulação da curcumina em polisorbato 80 (P80), apresenta ou não toxicidade, e se potencializa a eficiência farmacológica desse polifenol. Para isso, foram usadas *D. melanogaster* divididas em grupos de diferentes concentrações com curcumina livre e nanocápsulas carregadas com curcumina, expostas por 10 dias e avaliadas quanto à porcentagem de sobrevivência. Ao final foram realizados testes comportamentais, atividade da acetilcolinesterase (AChE), consumo de dieta e parâmetros de estresse oxidativo. Foi observado que exposição à curcumina livre e às nanocápsulas carregadas com curcumina revestidas com P80, nas concentrações avaliadas, não causou efeitos tóxicos em *D. melanogaster*, e que com baixas doses já é possível modular positivamente os marcadores avaliados (Artigo). Nos parâmetros abordados no manuscrito, quanto à avaliação do efeito protetor de nanocápsulas carregadas com curcumina, foi visto que a concentração de 10 µM, protegeu contra as alterações no perfil comportamental e moleculares induzidas pelo LPS em *D. melanogaster*, através da regulação das vias p38 MAPK/Nrf2. Podemos sugerir que nanoencapsulação de curcumina em p80 potencializou positivamente seus efeitos antioxidantes ao regular o equilíbrio redox e antiapoptótico, sugerindo que esse mecanismo de ação pode ser explorado em possíveis aplicações futuras no tratamento de doenças e agentes tóxicos que agem por mecanismo semelhante.

Palavras-chave: nanoencapsulação; polissorbato 80; lipopolissacarídeo; antiapoptótico; balanço redox.

ABSTRACT

Oxidative stress and inflammatory processes are induced by some diseases and toxic agents. Therefore, knowledge of compounds that have an effect on these markers may be an alternative for treating already established and untreated diseases. In this sense, curcumin deserves attention, a bioactive compound with a large presence of polyphenolic substances that give it antioxidant properties. Despite its properties, free curcumin can have toxic effects. In this context, nanoparticles are considered a promising strategy to reduce toxicity. The study aims to investigate a possible toxic effect of chronic exposure of *Drosophila melanogaster* to curcumin-loaded nanocapsules and evaluate their protective effect on toxicity induced by lipopolysaccharide (LPS). To verify whether or not the nanoencapsulation of curcumin in polysorbate 80 (p80) presents toxicity, and whether it enhances the pharmacological efficiency of this polyphenol. For this, *D. melanogaster* divided into groups of different concentrations of free curcumin and curcumin-loaded nanocapsules exposed for 10 days and evaluated for survival percentage. At the end, behavioral tests, acetylcholinesterase (AChE) activity, diet consumption, and oxidative stress parameters were carried out. It was observed that exposure to free curcumin and curcumin-loaded nanocapsules coated with P80, at the concentrations evaluated, did not cause toxic effects in *D. melanogaster*, and that with low doses it is already possible to positively modulate the markers evaluated (Article). In the parameters addressed in the manuscript, regarding the evaluation of the protective effect of curcumin-loaded nanocapsules, it was seen that the concentration of 10 µM protected against changes in the behavioral and molecular profile induced by LPS in *D. melanogaster*, through the regulation of the p38 MAPK/Nrf2 pathways. We can suggest that nanoencapsulation of curcumin in p80 positively potentiated its antioxidant effects by regulating redox and antiapoptotic balance, suggesting that this mechanism of action can be explored in possible future applications in the treatment of diseases and toxic agents that act through a similar mechanism.

Keywords: nanoencapsulation; polysorbate 80; lipopolysaccharide; antiapoptotic; redox balance.

LISTA DE FIGURAS

Referencial Teórico

LISTA DE ABREVIATURAS E SIGLAS

LPS – Lipopolisacarídeo

P80 – Polisorbato 80 (P80),

D. melanogaster – Drosophila melanogaster

AChE – Acetilcolinesterase

-OH – Hidroxila

DCNT – Doenças crônicas não transmissíveis

EMA – Agência Europeia de Medicamentos (Do inglês European Medicines Agency)

ANVISA – Agência Nacional de Vigilância Sanitária

DMSO – Dimetilsufóxido

SOD – Superóxido dismutase

CAT – Catalase

GST – Glutationa-S-transferase

GPx – Glutationa peroxidase

RS– Espécies reativas

TBARS – Substâncias reativas ao ácido tiobarbitúrico

GSH – Glutationa

GSTT1 – Glutationa S-transferase teta-1

Nrf2 – Fator nuclear derivado do eritroide 2

mRNA – Ácido ribonucleico mensageiro

NCUR – Curcuminóides

EROs – Espécies reativas de oxigênio

RROs – Receptores de reconhecimento de padrões

PAMPs – Padrões moleculares associados a patógenos

AMPs – Peptídeos antimicrobianos

LTA – Ácido lipoteicóico

Imd – deficiência imune

p38 MAPK – Proteína quinase ativada por mitógeno p38

E. coli – Escherichia coli

ACh – Acetilcolina

ATP –Adenosina trifosfato

FADH2 – Dinucleótido de flavina e adenina

NADH – Nicotinamida-adenina-dinucleótido reduzida

O_2 – Oxigênio molecular

$O2^{-\bullet}$ – ânion superóxido

H_2O_2 – Peróxido de hidrogênio

Fe^{2+} – Íon ferroso

OH – Radical hidroxila

Fe^{3+} – Íon férrico

Keap1 – Proteína 1 associada à ECH semelhante a Kelch

ERA – Elemento de resposta antioxidante

ERN – Espécies reativas de nitrogênio

MDA – Malondialdeído

UNIPAMPA – Universidade Federal do Pampa

UFSM – Universidade Federal de Santa Maria

SUMÁRIO

APRESENTAÇÃO

A presente tese foi dividida em três partes. Na parte I encontram-se a **INTRODUÇÃO, REFERENCIAL TEÓRICO** e os **OBJETIVOS**. Os itens INTRODUÇÃO e REFERENCIAL TEÓRICO são compostos por uma revisão da literatura, onde foram inseridos os temas abordados nesta tese.

Os resultados os quais compõem a parte II deste trabalho e fazem parte desta tese apresentam-se sob a forma de um **ARTIGO CIENTÍFICO** e um **MANUSCRITO CIENTÍFICO**, que constam as seções: Materiais e Métodos, utilizada para a produção dos Resultados obtidos, assim como Introdução, Discussão, Conclusão e Referências Bibliográficas. O manuscrito apresenta-se formatado de acordo com as normas da revista que será submetido *"Food and Chemical Toxicology".*

No final deste trabalho, encontram-se os itens **DISCUSSÃO** e **CONCLUSÕES** os quais compõem a parte III, que constam os principais achados e interpretações referentes ao artigo e manuscrito presentes nesta tese. Ainda, encontram-se as **REFERÊNCIAS BIBLIOGRÁFICAS**, referente a citações que aparecem nos itens introdução, referencial teórico e discussão apresentados na presente tese.

PARTE I

1. INTRODUÇÃO

A produção excessiva de radicais livres e os processos inflamatórios, produzidos pelo metabolismo celular, são induzidos por algumas doenças e agentes tóxicos (ANSARI; AHMAD; HAQQI, 2020; RAMOS-GONZÁLEZ *et al.*, 2024; SUL; RA, 2021). Assim, se faz necessário o estudo e conhecimento de compostos que tenham potencial ação sobre o estresse oxidativo e a inflamação, como uma alternativa para tratar ou aliviar sintomas de doenças já estabelecidas e sem tratamentos.

Muitos compostos bioativos, extraídos de plantas naturais têm sido investigados para fins terapêuticos. Entre eles, a curcumina que apresenta potenciais efeitos anti-inflamatórios, imunomoduladores, antivirais, antibacterianos e antioxidantes (EDWARDS *et al*., 2017; DAI *et al.*, 2018; PACIELLO *et al.*, 2020). A estrutura com o anel benzênico ligado a grupamentos hidroxilas (-OH) caracteriza a capacidade antioxidante da curcumina, essa característica tem sido alvo de pesquisas na linha de proteção ou atenuação de doenças crônicas não transmissíveis (DCNT) indutoras de estresse oxidativo (PANDEY; RIZVI, 2009). No entanto, a curcumina livre pode apresentar efeito tóxico (LÓPEZ-LÁZARO, 2008), dano hepatocelular dependente da dose em roedores (BALAJI; CHEMPAKAM, 2010), e doses mais altas também apresentam efeitos tóxicos em embriões (ALAFIATAYO *et al.*, 2019).

Nesse contexto, nanopartículas são consideradas uma estratégia, pois possibilitam a entrega de fármacos direcionados às células, maximizando o potencial terapêutico e minimizando a toxicidade, além de apresentar benefícios no tratamento de doenças respiratórias (DA SILVA *et al.*, 2013), proporcionam uma liberação controlada, menor toxicidade e é necessário concentrações menores para se ter efeitos farmacológicos (NAZILA *et al.*, 2016), pois a nanoencapsulação favorece uma maior interação com o tecido alvo (BENDER *et al.*, 2012). Um estudo realizado *in vitro* com nanocápsulas revestidas com polissorbato 80 (P80) contendo curcumina, não apresentou efeito citotóxico em relação à curcumina livre, e esse estudo o qual temos pacerias, incentivou testes *in vivo* (SANTOS *et al.*, 2021).

A *Drosophila melanogaster* é modelo de organismo modelo promissor que permite avaliar efeitos tóxicos com ênfase em dano oxidativo, assim como os efeitos

mediados por nanomateriais, além de ter baixo custo e rápido desenvolvimento (AHAMED *et al.*, 2010). Ainda, para avaliar o estresse oxidativo, estudos usam lipopolissacarídeo (LPS), uma endotoxina constituinte da membrana externa de bactérias gram-negativas para induzir estresse oxidativo, inflamação e apoptose (JOHN *et al.*, 2022; SAMPATH, 2018). O LPS pode atacar o sistema imunológico inato e as vias Toll, que são vias de respostas imunes em insetos, que irão responder à presença desse patógeno, e causar reações imunológicas também em invertebrados (RAO; YU, 2010; SAMPATH, 2018).

Devido ao fato de que a curcumina livre e nanocápsulas carregadas com curcumina podem apresentar toxicidade, se faz necessário determinar os seus possíveis efeitos tóxicos. E ainda, avaliar se o nanoencapsulamento pode melhorar a eficiência farmacológica e proteger contra um agente tóxico como o LPS.

2. REFERENCIAL TEÓRICO

2.1 Curcumina

Dentre os compostos bioativos com grande potencial terapêutico, podemos destacar a Curcumina, o principal polifenol encontrado no turmérico da *Curcuma longa L*, um arbusto perene representante da Família Zingiberacea, nativo da Índia, sendo o rizoma da planta o mais utilizado, e quando desidratado e moído, forma o pó de cor amarela (Figura 1), caracterizado como tumérico, utilizado na culinária, medicina e religião (SHAH *et al.*, 2022; SUETH-SANTIAGO *et al.*, 2015).

Figura 1 - Representação da fonte da curcumina

Fonte: adaptado de Banik *et al.*, 2017.

A *Curcuma longa,* também é denominada popularmente como tempero dourado e açafrão da Índia, e vem sendo usada na medicina tradicional desde o início da humanidade para tosses, dores de garganta e problemas respiratórios, e devido ao seu potencial, está sendo estudada na medicina moderna no tratamento de doenças

como câncer (ALLEGRA *et al.*, 2016), depressão e ansiedade (SAHEBKAR *et al.*, 2013), e doenças relacionadas ao estresse oxidativo e inflamatório (SLAVOVA-KAZAKOVA *et al.*, 20-21; WANG *et al.*, 2021).

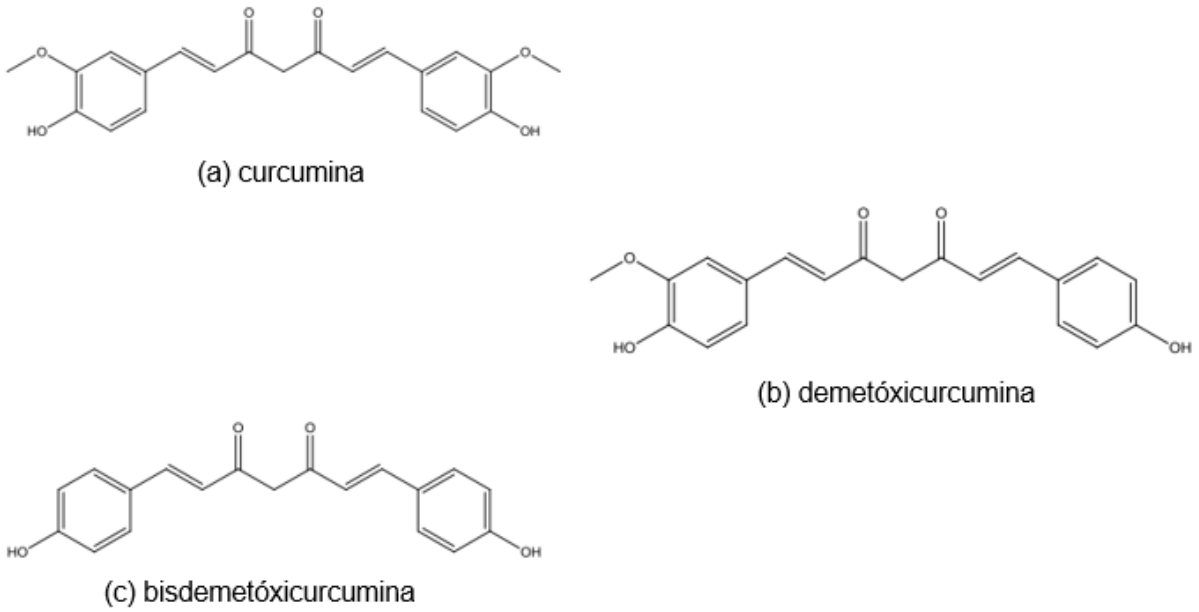
Os rizomas/raiz de curcuma são empregados na forma de chá de ervas para beber e em formas sólidas ou líquidas para tomar por via oral, segundo European Medicines Agency (EMA) (EMA, 2024). Outras formas de apresentação é a de infusão e tintura, de acordo com a World Health Organization (WHO, 1999), e em cápsula com a droga vegetal, segundo a Agência Nacional de Vigilância Sanitária (ANVISA, 2021).

O primeiro produto contendo *Curcuma longa* com registro na ANVISA é o Motore, comercializado sob a forma farmacêutica de cápsulas, contendo 250 mg do extrato seco de *Curcuma longa* (equivalente a 50 mg de curcuminoides), com registrado em 12/2012, está inserido na classe terapêutica de anti-inflamatórios/antirreumáticos (ANVISA, 2024a). Comercializado em embalagens com 15, 60 e 120 cápsulas, segundo o fabricante, as cápsulas ainda contêm polissacarídeos de soja, fosfato de cálcio, hiprolose, croscarmelose sódica, povidona, dióxido de silício e estearato de magnésio. A dose habitual para adultos é de 2 cápsulas a cada 12 horas, totalizando 500 mg de extrato de cúrcuma por dia, sendo indicado para o tratamento da osteoartrite e artrite reumatoide, apresentando ação anti-inflamatória e antioxidante (ACHÉ, 2024).

Outro produto com registro na ANVISA, é o CUMIAH, contendo o princípio ativo *Curcuma longa*, encontrasse na categoria de fitoterápicos, sendo comercializado sob a forma farmacêutica de cápsulas, contendo 250 mg do extrato seco de *Curcuma longa*, em embalagens com 15, 30, 60, 90 e 120 cápsulas, registrado em 03/2023, com validade de 24 meses (até 03/2025), também está inserido na classe terapêutica de anti-inflamatórios/antirreumáticos (ANVISA, 2024b).

Os curcuminóides presentes na *Curcuma longa* consistem em curcumina, demetóxicurcumina e bisdemetóxicurcumina (KIM; JANG, 2009) (Figura 2). A curcumina é considerada um composto polifenólico, com características hidrofóbicas solúvel em etanol e dimetilsufóxido (DMSO) (BANIK *et al.*, 2017).

Figura 2- Estrutura molecular dos componentes químicos presentes na *Curcuma longa*.



Fonte: adaptado de Kim; Jang (2009).

Nota: (a) Estrutura química da curcumina; (b) Estrutura química da desmetóxicurcumina; (c) Estrutura química da bisdemetóxicurcumina.

O anel benzênico ligado a um ou mais grupamentos hidroxilas (-OH) caracteriza a capacidade antioxidante da curcumina e essa propriedade tem sido alvo de pesquisas na linha de prevenção ou atenuação de DCNT causadores de estresse oxidativo (PANDEY; RIZVI, 2009). Os grupos fenólicos presentes em sua estrutura, que são capazes de reagir com espécies reativas, sequestrando óxido nítrico, ânion superóxido, peroxinitrito, radicais peroxila e hidroxila, e assim protegem contra o dano oxidativo, e podem regular as defesas antioxidantes, ativando as enzimas superóxido dismutase (SOD), catalase (CAT), glutationa-S-transferase (GST), glutationa peroxidase (GPx), glutationa redutase, γ-glutamil cisteína ligase (TRUJILLO *et al.*, 2013).

A curcumina apresentou efeito protetor contra o estresse oxidativo induzido com glicose oxidase em células estreladas hepáticas de ratos, e ainda, o tratamento com curcumina diminuiu significativamente os níveis de espécies reativas (RS) e substâncias reativas ao ácido tiobarbitúrico (TBARS) e aumentou os níveis de glutationa (GSH) (LIU *et al.*, 2016). Assim como a curcumina livre diminuiu a

peroxidação lipídica, desencadeada pelo estresse de contenção no cérebro, fígado e rim de ratos (SAMARGHANDIAN *et al.*, 2017).

No que se refere a ação da curcumina, as evidencias indicam prováveis mecanismos de ação da curcumina na modulação e ativação do fator nuclear derivado do eritroide 2 (Nrf2). O Nrf2 é um fator de transcrição, ou seja, é capaz de induzir a transcrição de uma variedade de genes, proteínas e enzimas que são citoprotetores, desintoxicantes e antioxidantes, que participam das defesas contra o estresse oxidativo e inflamação (BARBOSA *et al.*, 2019; GALI; GALIÈ; COVI, 2019).

Nesse sentido, a curcumina pode servir como um indutor de Nrf2, já que a conformação estrutural da curcumina favorece a eliminação de radicais livres e ativa o Nrf2, que restaura a homeostase redox, ativando um grupo de enzimas antioxidantes (TRUJILLO *et al.*, 2013).

A curcumina também foi sugerida no tratamento da COVID-19. O estudo realizado em 40 pacientes com infecção por COVID-19, mostrou que a suplementação de 160 mg de nanocurcumina em quatro cápsulas de 40 mg, diariamente por 14 dias, reduziu citocinas inflamatórias, principalmente a expressão de RNA mensageiro (mRNA) de IL-1β e lL-6. Ainda, foi observado redução significativa da taxa de mortalidade, sugerindo que a curcumina regulou processos inflamatórios e pode ser suplementada como um adjuvante ao tratamento medicamentoso da SARS-CoV-2 (VALIZADEH *et al.*, 2020).

Apesar do alto potencial terapêutico da curcumina, sua aplicação clínica é limitada. A baixa solubilidade aquosa da curcumina dificulta a administração intravenosa, (CHRISTINE SYNG-AI, A. LEELA KUMARI; KHAR, 2004), e intraperitoneal (IRVING *et al.*, 2011). Quando administradas por via intraperitoneal ou intravenosa ocorre a redução metabólica à di-hidrocurcumina, hexahidrocurcumina e octahidrocurcumina, curcumina glucuronida, DHC-glucuronida, THC-glucuronido e sulfato de curcumina. Sabe-se que esses metabólitos também podem apresentar propriedades anti-inflamatórias e antioxidantes, no entanto, independente da via de administração, a concentração plasmática da forma livre da molécula é muito baixa e a curcumina é mais ativa do que os seus metabólitos, sendo assim, os efeitos benéficos da curcumina são condicionados à fração livre (AGGARWAL; DEB; PRASAD, 2014).

Seu uso terapêutico também é limitado devido seu potencial tóxico, pois altas doses de curcumina podem causar toxicidade (LÓPEZ-LÁZARO, 2008), e dano hepatocelular dependente da dose em ratos (BALAJI; CHEMPAKAM, 2010). Do mesmo modo que, doses mais altas e prolongadas causam toxicidade, é visto que a administração de curcumina (100 mg/kg de peso corporal), cinco dias por semana e durante 90 dias, causou diminuição do peso corporal de ratos, toxicidade hepática e alterações no metabolismo lipídico em ratos (QIU *et al.*, 2016). Assim como foram observados efeitos nocivos da curcumina em concentrações mais elevadas no desenvolvimento do embrião nos estágios iniciais do desenvolvimento embrionário em modelo de zebrafish, produzindo deformidades do corpo físico, como cauda dobrada, tronco dobrado e edema aumentado do saco vitelino, mostrando que plantas bioativas em doses mais altas também podem ser tóxicas (ALAFIATAYO *et al.*, 2019).

2.1.1 Nanocápsulas carregadas com curcumina

Como mencionado acima, a curcumina pode apresentar efeito tóxico, em razão disso, as pesquisas têm buscado estratégias que possam ser incorporados ao composto, afim de proporcionar maior biodisponibilidade e menor toxicidade. Nesse sentido, as nanopartículas são consideradas uma estratégia na farmacoterapia, pois possibilitam a entrega de drogas direcionadas às células, maximizando o potencial terapêutico e minimizando a toxicidade (DA SILVA *et al.*, 2013).

Há mais de 50 anos, o desenvolvimento de polímeros biodegradáveis representa um avanço na medicina, possibilitam a entrega de nanofármacos com liberação controlada que são capazes de superar as limitações farmacológicas das formas de dosagem convencionais (NAZILA *et al.*, 2016). As nanopartículas poliméricas podem ser revestidas com P80, um surfactante não iônico sintético, composto de ésteres de ácidos graxos de polioxietileno sorbitano, usado como excipiente na formulação de medicamentos, tanto para prevenir a adsorção superficial, quanto como estabilizadores contra a agregação de proteínas (KERWIN, 2008; KHAN; MAHLER; KISHORE, 2015). A composição de ácidos graxos é principalmente pelo ácido oleico > 58% (KERWIN, 2008). No entanto, o principal constituinte do P80 é o monooleato de polioxietileno-20-sorbitano, com um peso molecular de 1309,7 Da e uma densidade

de 1,064 g/ml, componente estruturalmente semelhante aos polietilenoglicóis (TEN TIJE *et al.*, 2012).

Uma análise do perfil de liberação *in vitro* e a atividade antimalárica de diferentes revestimentos de nanocápsulas, incluindo P80, carregadas com curcumina, mostra uma liberação controlada e atividade antimalárica da curcumina nanoencapsulada em relação à curcumina livre, propondo que os mesmos podem ter uma importante aplicação biofarmacêutica *in vivo* (SANTOS *et al.*, 2021).

Da mesma forma, um estudo que avaliou o trans–resveratrol revestido com P80, aumentou a concentração do composto no tecido cerebral e proporcionou maior segurança gastrointestinal quando comparada com a forma livre, efeito atribuído ao fato de que o revestimento pode reduzir a absorção das nanocápsulas pelo sistema fagocitário, prolongando o tempo de circulação das nanopartículas (FROZZA *et al.*, 2010). Além disso, os polímeros aniônicos como o P80 apresentam características surfactantes, podem penetrar e saturar a membrana levando à solubilização dos lipídios e proteínas, favorecendo uma maior interação com o tecido alvo (BENDER *et al.*, 2012).

Outro estudo avaliou a eficácia de nanomicelas contendo curcuminóides (NCUR) e curcumina natural contra o dano oxidativo induzido por Diazinon em ratos machos. Os autores constataram que a capacidade antioxidante da NCUR também supera o uso de curcumina natural, o resultado é associado a melhor biodisponibilidade em soluções aquosas, a maior estabilidade física e menor predisposições citotóxicas (ABDOLLAHZADEH ESTAKHRI *et al.*, 2019). Assim como curcumina nanoencapsulada apresentou efeitos terapêuticos aprimorados em comparação à curcumina livre, melhora a memória em um modelo de doença de Alzheimer e reduz neuroinflamação em ratos (SAVALL *et al.*, 2024).

A utilização de nanopartículas vem sendo cada vez mais empregada, pois pode elevar consideravelmente a eficácia dos medicamentos e oferecer uma melhor qualidade de vida aos pacientes (ZHANG *et al.*, 2017). Diante do exposto, e visado superar as desvantagens da curcumina livre, nesse estudo ela foi nanoencapsulada em no dispositivo manométrico, sendo este o P80.

2.2 Doenças e agentes tóxicos aumentam o estresse oxidativo

Algumas doenças e agentes tóxicos atuam através de diferentes mecanismos que aumentam o estresse oxidativo e os processos inflamatórios (ANSARI *et al.*, 2020; RAMOS-GONZÁLEZ *et al.*, 2024; SUL; RA, 2021). Neste sentido, o conhecimento de compostos que tenham potencial ação sobre o estresse oxidativo e a inflamação pode ser uma alternativa para tratar ou aliviar sintomas de doenças já estabelecidas e não tratadas, como a osteoartrite e a esclerose múltipla (ANSARI *ET AL.*, 2020; RAMOS-GONZÁLEZ *et al.*, 2024), ou doenças emergentes, como o Coronavírus 2019 (COVID-19), que está associada ao estresse oxidativo (HUANG et al., 2020; KHOMICH *et al.*, 2018; POLONIKOV, 2020).

Alguns medicamentos usados no tratamento de outras doenças, são algumas das opções propostas para tratar a COVID-19 como a hidroxicloroquina, cloroquina e ivermectina, porém as diretrizes para o tratamento farmacológico da COVID-19, não recomendam o uso desses medicamentos, pois o uso não reduz a mortalidade, podem ser tóxicos e agravar a doença (REIS *et al.*, 2022a; SIEMIENIUK *et al.*, 2020). Nesse sentido, algumas revisões apontaram o papel do sistema redox na fisiopatologia da infecção por SARS-CoV-2, propondo que o aumento excessivo de espécies reativas de oxigênio (EROs) e a redução das defesas antioxidantes são cruciais para a replicação viral (DELGADO-ROCHE; MESTA, 2020; FAKHRI *et al.*, 2020). Outra doença sem tratamento é a causada pelo vírus Zika (ZIKV), um agente infeccioso que também foi associado a doenças inflamatórias e estresse oxidativo (FRANÇA *et al.*, 2023; MORAIS *et al*, 2020).

O estresse oxidativo é causado pelo desequilíbrio entre as moléculas oxidantes e as defesas antioxidantes, em favor dos oxidantes, que geralmente tem como causa os distúrbios na produção, distribuição ou geração exacerbada de EROs, desencadeado por agentes endógenos ou fatores ambientais, que causam danos severos nas células (NOVAES *et al.*, 2014; SILVA; FERRARI, 2011). EROs são moléculas que reagem com várias macromoléculas nas células, altamente reativa devido ao número ímpar de elétrons em sua última camada eletrônica, tornando-o livre (BIRBEN *et al*., 2012; COTINGUIBA *et al.*, 2013), têm sido caracterizadas como subprodutos metabólicos tóxicos e que causam várias patogenicidades (HAMANAKA; CHANDEL, 2010). Um exemplo de fatores ambientais é a exposição ao LPS, uma

endotoxina que pode atacar o sistema imunológico inato e as vias Toll, que irão responder à presença desse patógeno, e causar reações imunológicas (RAO; YU, 2010; SAMPATH, 2018).

2.2.1 Endotoxina Lipopolissacarídeo (LPS)

O sistema imunológico inato desempenha um papel crucial no combate a infecções microbianas, incluindo respostas humorais e mediadas por células a microrganismos invasores. As respostas imuno inata do hospedeiro é ativada por receptores de reconhecimento de padrões (RRPs), que reconhecemos componentes microbianos (PERI *et al.*, 2010). Podemos citar alguns RRPs já identificados, como as classes dos receptores do tipo proteínas de reconhecimento de peptidoglicano e proteínas de ligação gram-negativas (PAL; WU, 2009). Os RRPs podem reconhecer agentes microbianos, caracterizados como padrões moleculares associados a patógenos (PAMPs), como o lipopolissacarídeo (LPS) bacteriano, peptidoglicano, ácido lipoteicóico (LTA) e β-1, 3-glucanos fúngicos, presentes apenas em patógenos, mas não em células hospedeiras (RAO; YU, 2010).

Componentes microbianos podem ativar respostas imunes humorais e celulares em insetos, as respostas imunes incluem fagocitose mediada por hemócitos, formação de nódulos, cascatas enzimáticas que regulam a coagulação e a melanização da hemolinfa e a produção de espécies reativas de oxigênio (ROS) e espécies reativas de nitrogênio (RNS), e síntese de peptídeos antimicrobianos (AMPs) (LEMAITRE; HOFFMANN, 2007; MARMARAS; LAMPROPOULOU, 2009).

Os AMPs são produzidos no corpo gorduroso *D. melanogaster*, que corresponde ao fígado dos mamíferos, e são secretados na hemolinfa, onde destroem os microrganismos invasores (HOFFMANN; REICHHART, 2002). Em *D. melanogaster*, a ativação de AMPs é regulada pelas vias Toll e via deficiência imune (Imd). A via imd é ativada por LPS, de bactérias gram-negativas (GEORGEL *et al*., 2001; POTTER *et al.*, 2021), enquanto a via Toll é ativada por exemplo, por peptidoglicano e LTA de bactérias gram-positivas, (RAO; YU, 2010).

O LPS é uma importante endotoxina presente na parede celular de bactérias gram-negativas, composto pela âncora hidrofóbica conhecida como lipídio A (endotoxina), um oligossacarídeo (carboidrato) no centro, e um polissacarídeo distal,

conhecido também como antígeno específico de O (O-polissacarídeo), ainda, o lipídio A, é bem caracterizado em *Escherichia coli* (*E. coli)*, e necessário para o crescimento desta e da maioria das bactérias gram-negativas (RAETZ; WHITFIELD, 2002). O LPS se liga a receptores específicos nas células hospedeiras, induzindo a resposta imune em modelos experimentais (BING; LIU, 2011), assim como, induz estresse oxidativo, inflamação e apoptose (HOU; YANG; YIN, 2019).

Inquestionavelmente, os elétrons transportados na cadeia respiratória mitocondrial, corresponde a principal fonte de geração de EROs. Nesse sentido, sua disfunção resulta no aumento da geração de EROs, que desenvolve o estresse oxidativo (GALLEY, 2011; LAMBERT; BRAND, 2009). Sabe-se ainda, que a diminuição na atividade do complexo I pode ser devido à perda de função ocasionada pelo estresse oxidativo (KEENEY *et al.*, 2006). Em vista disso, o estudo em ratos, verificou que os efeitos do LPS, causaram inibição na atividade dos complexos I, III e IV (CIMOLAI *et al.*, 2014). Assim como, a exposição intrauterina em ovelhas ao LPS, induz estresse oxidativo e inibição complexo II e IV (SONG *et al.*, 2013). Além disso o LPS aumenta geração de óxido nítrico e a imunoreatividade da proteína quinase ativada por mitógeno p38 (p38 MAPK), proteína responsável por regular o estresse oxidativo (XING *et al.*, 2008). Nesse sentido, esses dados nos confirmam o envolvimento do LPS com a disfunção da cadeia transportadora de elétrons, com a inibição dos complexos e consequente aumento do estresse oxidativo.

2.3 Marcadores de estresse oxidativo

A avaliação da toxicidade é caracterizada por identificar o efeito de uma determinada substância em causar danos após a exposição/administração a uma dose, onde os efeitos adversos ocorridos em determinado intervalo de tempo são analisados (STRICKLAND *et al.*, 2018). A exposição à produtos tóxicos podem ocasionar alterações na atividade da Acetilcolinesterase (AChE) e ocasionar a reprodução de comportamentos atípicos, assim como alterar as defesas antioxidantes enzimáticas e não enzimáticas, sendo assim, a avaliação do desequilíbrio redox vem sendo estudado (FERNANDES *et al.*, 2021; MUSACHIO *et al.*,2020).

O estudo direcionado à modulação da enzima AChE pode ser para fins de investigar meios de inibir a atividade da AChE frente à exposição de diferentes

compostos, podendo ser por meio da indução da toxicidade (AKINYEMI *et al.*, 2017). A AChE é a enzima responsável por hidrolisar a acetilcolina (ACh) em colina e acetato, a ACh é um neurotransmissor presente nas sinapses colinérgicas que atua na transmissão sináptica no sistema nervoso central, e tem papel vital na regulação das atividades locomotoras e, portanto, alterações na AChE pode comprometer a neurotransmissão colinérgica (CRAIG; HONG; MCDONALD, 2011). Sabe-se que a AChE está diretamente relacionada ao comportamento locomotor e exploratório em *D. melanogaster* e que esta enzima é sensível a condições de estresse oxidativo (DE FREITAS COUTO *et al.*, 2019; FERNANDES *et al.*, 2021). Diante disso, evidencia-se a importância dessa enzima no entendimento das questões relacionadas as *alterações* motoras e toxicidade. Assim, são de suma importância estudos que avaliem a ação de compostos bioativo capazes de regular a atividade da AChE (ZHU *et al.*, 2008).

Organismos aeróbicos geram EROs como consequência da respiração e da oxidação de substratos, em níveis baixos são de grande importância para as células por possuírem funções em muitos processos bioquímicos, porém, níveis altos induz estresse oxidativo, podendo causar danos severos à célula e até morte celular (YANG; LEE, 2015). As EROs incluem substâncias tóxicas contendo um ou mais átomos de oxigênio ativo, decorrente da redução do oxigênio (ARAÚJO, 2018). Níveis de RS e TBARS são exemplos de metabólitos da oxidação de substratos, e são marcadores de toxicidade não enzimáticos amplamente utilizados, seus níveis elevados são indicadores de peroxidação lipídica e dano oxidativo (NIU *et al.*, 2008).

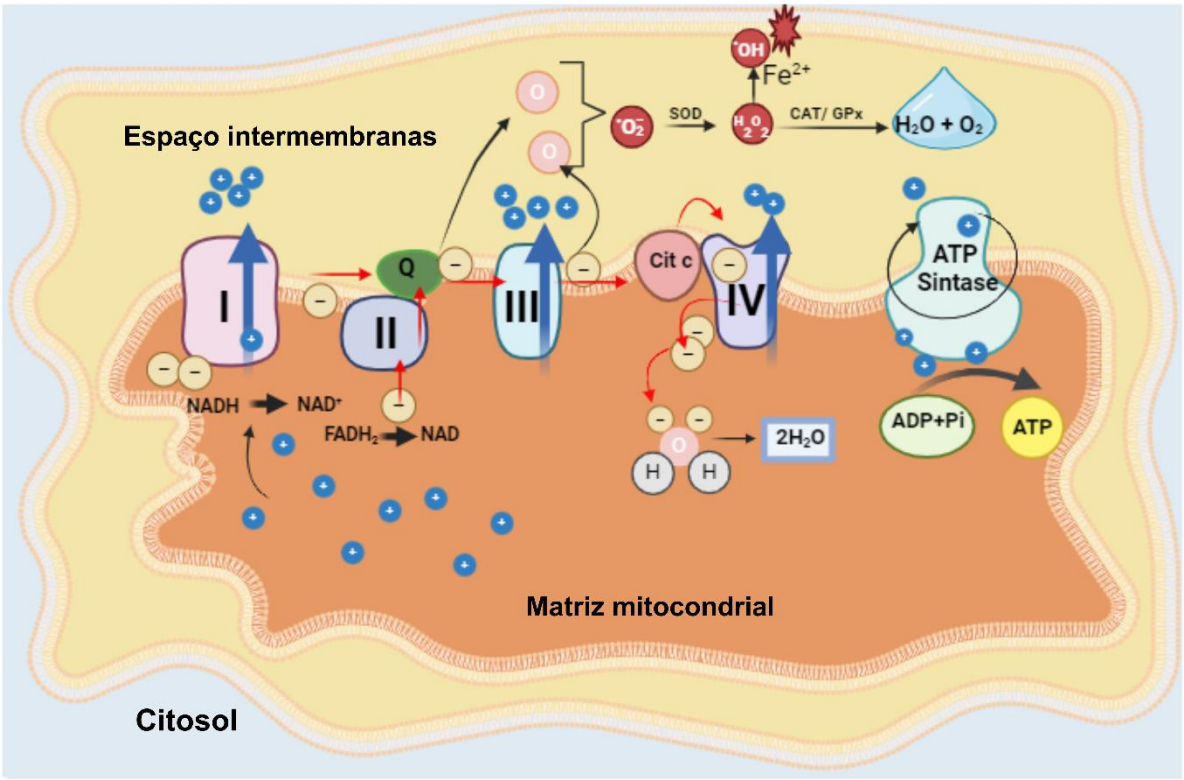
O estresse oxidativo é uma situação fisiológica caracterizada pelo desequilíbrio entre a produção de espécies reativas de oxigênio e as defesas antioxidantes do organismo. O *desequilíbrio* entre EROs e defesa antioxidante é responsável por causar danos severos na célula, nessa situação crítica, são produzidas EROs de forma exacerbada como um subproduto da respiração aeróbica, uma vez que, a mitocôndria a principal fonte endógena de EROs (FINKEL; HOLBROOK, 2000).

A mitocôndria é a organela celular encarregada pela produção de adenosina trifosfato (ATP) nas células eucarióticas. No espaço intermembranas, estão presentes as enzimas como a SOD, CAT e GPx, que integram a cadeia transportadora de elétrons, onde equivalentes reduzidos doam seus elétrons, oriundos de moléculas

orgânicas, para converter adenosina difosfato em ATP, através da fosforilação oxidativa (Figura 3).

O complexo I é responsável pela oxidação e transporte de nicotinamida-adenina-dinucleótido reduzida (NADH) e dinucleótido de flavina e adenina (FADH2), utilizando a ubiquinona (Q) como aceitador de elétrons, processos que contribuem para fornecimento de energia pelo metabolismo aeróbico. Durante o transporte dos elétrons, entre os complexos, o oxigênio molecular (O_2) é usado como aceptor final de elétrons. Esse transporte gera o bombeamento de prótons da matriz mitocondrial para o espaço intermembranas, gerando o potencial eletroquímico necessário para a síntese de ATP no processo de fosforilação oxidativa (FERREIRA; AGUIAR; VILARINHO, 2008).

Figura 3. Defesas antioxidantes enzimáticas presentes na cadeia transportadora de elétrons e fosforilação oxidativa.



Fonte: Arquivo pessoal.

Segundo estimativas, em condições normais, cerca de 0,5% dos elétrons escapam, principalmente nos complexos I e III, logo, reagem com o O_2, formando o

ânion superóxido, o qual pode reagir com proteínas e lipídios de membrana, acarretando em disfunções e danos celulares (MURPHY, 2009).

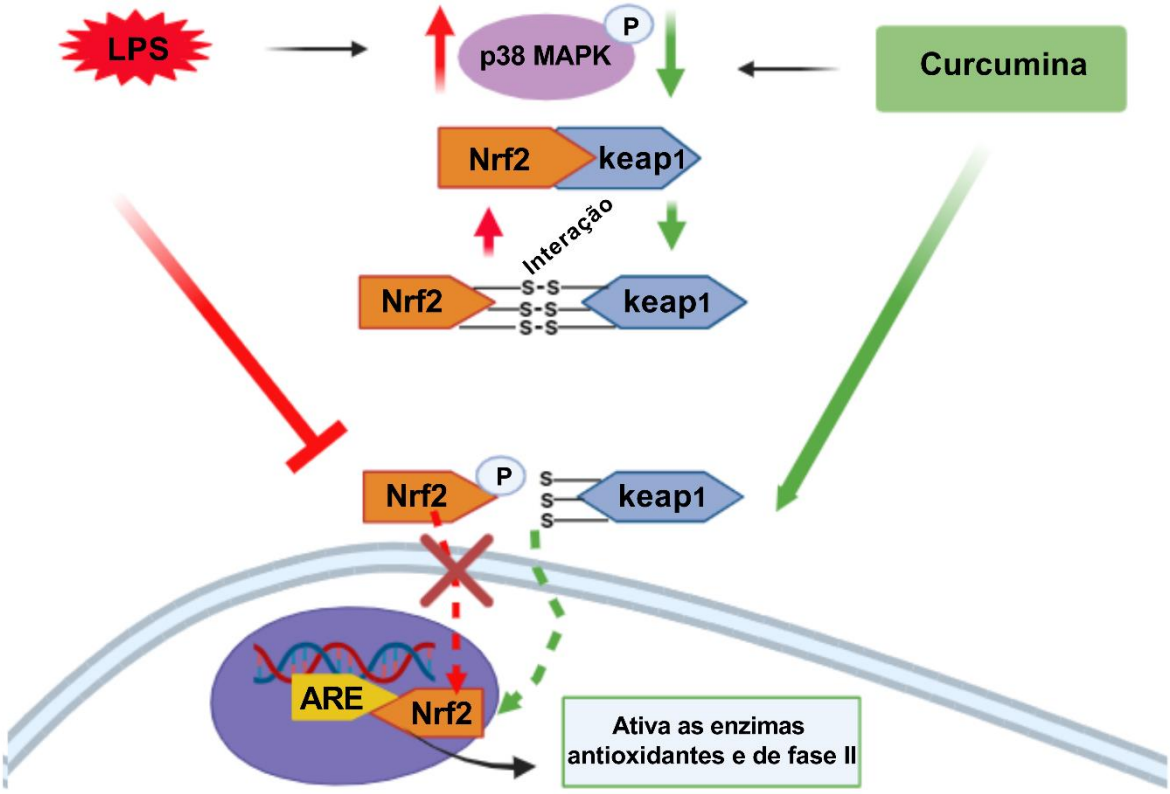
No entanto, as defesas antioxidantes enzimáticas, são responsáveis por impedir o estresse oxidativo, como a enzima antioxidante SOD, que é responsável por catalisar a conversão do ânion superóxido ($O2^{-\bullet}$) em peróxido de hidrogênio (H_2O_2), espécie menos reativa (WANG *et al.*, 2018). Já o H_2O_2 gerado, pode reagir com o íon ferroso (Fe^{2+}), formando o radical hidroxila (OH) e o íon férrico (Fe^{3+}), evento conhecido como reação de Fenton, e sabe-se que o OH, apresenta uma alta instabilidade, sendo altamente reativo, que provoca danos oxidativos em proteínas, peroxidação lipídica e alterações no DNA, além disso, não existindo uma enzima que possa catalisar sua degradação (IMLAY; CHIN; LINN, 1988). Nesse caso, a enzima CAT, catalisa a decomposição de H_2O_2 em oxigênio e água, e desempenha papéis críticos na proteção das células contra o estresse oxidativo (GLORIEUX; CALDERON, 2017). A concentração destes radicais é mantida pelo balanço entre a sua produção e sua eliminação por antioxidantes (DRÖGE, 2002).

Ainda, podemos citar a enzimas de detoxificação GST, uma família de enzimas de fase II que catalisam a conjugação de intermediários reativos de compostos eletrofílicos com a GSH citosolica, processo importante na desintoxicação de xenobiótico, defesa contra o estresse oxidativo (OYETAYO *et al.*, 2020), e modula da proliferação celular (PALLARDÓ *et al.*, 2009). Achados revelam que os genótipos nulos de glutationa S-transferase teta-1 (GSTT1), uma classe de GST pode ser um fator crucial em relação à mortalidade e letalidade da COVID-19 (SAADAT, 2020). Níveis significativamente mais baixos das enzimas SOD, CAT, GSH e GPx são relatados em pacientes com COVID-19 comparados aos indivíduos saudáveis, os quais exibem maior chance de apresentar níveis reduzidos de defesas antioxidantes devido ao aumento de sua utilização para combater o estresse oxidativo na doença (MUHAMMAD *et al.*, 2021). Ainda, a atividade dessas enzimas pode ser regulada pelo Nrf2, o fator de transcrição incumbido em ativar diversas enzimas antioxidantes, atuando quando há uma grande quantidade de EROs nas células (ISHII *et al.*, 2000).

O Nrf2, é uma proteína que atua como um regulador chave do sistema de defesa antioxidante, está ligado à proteína 1 associada à ECH semelhante a Kelch (Keap1) no citoplasma (JI *et al.*, 2013). Foram descritas vias apoptóticas que regulam negativamente o Nrf2, incluindo a proteína p38 MAPK (YAMAMOTO; KENSLER;

MOTOHASHI, 2018). O estresse oxidativo causado, causado por agentes tóxicos, incluindo o LPS (XING et al., 2008), pode agir diretamente e induzir apoptose (SAMARGHANDIAN; AZIMI-NEZHAD; SAMINI, 2014; XING *et al.*, 2008), através das vias quinases, incluindo a p38 MAPK, que é responsável por fosforilar o Nrf2, em três resíduos Ser (Ser215, Ser408 e Ser577), melhorando a interação Nrf2-Keap1, e inibindo a translocação de Nrf2 para o núcleo e consequentemente, reduzindo o acúmulo de Nrf2 no núcleo celular (KEUM *et al.*, 2006; YAMAMOTO; KENSLER; MOTOHASHI, 2018). No entanto, a inibição da atividade das quinases p38 MAPK, favorece a separação de Nrf2/keap1, e o fator de transcrição Nrf2 tem maior facilidade de translocar-se para o núcleo (NAIDU *et al.*, 2009), onde o Nrf2 se liga ao elemento de resposta antioxidante (ERA) e ativa enzimas antioxidantes ou de detoxificação de fase II (BALOGUN *et al.*, 2003). A inibição de p38 MAPK pode ser induzida pela Curcumina (UZUN-GOREN; UZ, 2022).Esquema mostrado na figura 4.

Figura 4. Esquema da inibição de p38 MAPK por LPS e aumento de Nrf-2 induzido pela curcumina



Fonte: Arquivo pessoal.

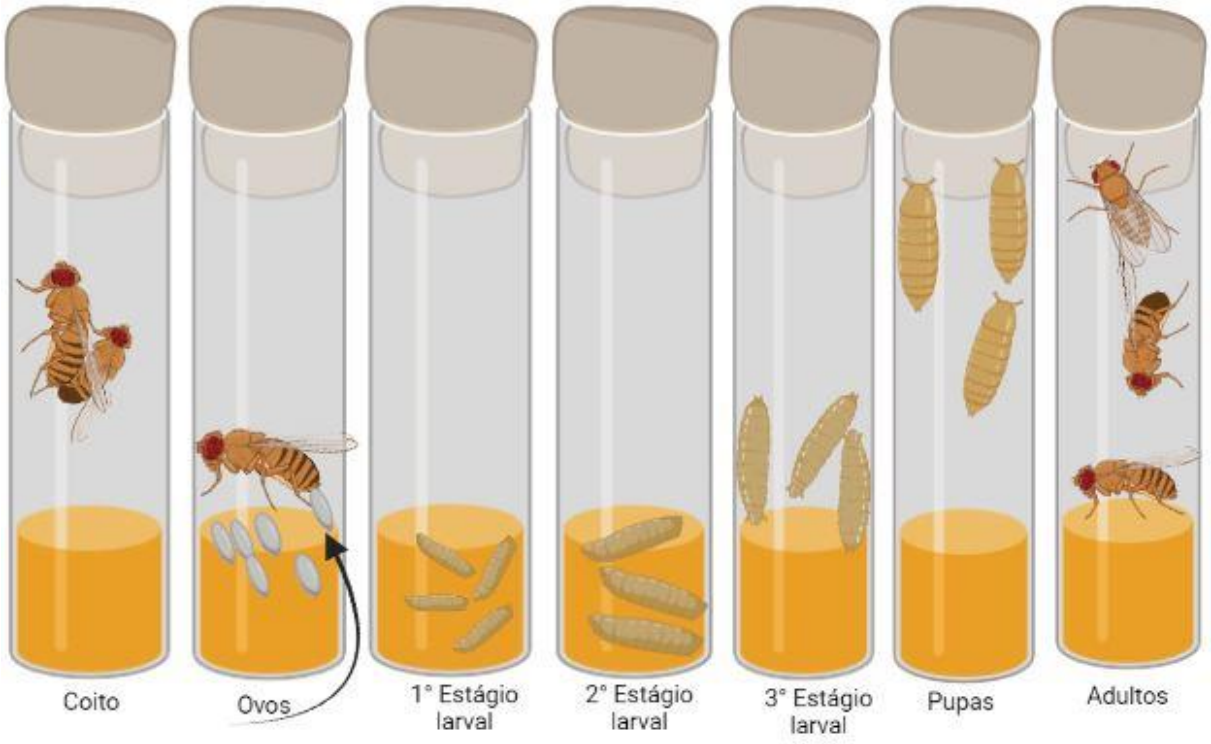
Assim, com base na literatura, pode-se especular que a diminuição de P38 MAPK e aumento de Nrf2, e consequentemente a atividade aumentada da SOD, CAT e GST, pode ter efeitos benéficos, e que a curcumina quando administrada, poderia modular essas vias, e consequentemente regular a resposta imune.

2.4 Modelo de *Drosophila melanogaster*

Entre os insetos, a mosca *D. melanogaster* tem sido usada como um modelo único e grandioso para estudos de genética e doenças humanas. Esse modelo, apresenta cerca de 75% de homologia genética à mamíferos, incluindo estrutura e funcionamentos cerebrais, somado a isso, o modelo pode ser utilizado para elucidar mecanismos de efeitos de substâncias tóxicas a humanos (NAGOSHI, 2018; PANDEY; NICHOLS, 2011). Além disso, o seu ciclo de desenvolvimento rápido possibilita a replicação de vários experimentos, em tempo relativamente curto, e assim contribui para uma avaliação dos efeitos toxicológicos dos compostos (PANDEY; NICHOLS, 2011).

Nesse sentido, sabe-se que o desenvolvimento da *D. melanogaster* é composto por 4 estágios: ovo (embrião), larva (1°, 2° e 3° estágio), pupa e mosca adulta, o desenvolvimento embrionário da acontece no meio em que o ovo é oposto (BROOKHEART; DUNCAN, 2016), conforme é mostrado na figura 5. O embrião se desenvolve no ovo por cerca de 1 dia. Após, eclode como uma larva de 1° estágio, após mais 1 dia se desenvolve a larva de 2° estágio, depois de mais 1 dia, muda para a larva de 3° estágio. A larva de 3° estágio fica no meio alimentar e continua se alimentando, mas começa a subir e explorar as laterais do frasco da dieta, procurando um ambiente mais seco, após 2 ou 3 dias, se desenvolve em pupa. Durante essa fase de pupa, a larva metamorfoseia em mosca adulta, ao longo de 4 dias (JENNINGS, 2011). Em torno de 12 horas após eclodir a fêmea pode acasalar e em poucos dias pode colocar centenas de ovos (NICHOLS, 2006) (NICHOLS,2006).

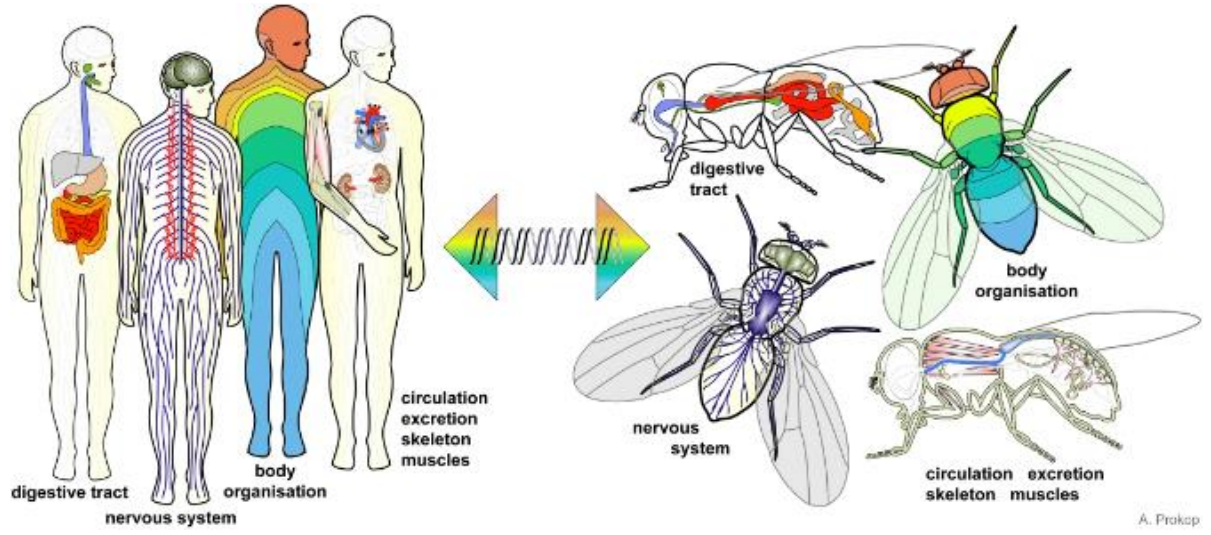
Figura 5 - Desenvolvimento da *Drosophila melanogaster*



Fonte: Arquivo pessoal.

As moscas têm requisitos fisiológicos semelhantes aos humanos, como organização corporal, sistema nervoso, movimento, respiração, digestão e excreção (HELD, 2017), como ilustrado na Figura 6.

Figura 6 - Homologia entre órgãos humanos e moscas



Fonte: adaptado de https://droso4schools.wordpress.com/organ

A *Drosophila* é modelo de organismo promissor para avaliar efeitos tóxicos com ênfase em dano oxidativo, como os mediados por nanomateriais, devido à genética bem documentada e biologia do desenvolvimento que permite avaliar os efeitos de longo prazo em um curto período de tempo, e por apresentar menos objeções éticas e ter custo relativamente mais baixo, comparado a outros modelos experimentais (AHAMED *et al.*, 2010). *Drosophila* é depende de sistema imunológico de reações imunes inatas para sua defesa, sendo um modelo prestigiado para estudar a imunidade inata, que permanece conservada e bem desenvolvida, que permite uma resposta geral e rápida aos agentes infecciosos em moscas (HOFFMANN; REICHHART, 2002). O sistema imune inato de *Drosophila* depende de respostas humorais que incluem vários peptídeos antimicrobianos, cascatas enzimáticas que regulam a coagulação e melanização da hemolinfa, assim como, produção de ROS e espécies reativas de nitrogênio (ERN). Já as respostas imunes celulares, incluem fagocitose, nodulação e encapsulamento (MARMARAS; LAMPROPOULOU, 2009).

3. JUSTIFICATIVA E HIPÓTESE

O conhecimento de compostos que tenham potencial ação sobre o estresse oxidativo e a inflamação pode ser uma alternativa para tratar ou aliviar sintomas de doenças já estabelecidas e sem tratamentos. Devido ao fato de que a curcumina livre e nanoencapsulada poderem apresentar toxicidade. Se faz necessário avaliar se nanocápsulas carregadas com curcumina apresenta toxicidade, assim como potencializar seus efeitos terapêuticos. Desse modo, levantamos a hipótese, de que seria de fundamental importância, verificar se a nanoencapsulação da curcumina em P80, apresenta ou não toxicidade, e se potencializa os efeitos terapêuticos desse polifenol, em modelo pré-clínico induzido por LPS. Para que posteriormente, este estudo possa alicerçar outros que permitam a aprovação dessa nano formulação como alternativa terapêutica segura. Dentre as estratégias que foram abordadas neste estudo, encontram-se avaliação toxicológica da curcumina livre e associada à utilização de nanocarreadores P80, e o efeito dessa nano formulação sobre os danos toxicológicos induzidos por LPS, visando explorar o potencial de ação das nanocápsulas carregadas com curcumina e possíveis aplicações futuras.

4. OBJETIVOS

4.1 Objetivo geral

Investigar um possível efeito tóxico da exposição crônica de *D. melanogaster* à nanocápsulas carregadas com curcumina e avaliar seu efeito protetor sobre a toxicidade induzida por LPS.

4.2 Objetivos específicos

- Investigar uma possível toxicidade induzida pela exposição crônica de *D. melanogaster* à nanocápsulas carregadas com curcumina, através avaliação do consumo da dieta, porcentagem de sobrevivência, testes de desempenho locomotor e exploratório e atividade da enzima AChE;
- Investigar uma possível toxicidade induzida pela exposição crônica de *D. melanogaster* à nanocápsulas carregadas com curcumina, através da determinação de indicadores de estresse oxidativo;
- Avaliar o efeito protetor de nanocápsulas carregadas com curcumina sobre a toxicidade induzida por LPS, através da avaliação do consumo da dieta, porcentagem de sobrevivência, testes comportamentais de desempenho locomotor e exploratório;
- Avaliar o efeito protetor de nanocápsulas carregadas com curcumina sobre a toxicidade induzida por LPS, através da determinação de indicadores de estresse oxidativo;
- Avaliar o efeito protetor de nanocápsulas carregadas com curcumina sobre a toxicidade induzida por LPS, através da avaliação da imunoreatividade de Nrf2 e p38 MAPK.

PARTE II

5. RESULTADOS

Os resultados os quais fazem parte desta tese apresentam-se sob a forma de um artigo, o qual encontra-se aqui sob o título: “Evaluation of oxidative stress indicators as toxicity parameters after chronic exposure of *Drosophila melanogaster* to free curcumin and curcumin-loaded nanocapsules”. O artigo já foi publicado no ano de 2023 na revista “*Food and Chemical Toxicologi*”.

Além disso, os resultados apresentam-se sob a forma de um MANUSCRITO CIENTÍFICO, o qual encontra-se aqui estruturado sob o título: Curcumin-loaded nanocapsules attenuate LPS-induced toxicity in *Drosophila melanogaster*: through the mechanism of regulation of apoptosis and balance redox. Ainda constam nesse item, a seções: Materiais e Métodos, utilizada para a produção dos Resultados obtidos, assim como Introdução, Discussão, Conclusão e Referências Bibliográficas. O manuscrito apresenta-se formatado de acordo com as normas da revista que será submetido “*Food and Chemical Toxicologi*”

5.1 ARTIGO CIENTÍFICO

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Evaluation of oxidative stress indicators as toxicity parameters after chronic exposure of *Drosophila melanogaster* to free curcumin and curcumin-loaded nanocapsules

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ABSTRACT

We investigated a possible toxic effect induced by chronic exposure to free curcumin and curcumin-loaded nanocapsules in Drosophila melanogaster, enabling safe applications. Flies of both sexes were divided into groups: control group; free curcumin at concentrations of 10, 30, 100, 300, 900, and 3000 μM; curcumin-loaded nanocapsules at concentrations of 10, 30, 100, and 300 μM. Initially, the diet consumption test was evaluated in flies exposed to different concentrations. During the 10-day treatment, the flies were evaluated for percentage survival. After the treatment, behaviors (geotaxis negative and open field), acetylcholinesterase activity (AChE), and oxidative stress parameters (reactive species (RS) and thiobarbituric acid reactive substances (TBARS) levels, Glutathione-S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT) enzymes activity, erythroid-derived nuclear factor 2 (Nrf2) immunoreactivity, and cellular metabolic capacity, were assessed. No significant difference in diet consumption, indicating that the flies equally consumed the different concentrations of free curcumin and the curcumin-loaded nanocapsules. Was observed that free curcumin and curcumin-loaded nanocapsules increased survival, locomotor and exploratory performance, decreased AChE activity, RS and TBARS levels, increased GST, SOD and CAT activity, Nrf2 and viable cells compared to the control. The chronic treatment did not cause toxicity, suggesting that nanoencapsulation of curcumin could be explored.

1. Introduction

Oxidative stress is a physiological condition that affects organisms with an imbalance between the production of reactive oxygen species (ROS) and antioxidant capacity. In this process, intracellular ROS levels exceed the capacity of enzymes and endogenous antioxidant molecules to neutralize or eliminate them from the body, resulting in the oxidation of macromolecules and nucleic acids, causing damage and even cell death (Hajam et al., 2022). Cellular dysfunction induced by oxidative stress alters the physiological environment of metabolic, oxidative, inflammatory and apoptosis pathways, acting as an etiological factor of several pathologies (Rani et al., 2016). Therefore, alternatives that protect the body against oxidative stress are relevant. In this context, bioactive compounds extracted from plants and with antioxidant, anti-cancer, antimicrobial, anti-inflammatory and neuroprotective action, such as curcumin, a curcuminoid of yellow color with hydrophobic characteristics found in the rhizome of *Curcuma longa*, a representative shrub of the Zingiberaceae family, have been investigated as adjuvant alternative therapies (Sueth-Santiago et al., 2015; Yavarpour-Bali et al., 2019).

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Our research group has developed curcumin-loaded nanocapsules using poly(ε-caprolactone) (PCL) as polymer and polysorbate 80 as nonionic surfactant (Santos et al., 2021). The nanosuspension showed anionic surface charge and able to controlled curcumin *in vitro* release. Curcumin-loaded nanocapsules have not been demonstrated to alter organogenesis in pregnant rats (Giacomeli et al., 2020) and have not induced teratogenesis in a Chick embryo modelo (de Carvalho et al., 2021).

However, despite the beneficial properties of bioactive compounds, they can present toxicity, depending greatly on the duration of the treatment and the doses used. High doses of curcumin produce prooxidative, toxic, and carcinogenic effects (López-Lázaro, 2008), hepatocellular damage in rodents (Balaji and Chempakam, 2010), cytotoxic damage to human gastric cancer cell lines (Li et al., 2017) and non-cancerous cell lines (Nelson et al., 2017). It has also increased the mortality of zebrafish embryos, and deformities of the physical body of larvae were observed among the hatched embryos at higher concentrations (Alafiatayo et al., 2019). Thus, a definition of the toxicological profile of bioactive compounds, especially for long-term treatments, is extremely important.

In this sense, nanoparticles emerge as a strategy to increase the bioavailability of compounds, obtaining therapeutic effects from smaller doses and, in most cases, presenting less toxicity (Nazila et al., 2016).

Due to the toxicological risks of free curcumin, safe alternatives are an extreme necessity, and assessing whether nanoencapsulation can improve biological activity and decrease toxicity. In that context, the fly *Drosophila melanogaster* is efficient for toxicity studies (Ahamed et al., 2010). Thus, the present study aims to investigate a possible toxic effect induced by chronic exposure to free curcumin and curcumin-loaded nanocapsules in *D. melanogaster*, enabling future investigations and safe applications of curcumin.

2. Materials and methods

2.1. Materials

Free curcumin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and diluted in 98% ethanol. To overcome the disadvantages of free curcumin, it was nanoencapsulated in nanometric devices. The polymer used was Polysorbate 80 (P80), which was purchased from Sigma-Aldrich (St. Louis, MO, USA). All the other chemicals and solvents utilized were of analytical grade.

2.2. Development of curcumin-loaded nanocapsules

Curcumin-loaded nanocapsules formulations were provided by the Nanobiotechnology Laboratory of the Federal University of the Pampa, UNIPAMPA, Uruguaiana campus, RS, Brazil. Curcumin-loaded nanocapsules were prepared with poly (ε-caprolactone (PCL) as polymer and coated with polysorbate 80 (P80) as nonionic surfactant, through the interfacial polymer deposition method, according to previous studies published by the research group (Santos et al., 2021). Following this method, the organic phase was composed of Curcumin, PCL (1% w/v), medium chain triglycerides (TCM) (3.3% w/v), and Span 60® (0.78% w/v), dissolved in acetone, at 40 ± 2 °C, was injected into the aqueous phase composed of distilled water and P80 (0.78% w/v), which were maintained under magnetic stirring for 10 min. Subsequently, a rotary evaporator was used to evaporate the acetone and to obtain a final desired concentration (Curcumin 0.6 mg mL^{-1}). Nanocapsules were characterized in terms of diameter, polydispersity index (SPAN), pH, drug content and encapsulation efficiency (Santos et al., 2021). Analyzes were performed in triplicate. The diameter of the nanoparticles and the polydispersity were analyzed by laser diffraction at room temperature and dilution of the sample in deionized water, using the Mastesizer 2000 equipment (Malvern®), and by dynamic light scattering in which the suspensions were diluted in NaCl 0.9% (w/v) (previously filtered) (NanoBrook 90 Plus Zeta, BrookHaven®). The pH of the formulations was determined through measurements of the formulations, using a potentiometer (HANNA®) previously calibrated with pH 7.01 and 4.01. To determine the drug content, the formulations were dissolved in methanol, filtered and quantified by high-performance liquid chromatography with diode array detector (HPLC-PDA), using a method previously validated by the research group (de Oliveira Pacheco et al., 2022). The encapsulation efficiency was determined by the ultrafiltration/centrifugation technique (Ultrafree-Millipore®), in which the suspension was centrifuged at 1960 g for 10 min at 4 °C, and the ultrafiltrate was quantified by HPLC-PDA, determining the concentration of curcumin. All analyzes were performed in triplicate.

2.3. Drosophila melanogaster stock and culture

This study used flies (*Drosophila melanogaster* – Harwich strain) obtained from the Laftambio laboratory (Unipampa, Itaqui-RS). The *D. melanogaster* were kept in glass flasks, under controlled conditions of light (light/dark cycle of 12/12 h), temperature (25 ± 1 °C), and humidity (60–70%), and fed with a standard diet containing, 76.59% corn flour, 8.51% wheat germ, 7.23% sugar, 7.23% milk powder, 0.43% salt, 0.08% methylparaben.

2.4. Experimental methodological schedule

2.4.1. Comparison between the effect of chronic exposure to free curcumin and curcumin-loaded nanocapsules in the modulation of diet consumption, survival, behavioral tests, AChE activity, and oxidative stress markers

To evaluate the toxicity of chronic exposure to free curcumin and curcumin-loaded nanocapsules, cohorts of adult *D. melanogaster* (1–3 days old) of both sexes were used, containing 25 female and 25 male in each group. The free curcumin and curcumin-loaded nanocapsules were dissolved in 0.5% ethyl alcohol and water, respectively, and then mixed in 10 mL of the standard diet. The experimental groups were subdivided into a control group (standard diet containing 0.5% ethyl alcohol), groups of different free curcumin concentrations (10, 30, 100, 300, 900, and 3000 μM), another control group (standard diet containing only water), and groups of different curcumin-loaded nanocapsules concentrations (10, 30, 100, and 300 μM). The flies were kept under treatment for 10 days. The curcumin doses used in this study were based on the study by Akinyemi et al. (2017), which showed that free curcumin concentrations up to 1 mg/g of diet did not cause obvious signs of toxicity in *D. melanogaster*, as evidenced in the survival curve and biochemical assays. Initially, the diet consumption test was evaluated in flies exposed to different concentrations for 30 min. The flies were evaluated for survival percentage during the 10 days of exposure. After the treatment period, behavioral tests of locomotor and exploratory performance (negative geotaxis and open field) were performed, as well as biochemical evaluations of acetylcholinesterase activity (AChE); oxidative stress markers, such as levels of reactive species (RS) and thiobarbituric acid reactive substances (TBARS); activity of the detoxification enzyme Glutathione-S-transferase (GST), and the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), erythroid-derived nuclear factor 2 (Nrf2) immunoreactivity; and cellular metabolic capacity by the resazurin test. These assessments are demonstrated in the experimental methodological schedule (Fig. 1).

2.5. Diet consumption

The estimate of diet consumption containing the treatment was performed according to Sun et al. (2013). Groups of 5 flies were transferred to each flask containing the treatment medium with the addition of 0.5% dye (FD&C Blue dye #1, Sigma Aldrich), and allowed to feed on this medium for 30 min. After that time, feeding was interrupted and the flies were immediately transferred to an microcentrifuge tube and euthanized on ice, and soon the body was separated from the head. The

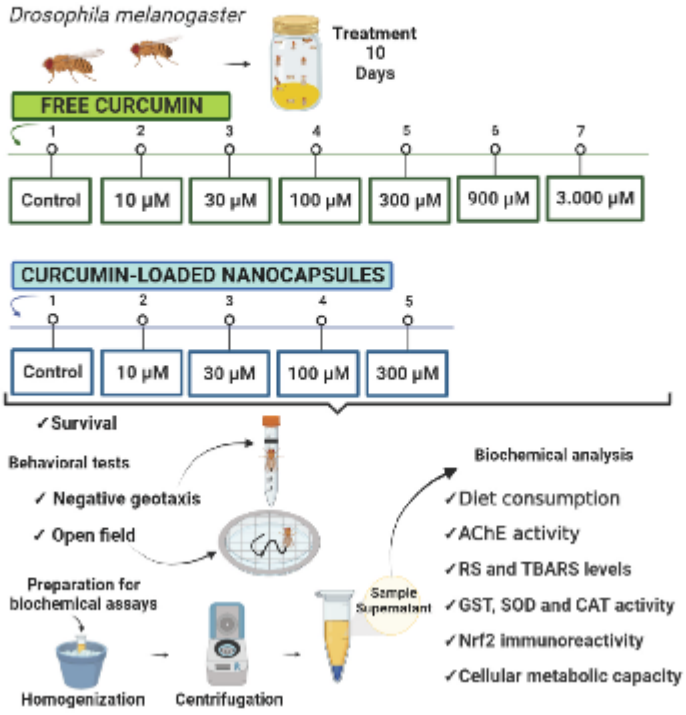

Fig. 1. Experimental methodological schedule. Effect of chronic exposure to free curcumin and curcumin-loaded nanocapsules for 10 days on the modulation of diet consumption, survival, behavioral tests, AChE activity, and oxidative stress markers in *D. melanogaster*.

5 bodies from each group were homogenized in 200 μL of distilled water. The homogenate was centrifuged at 12,000 g for 2 min, and the supernatant was pipetted in duplicate. Absorbance was read on a microplate reader at 625 nm. The absorbance of the fly supernatant who consumed the diet without dyes was used as a standard curve. The absorbance results of five independent experiments were expressed as percentage in relation to the control group.

2.6. Survival percentage

The percentage of survival after exposure to free curcumin and curcumin-loaded nanocapsules was analyzed by counting once a day the number of live *D. melanogaster* adults until the end of the experimental period of 10 days. Each group contained 50 adult flies of both sexes. Five to six independent experiments were performed. The data were compared using the Log-rank test (Mantel-Cox), expressed as percentage of surviving flies (Fernandes et al., 2021).

2.7. Behavioral tests

2.7.1. Negative geotaxis assay

To assess the locomotion and climbing ability of *D. melanogaster*, the negative geotaxis test was performed as described by Feany and Bender (2000), with some modifications. After 10 days of exposure to the treatment, the assay was performed with five flies from each group. The flies were briefly anesthetized on ice and individually transferred to clear tubes held vertically. After 10 min of recovery, the tubes containing the flies were tapped lightly on a bench so that the flies remained at the bottom of the tube, and then, with the aid of a stopwatch, the time spent by each fly to reach the 8 cm mark at the top of the tube was recorded. The maximum evaluation time was 120 s for each fly. The test was repeated five times for each fly, with a time interval of 3 min between repetitions, and the data were analyzed according to individual time. Data are expressed as the time mean of five to seven independent experiments.

2.7.2. Open field test

The exploratory activity was performed through the open field task according to the method of Connolly (1966) with modifications by Musachio et al. (2020). The flies were briefly anesthetized on ice, then five flies from each group were separated and individually added to a transparent polycarbonate Petri dish measuring 9 mm in diameter, divided by squares measuring 1 cm^2 each. At the end of the 2 min recovery period, the test was started. The score of crossings traveled by the flies was recorded manually and determined during 60 s, timed with the aid of a stopwatch. The activity was obtained by counting the number of squares that each fly traveled in 1 min. The test was performed in duplicate and the mean values were calculated. Data represent the mean of five to six independent experiments.

2.8. Homogenized preparation for biochemical assays

After the end of the treatment period of 10 days, the flies were euthanized on ice, homogenized, and centrifuged according to each analysis to be performed. The resulting supernatant was used for the biochemical determinations.

2.9. Determination of protein concentration

The determination of protein concentration in the samples was carried out according to the method of Bradford (1976), using bovine serum albumin (BSA) as a standard. Samples were read in duplicate, using a wavelength of 595 nm.

2.10. Acetylcholinesterase enzyme activity

The activity of the acetylcholinesterase (AChE) enzyme was determined according to the previously described method by Ellman et al. (1961). For this, 10 flies were homogenized in 500 μL of HEPES buffer (20 M, pH 7.0), and centrifuged at 78 g for 10 min. A reaction mixture was prepared with 0.1 M Kpi buffer (pH 8.0), 5,5-dithio-bis-2-nitrobenzoic acid (DTNB/5 mM), and distilled water. In the moment of the analysis, 20 μL of the sample supernatant and 170 μL of the mixture were added, and the reaction was started with 10 μL of acetylthiocholine (AcSCh) (7.25 mM) used as a substrate, which was added to the microplate. The rate of hydrolysis of acetylthiocholine iodide was measured at 412 nm for 2 min. Each sample was evaluated in duplicate. Five independent experiments were performed, and the results were expressed as μmol AcSCh/min/mg protein.

2.11. Quantification of levels of reactive species

The levels of reactive species (RS) were quantified using the method proposed by Pérez-Severiano et al. (2004), which consists of the oxidation of 2,7-dichlorofluorescein diacetate (DCFHDA) in its form (DCFH), in which emits fluorescence for reading. Ten flies were homogenized in N-(2-hydroxyethyl) piperazine-N-(2-ethane sulfonic acid) buffer (20 M HEPES, pH 7.0), and then centrifuged at 1000 g for 5 min at 4 °C. Sample supernatant tissue was transferred to test tubes, together with 2890 μL of HEPES buffer and finally 10 μL of DCFDA (1 mM). The reaction mixture was incubated for 1 h in the dark at room temperature. In the end, the reading was performed in a fluorimeter, at an excitation wavelength of 488 nm and 520 nm of emission, using a light beam of 2.5 mm. Six to seven independent experiments were performed and the results were expressed as percentage in relation to the control group.

2.12. Evaluation of thiobarbituric acid reactive substances levels

The analysis of thiobarbituric acid reactive species (TBARS) levels was used to evaluate lipid peroxidation, through the quantification of malondialdehyde (MDA), according to Ohkawa et al. (1979), followed by some adaptations for *D. melanogaster*. Eight flies were homogenized in 400 μL of 20 mM HEPES buffer (pH 7.0), and the homogenate was centrifuged at 78 g for 10 min. Sequentially, 250 μL of thiobarbituric acid (TBA, 0.8%, pH 3.2), 250 μL of 0.45 M acetic acid (pH 3.4), 50 μL of distilled water, 100 μL of sample supernatant, and 100 μL of sodium dodecyl sulfate (1.2%) were added to a test tube. The reaction mixture was incubated for 2 h in a water bath at 95 °C. After that time, the samples were cooled to room temperature and transferred to microplates for the readings, which were performed at a wavelength of 532 nm. Absorbance was corrected to protein value, and the results of six to nine independent experiments were expressed as nmol MDA/mg protein.

2.13. Evaluation of enzymatic activity: Glutathione-S-transferase, superoxide dismutase, and catalase

The preparation of samples to evaluate the activity of the detoxification enzyme GST, and the antioxidant enzymes SOD and CAT followed the same procedures. Thus, 12 flies were homogenized in 600 μL of a 20 M HEPES buffer (pH 7.0), and centrifuged at 78 g for 10 min. The supernatant obtained was used for the analysis, according to the methodology used for each enzyme.

The assessment of GST activity was performed as described by Habig and Jakoby (1981). From the obtained supernatant, 10 μL was transferred to a microplate, and then 185 μL of the mixture was added, composed of 0.25 M Kpi/2.5 mM EDTA buffer (pH 7.0), 100 mM GSH, and distilled water. Finally, 5 μL of 50 mM 1-chloro-2-4-dinitrobenzene (CDNB) was added as a reaction initiator. The reading was performed at a 340 nm wavelength for 2 min. Absorbance results were corrected to protein value, and the results of six to eight independent experiments were expressed as mU/mg protein.

The evaluation of SOD activity was performed according to the protocol described by Misra and Fridovich (1972), with modifications by Sun and Zigman (1978). An aliquot of the sample supernatant was diluted in HEPES buffer, in a 1:10 ratio, respectively. The assay was performed in microplates, where 6, 12, and 18 μL of the sample were pipetted, as well as 254, 248, and 242 μL of Na_2CO_3 sodium carbonate buffer, 57.7 mM (pH 11.40, kept at a temperature of 30 °C), respectively, and 30 μL of epinephrine (6 mM), dissolved in 0.1M HCl (200 μL of HCl, over 9.8 μL of distilled water, pH 2.0). To prepare the blank, 260 μL of Na_2CO_3 buffer was used, plus 30 μL of the epinephrine solution. This method is based on the inhibition of the superoxide anion reaction in the autoxidation of epinephrine. The absorbance of different sample volumes was determined by evaluating the formation of a pink adrenochrome product in a kinetic cycle with 12 readings of 10 s, at a wavelength of 480 nm. The results were corrected to the protein value of the sample, and the results six to eight independent experiments were expressed as U/mg of protein.

For the evaluation of CAT activity, the method described by Aebi (1984) was used, with some modifications. Hence, 5 μL of the sample was added to the microplate along with 195 μL of the mixture composed of Kpi buffer 0.25 M/EDTA 2.5 mM pH 7.0, distilled water, 30% of hydrogen peroxide (H_2O_2), and Triton X100 (0.012%). The reading of the decomposition of H_2O_2 was performed at a wavelength of 240 nm, for 120 s. The results were expressed as U/mg of protein, where one CAT unit (U) corresponds to the enzymatic activity required to decompose 1 μmol of H_2O_2/min at 37 °C. Results represent the mean of six to seven independent experiments in each group.

2.14. Western blot

Western blot analysis was performed as described previously by Guerra et al. (2012) with slight modifications by Maitra et al. (2019). Thirty flies were rapidly euthanized and homogenized in an ice-cold buffer consisting of KCl (10 mM), $MgCl_2$ (2 mM), EDTA (1 mM), NaF (1 mM), aprotinin (10 μg/mL), β-glycerolphosphate (10 mM), PMSF (1 mM), DTT (1 mM), and sodium orthovanadate (2 mM) in HEPES (10 mM, pH 7.9). The samples were then incubated on ice for 15 min and centrifuged at 16,000 g for 45 min at 4 °C. The supernatant was collected for further processing, and the protein concentration was determined using the method Bradford (1976). Equivalent amounts of protein (80 μg) were mixed with a concentrated loading buffer composed of Tris (200 mM), glycerol (10%), SDS (2%), β-mercaptoethanol (2.75 mM), and bromophenol blue (0.04%) in a 0.2:1 ratio, and boiled for 10 min.

The proteins were then separated by 12% SDS–PAGE, and the gels were transferred onto Amersham™ Protran® Premium Western blotting nitrocellulose membranes, using Transfer-Blot® Turbo™ Transfer System (1.0 mA; 30 min). Ponceau staining was utilized as a positive control, an alternative to b-actin, as described by Romero-calvo et al.

(2010). After blocking the membrane with 1% BSA in TBS-T (0.05% Tween 20 in Tris-borate saline), a specific primary polyclonal antibody anti-Nrf2 (1:1000, anti-mouse, sc-365949; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was incubated overnight. The membrane was then washed three times with TBS-T and incubated with Horseradish peroxidase-conjugated secondary antibody (1:5000, anti-mouse IgG-HRP; sc-516102; Santa Cruz Biotechnology, Inc.) for 2 h.

Protein bands were visualized using 3,3′,5,5′-Tetramethylbenzidine (TMB; Sigma-Aldrich), the TMB was left at room temperature, added enough to the membranes to cover them, and allowed to react for 15 min in the dark. Manufacturer's recommendation (Sigma Aldrich). Finally, the membranes were dried, scanned, and quantified using the ImageJ PC version (NIH, Bethesda, MD, USA). The results were normalized by setting the densitometry of the control group as 100%.

2.15. Assessment of cellular metabolic capacity

Cellular metabolic capacity was assessed according to the methodology used by Franco et al. (2009), where viable cells present in the sample, through dehydrogenases, can reduce resazurin to resorufin, a fluorescent molecule, which can be quantified. First, 10 flies were homogenized in 500 μL of 20 M HEPES buffer (pH 7.0) and centrifuged at 1000 g for 10 min. An aliquot of 20 μL of supernatant was used, it was transferred to a microplate, and combined with a mixture of 180 μL of HEPES and 10 μL of resazurin. The samples were kept in the dark at room temperature for 1 h and then read at a wavelength of 573 nm. The fluorescence intensity result of five independent experiments was expressed as percentage in relation to the control group.

2.16. Statistical analysis

For statistical analysis of the data, the GraphPad Prism software version 8 (San Diego, CA, USA) was used, and a one-way analysis of variance (ANOVA) was applied. Data normality was analyzed using the Shapiro-Wilk test. For data that presented a normal distribution, the Bonferroni test of multiple comparisons was used, and for data that did not have a normal distribution, the Kruskal-Wallis test was applied. Survival percentage was determined using the log-rank test (Mantel-Cox). Values of $P < 0.05$ were considered statistically significant. All values were expressed as mean ± standard deviation (SD).

3. Results

3.1. Nanocapsules development and characterization

Nanocapsules were successfully developed by interfacial polymer deposition method. Characterization is showed in Table 1. Both formulations showed nanometric size, population monodisperse and pH slightly acid. Drug content was close 100% as well encapsulation rate.

3.2. Effect of free curcumin and curcumin-loaded nanocapsules on diet consumption and percentage of survival

The statistical analysis showed that the estimated diet consumption containing free curcumin (Fig. 2A) and curcumin-loaded nanocapsules (Fig. 2B) at different concentrations did not differ significantly compared with the control group. The Log-rank test (Mantel-Cox) revealed that exposure to free curcumin [$P = 0.0029$] and curcumin-loaded nanocapsules [$P < 0.0001$], significantly increased the survival percentage. Comparisons showed that exposure to free curcumin at the concentrations of 300 μM; [$P = 0.0002$]and 900 μM; [$P = 0.0070$] (Fig. 2C), and curcumin-loaded nanocapsules at the concentrations of 10 μM; [$P < 0.0001$], 30 μM; [$P = 0.0002$], 100 μM; [$P = 0.0015$], and 300 μM; [$P = 0.0052$] (Fig. 2D) improved the survival percentage compared with the control group.

Table 1
Characterization of nanocapsules.

Parameter	Curcumin-loaded nanocapsules
Diameter (nm)	255 ± 3
Índice de polidispersão	0.098 ± 0.041
pH	6.6 ± 0.2
Encapsulation rate	97 ± 1
Drug content	100 ± 1

3.3. Performance in the negative geotaxis and open field behavioral tests

To evaluate the locomotor and exploratory performance, negative geotaxis and open field tests were performed. The statistical analysis (one-way ANOVA) showed that at the end of the 10th day of exposure to free curcumin [$F_{(6,39)} = 4.05$; $P = 0.0081$] and curcumin-loaded nanocapsules [$F_{(4,28)} = 7.53$; $P < 0.0003$], there was a significant improvement in the exploratory capacity in the negative geotaxis. The post hoc comparisons showed that free curcumin at the concentrations of 900 μM [$P = 0.0489$] and 3000 μM [$P = 0.0059$]; (Fig. 3A) and curcumin-loaded nanocapsules at the concentrations of 30 μM; [$P = 0.0031$] and 100 μM; [$P = 0.0070$] vs Control; (Fig. 3B) decreased the climbing time compared with the control group.

The statistical analysis (one-way ANOVA) showed that at the end of the 10th day of exposure to free curcumin [$F_{(6,28)} = 12.71$; $P < 0.0001$] and curcumin-loaded nanocapsules [$F_{(4,25)} = 5.05$; $P = 0.0040$], there was a significant improvement in locomotion and exploratory capacity in the open field test. The post hoc comparisons showed that free curcumin at the concentrations of 300 μM; [$P = 0.0011$], 900 μM; [$P = 0.0022$], and 3000 μM [$P = 0.0002$] vs Control; (Fig. 3C) and curcumin-loaded nanocapsules at the concentrations of 30 μM; [$P = 0.0020$] and 100 μM; [$P = 0.0323$] vs Control; (Fig. 3D) increased the number of crossings compared with the control group.

3.4. Effect of free curcumin and curcumin-loaded nanocapsules on AChE activity

The statistical analysis (one-way ANOVA) showed that the exposure of flies to free curcumin [$F_{(5,28)} = 3.370$; $P = 0.0125$] and curcumin-loaded nanocapsules [$F_{(4,20)} = 8.78$; $P = 0.0003$ vs Control], for 10 days, reduced AChE activity. The post hoc comparisons showed that exposure to free curcumin at the concentrations of 900 μM; [$P = 0.0295$] and 3000 μM; [$P = 0.0179$] vs Control; (Fig. 4A) and curcumin-loaded nanocapsules at the concentrations of 30 μM; [$P = 0.0037$] and 100 μM; [$P = 0.0119$] vs Control; (Fig. 4B) decreased AChE activity.

3.5. Effect of free curcumin and curcumin-loaded nanocapsules on oxidative stress biomarkers and antioxidant defenses

3.5.1. Levels of reactive species (RS) and thiobarbituric acid reactive species (TBARS)

Fig. 5 shows the effect of the exposure of flies to free curcumin and curcumin-loaded nanocapsules on oxidative stress indicators. The statistical analysis (one-way ANOVA) revealed that at the end of the exposure period of 10 days to free curcumin [$F_{(5,35)} = 6.38$; $P < 0.0001$] and curcumin-loaded nanocapsules [$F_{(4,30)} = 23.23$; $P = 0.0001$], there was a statistically significant reduction in RS levels. The post hoc comparisons showed that exposure to free curcumin (900 μM; [$P = 0.0006$] and 3000 μM [$P < 0.000$] vs Control; Fig. 5A) and curcumin-loaded nanocapsules (30 μM; [$P < 0.0001$] and 100 μM; [$P < 0.000$] vs Control; Fig. 5B) decreased RS levels.

Furthermore, the statistical analysis (one-way ANOVA) revealed that exposure for 10 days to free curcumin [$F_{(6,35)} = 7.38$; $P < 0.0001$] and curcumin-loaded nanocapsules [$F_{(4,40)} = 6.32$; $P < 0.0001$] reduced TBARS levels. The post hoc comparisons showed that exposure to free curcumin (300 μM [$P = 0.0001$], 900 μM; [$P = 0.0005$], and 3000 μM;

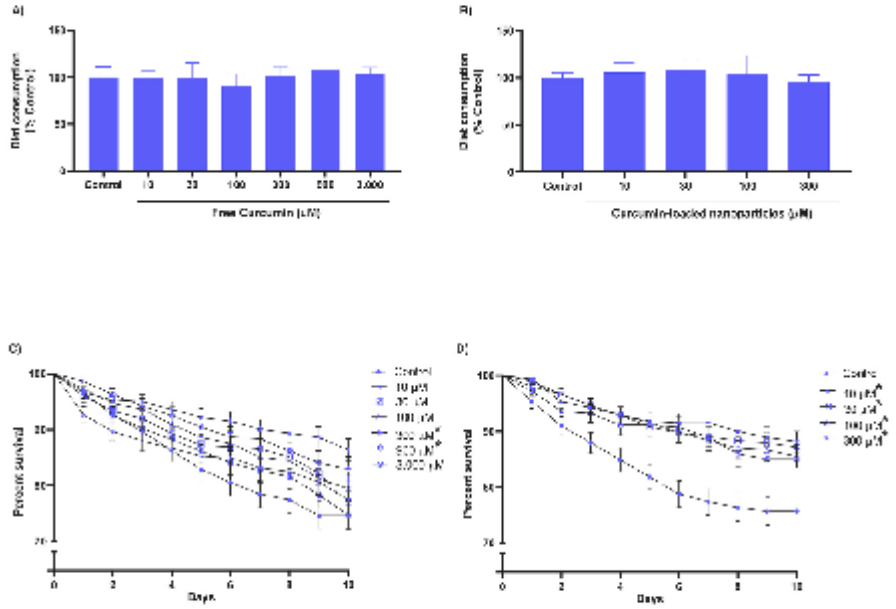

Fig. 2. Evaluation of the exposure to free curcumin (A) at the concentrations of 10, 30, 100, 300, 900, and 3000 μM, and curcumin-loaded nanocapsules (B) at the concentrations of 10, 30, 100, and 300 μM in diet consumption. This analysis was carried out on the first day of treatment, 30 min after the flies were placed in the bottles containing the diet, it was verified whether the consumption of the respective diets was actually occurring. Graph C and D express the survival curve in percentile of flies exposed to different concentrations of free curcumin and curcumin-loaded nanocapsules by 10 days, respectively. Data are expressed as mean ± standard deviation (SD), for n = 5–6 in each treatment-exposed group. Significance is presented as * $P < 0.05$ vs Control.

[$P = 0.0074$] vs Control; Fig. 5C); and curcumin-loaded nanocapsules (10 μM; [$P = 0.0009$], 30 μM [$P = 0.0005$], 100 μM; [$P = 0.0012$], and 300 μM; [$P = 0.0188$] vs Control; Fig. 5D); reduced TBARS levels.

3.5.2. *Enzymatic activity: GST, SOD, and CAT*

The effect of exposure to free curcumin and curcumin-loaded nanocapsules on the enzymatic activity of GST, SOD, and CAT in the flies was investigated and the results are presented in Fig. 6. The statistical analysis (one-way ANOVA) revealed that exposure for 10 days to free curcumin caused a significant increase in GST [$F_{(6,40)} = 3.61$; $P = 0.0059$], SOD [$F_{(6,42)} = 32.19$; $P < 0.0001$], and CAT activity [$F_{(6,35)} = 14.61$; $P < 0.0001$], as well as curcumin-loaded nanocapsules for GST [$F_{(4,27)} = 6.62$; $P = 0.0008$], SOD [$F_{(4,27)} = 15.32$; $P < 0.0001$], and CAT activity [$F_{(4,29)} = 22.48$; $P < 0.0001$]. The post hoc comparisons showed that exposure to free curcumin (900 μM; [$P = 0.0054$] and 3000 μM; [$P = 0.0289$] vs Control; Fig. 6A) increased GST activity, and (300, 900 e 3000 μM; [$P < 0.0001$] vs Control; Fig. 6C) increased SOD activity, and (900 μM; [$P = 0.0477$] and 3000 μM; [$P < 0.0001$] vs Control; Fig. 6 E) increased CAT activity, and curcumin-loaded nanocapsules (30 μM; [$P = 0.0004$] and 300 μM; [$P = 0.0107$] vs Control; Fig. 6B), increased GST activity, and (30 μM [$P < 0.0005$], 100 μM; [$P = 0.0013$] and 300 μM; [$P = 0.0001$] vs Control; Fig. 6D) increased SOD activity, and (30 μM; [$P < 0.0001$] and 300 μM; [$P = 0.0375$] vs Control; Fig. 6 F), increased CAT activity.

3.5.3. *Western blotting analysis*

Fig. 7 shows the effect of the exposure of flies to free curcumin and curcumin-loaded nanocapsules on the Nrf2 immunoreactivity. The statistical analysis (one-way ANOVA) revealed that at the end of the 10-day exposure period to free curcumin 3000 μM; [$F_{(6,21)} = 5.41$; $P < 0.0016$] and curcumin-loaded nanocapsules 300 μM; [$F_{(4,23)} = 5.53$; $P = 0.0028$], there was a statistically significant increase in Nrf2 immunoreactivity. The post hoc comparisons showed that exposure to free curcumin (3000 μM; Fig. 7A), and curcumin-loaded nanocapsules (300 μM; Fig. 7B), increased Nrf2 immunoreactivity vs Control.

3.5.4. *Effect on cellular metabolic capacity*

Fig. 8 shows the effect of the exposure of flies to free curcumin and curcumin-loaded nanocapsules on cellular metabolic capacity, through the resazurin reduction method. The statistical analysis (one-way ANOVA) revealed that at the end of the 10-day exposure period to free curcumin [$F = 15.09$; $P = 0.0196$] and curcumin-loaded nanocapsules [$F_{(4,20)} = 5.68$; $P = 0.0032$], cellular metabolic capacity increased. The post hoc comparisons showed that exposure to free curcumin (300 μM; [$P < 0.0091$] vs Control; Fig. 8A) and curcumin-loaded nanocapsules (30 μM [$P < 0.0122$], 100 μM; [$P < 0.0102$], and 300 μM; [$P < 0.0141$] vs Control; Fig. 8B); increased cellular metabolic capacity.

4. Discussion

The present study evaluated the potential toxic effect induced by chronic exposure to free curcumin and curcumin-loaded nanocapsules coated with P80 *in vivo* models of *D. melanogaster*. Knowing that free curcumin can be toxic, in this sense, nanoparticles emerge as a alternative to obtain therapeutic effects from smaller doses and with lower toxicity.

The characterization of curcumin-loaded nanocapsules developed in our study, showed nanometric size (255 ± 3 nm), polydispersion index <2 (0.098 ± 0.041), negative zeta potential, pH slightly acid (6.6 ± 0.2) and drug loading close to 100%. Initially, diet consumption was

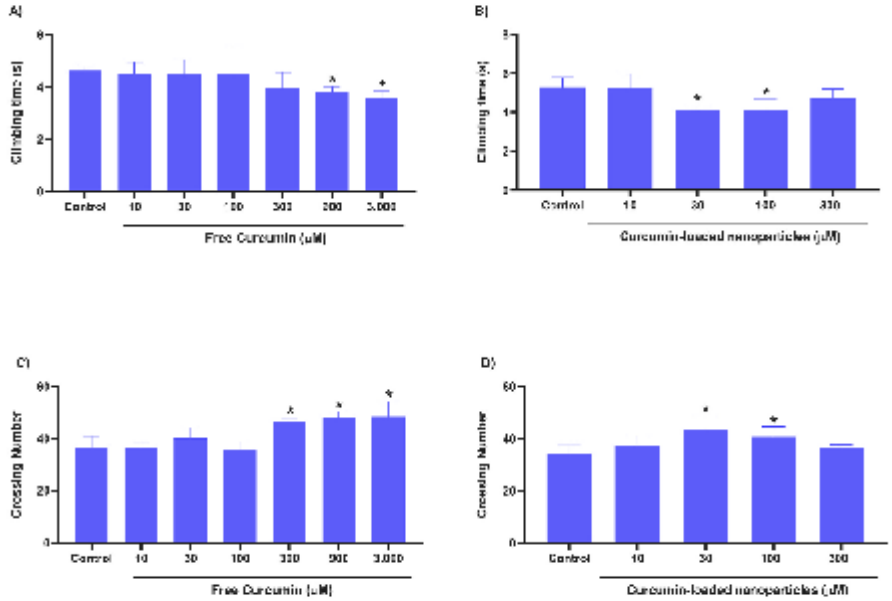

Fig. 3. Evaluation of the effect of exposure to free curcumin (10, 30, 100, 300, 900, and 3000 μM) and curcumin-loaded nanocapsules (10, 30, 100, and 300 μM) for 10 days. Effect of free curcumin in the negative geotaxis test (A) and the open field test (C). Effect of curcumin-loaded nanocapsules in the negative geotaxis test (B) and the open field test (D). Data are expressed as mean ± standard deviation (SD), for n = 5-7 in each treatment exposed group. Significance is presented as * $P < 0.05$ vs Control.

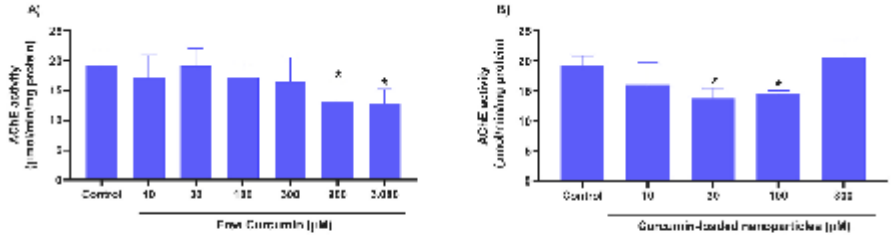

Fig. 4. Evaluation of the exposure to free curcumin (A) at the concentrations of 10, 30, 100, 300, 900, and 3000 μM, and curcumin-loaded nanocapsules (B) at the concentrations of 10, 30, 100, and 300 μM in the AChE enzyme activity. Data are expressed as mean ± standard deviation (SD), for n = 5 in each treatment-exposed group. Significance is presented as * $P < 0.05$ vs Control.

investigated to verify whether the free curcumin and curcumin-loaded nanocapsules would be readily accepted by the flies based on taste. Our results showed that there was no significant difference in diet consumption between the evaluated groups. This is a favorable outcome, as it indicates there was neither preference nor aversion towards the treatments. Therefore, any subsequent significant difference observed in the experiments cannot be attributed to greater or smaller consumption of curcumin by the flies.

Curcumin has been found to exhibit toxicity when administered in high doses and with prolonged exposure (Alafiatayo et al., 2019). However, in this study, exposure to free curcumin and curcumin-loaded nanocapsules increased survival rates. This not only suggests the absence of any toxic effects caused by curcumin but also indicates its potential to enhance the survival of healthy flies when compared to the control group. This finding aligns with the study conducted by Akinyemi et al. (2017), which showed that exposing *D. melanogaster* to 1 mg/g of free curcumin for 14 days did not result in any toxic effects. Likewise, a dose-dependent increase in the lifespan of *D. melanogaster* was observed with a diet containing 1 mg/g of curcumin (Suckow and Suckow, 2006). Moreover, our study is consistent with Santos et al. (2021), who evaluated the *in vitro* action of different coatings of curcumin-loaded nanocapsules, including those coated with the anionic polymer P80, and found no toxic effects. However, since *in vitro* studies may not accurately reflect *in vivo* effects, our *in vivo* study becomes crucial. The results demonstrate that curcumin-loaded nanocapsules exhibit no toxic effect in *D. melanogaster*.

Behavioral assessment related to locomotor and exploratory performance in *D. melanogaster* is an important toxicity indicator (Musachio et al., 2020). In the negative geotaxis test, it was observed that both free curcumin and curcumin-loaded nanocapsules reduced the climbing time

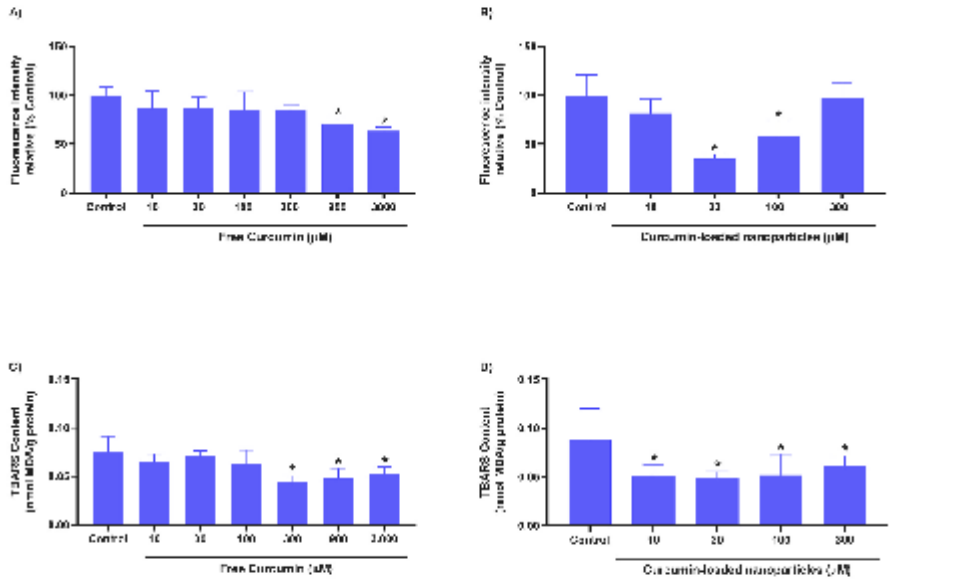

Fig. 5. Evaluation of the effect of exposure to free curcumin (10, 30, 100, 300, 900, and 3000 μM) and curcumin-loaded nanocapsules (10, 30, 100, and 300 μM) for 10 days. Effect of free curcumin in the levels of reactive species (RS) (A) and thiobarbituric acid reactive substances (TBARS) (C). Effect of curcumin-loaded nanocapsules in the levels of RS (B) and TBARS (D). Data are expressed as mean ± standard deviation (SD), for n = 6–9 in each treatment exposed group. Significance is presented as * $P < 0.05$ vs Control.

of the flies. This test evaluates the ability of flies to climb, considering factors such as reflexes and muscle tone (Musachio et al., 2020). Not only were these characteristics unaffected, but an improvement in reflexes and climbing ability was actually observed. The exploratory capacity of the flies was also evaluated through the open field test, where an increase in the number of crossings was observed with the free curcumin and curcumin-loaded nanocapsules. To complement these observations, AChE activity was also assessed, which is a widely used tool in toxicological studies, in addition to predicting exploratory behaviors (Fernandes et al., 2021). In our study, free curcumin in higher concentrations and curcumin-loaded nanocapsules in lower concentrations, decreased AChE activity, showing that nanoencapsulated curcumin already in low concentrations has effect, which may elucidate the result from the open field test. A decrease in AChE activity may lead to an increase in acetylcholine in the synaptic cleft, a neurotransmitter responsible for modulating motivation and attention behaviors (exploratory behaviors) (Faure et al., 2014), which may explain the improved behavioral activity observed in this study through the negative geotaxis and open field tests. Locomotor alterations can also be developed due to the exacerbated production of Reactive oxygen species (ROS), as observed in models of degenerative diseases (de Freitas Couto et al., 2019; Musachio et al., 2020) making the investigation of this marker quite pertinent as a toxic mechanism.

ROS have unpaired electrons in their structure that react with various macromolecules in cells (Birben et al., 2012). According to Hamanaka and Chandel (2010), ROS are toxic metabolic by-products that can trigger pathologies. Aerobic organisms possess enzymatic and non-enzymatic antioxidant systems that generally protect against the toxic effects of ROS (Birben et al., 2012). Therefore, it is relevant to evaluate molecules with antioxidant mechanisms, such as curcumin. In the present study, the groups of flies exposed to free curcumin and curcumin-loaded nanocapsules showed decreased levels of the non-enzymatic markers RS and TBARS. These results suggest that curcumin exposure is positively associated with the ability to neutralize reactive species and reduce lipid peroxidation. In a study with rodents, Abdollahzadeh Estakhri et al. (2019), showed that curcumin nanomicelles decreased lipid peroxidation and RS, and attributed this effect to the better direction of action of the substance when nanoencapsulated.

In addition, the enzymatic activity of GST, SOD, and CAT, and the immunoreactivity of Nrf2 were evaluated in order to observe the defenses against exposure to free curcumin and curcumin-loaded nanocapsules, considering that the suppression of these enzymes and Nrf2 indicates a toxic effect. The results showed that exposure to free curcumin and curcumin-loaded nanocapsules increased GST, SOD, and CAT activities. Studies have shown that free curcumin has a protective effect against oxidative damage in both rats and flies, increasing the activity of antioxidant and detoxifying enzymes such as SOD, CAT, and GST (Oyetayo et al., 2020; Samarghandian et al., 2017).

Additionally, free curcumin and curcumin-loaded nanocapsules increased Nrf2 immunoreactivity. Furthermore, it is relevant to note that curcumin-loaded nanocapsules at a concentration 10 times lower than free curcumin was able to increase Nrf2 immunoreactivity. At large, lower concentrations tend to have less toxic effects. In this sense, it is seen that curcumin can serve as an inducer of Nrf2 activation, as it is known that its structural conformation favors the elimination of free radicals, restoring redox homeostasis, transactivating a group of antioxidant enzymes and preventing inflammatory migration (Trujillo et al., 2013). The effect of free curcumin is already well described in the literature, however, the effect of curcumin nanoencapsulated in P80 polymer on the activity of these enzymes and of Nrf2 evaluated in our study had not yet been elucidated for *in vivo* models, which makes these data unprecedented.

Moreover, in the resazurin reduction assay to evaluate the cellular metabolic capacity, it was possible to observe that free curcumin and curcumin-loaded nanocapsules, increased the cellular metabolic

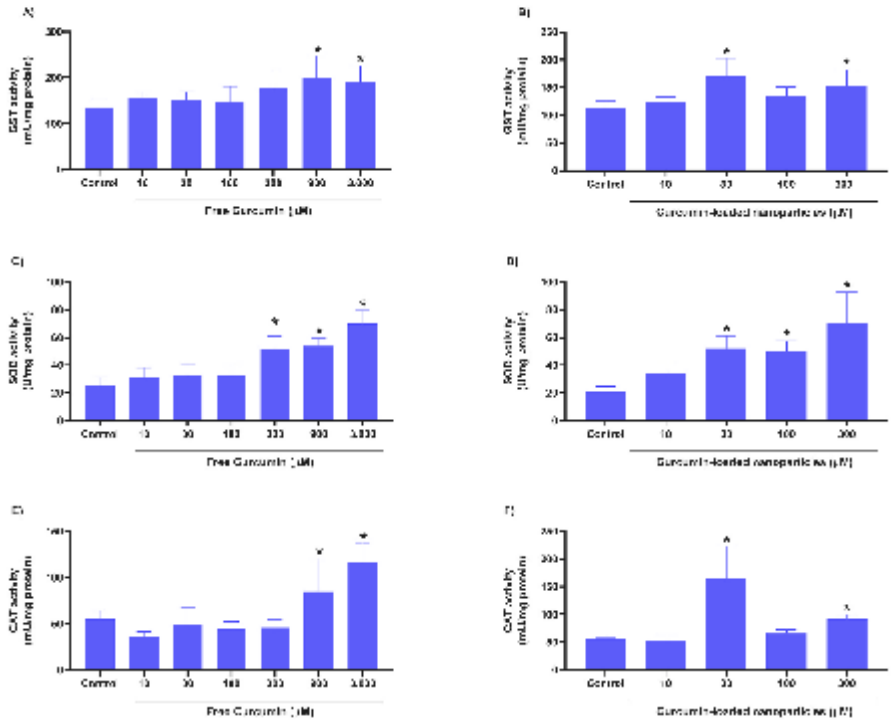

Fig. 6. Evaluation of the effect of exposure to free curcumin (10, 30, 100, 300, 900, and 3000 μM) and curcumin-loaded nanocapsules (10, 30, 100, and 300 μM) for 10 days in the markers of oxidative stress. Effect of free curcumin in the GST activity (A), SOD activity (B), and CAT activity (C). Effect of curcumin-loaded nanocapsules in the GST activity (D), SOD activity (E), and CAT activity (F). Data are expressed as mean ± standard deviation (SD), for n = 6–8 in each treatment exposed group. Significance is presented as * $P < 0.05$ vs Control.

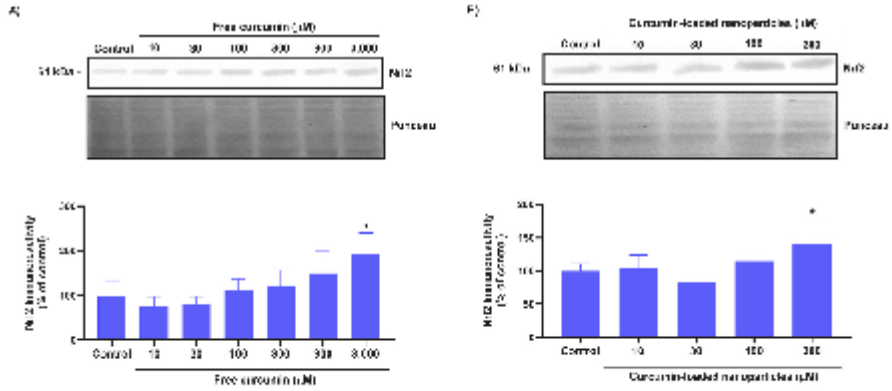

Fig. 7. Evaluation of the exposure to free curcumin (A) at the concentrations of 10, 30, 100, 300, 900, and 3000 μM, and curcumin-loaded nanocapsules (B) at the concentrations of 10, 30, 100, and 300 μM in the level of Nrf2 immunoreactivity. Data are expressed as mean ± standard deviation (SD), for n = 4–6 in each treatment-exposed group. Significance is presented as * $P < 0.05$ vs Control.

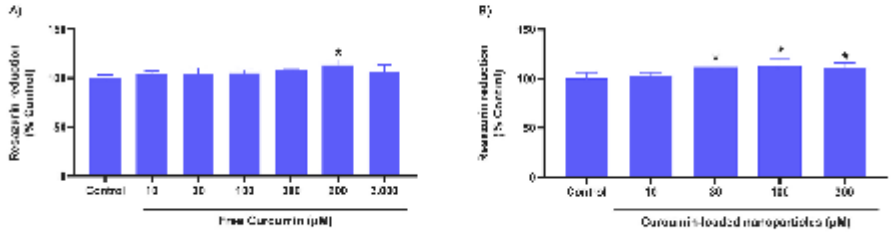

Fig. 8. Evaluation of the exposure to free curcumin (A) at the concentrations of 10, 30, 100, 300, 900, and 3000 μM, and curcumin-loaded nanocapsules (B) at the concentrations of 10, 30, 100, and 300 μM in the cellular metabolic capacity. Data are expressed as mean ± standard deviation (SD), for n = 5 in each treatment-exposed. Significance is presented as * $P < 0.05$ vs Control.

capacity, excluding any evidence of toxicity. These data corroborate the study by Abdollahzadeh Estakhri et al. (2019) where curcumin nanomicelles had greater antioxidant activity compared to free curcumin, due to better bioavailability and stability, reducing cytotoxic predisposition. The effects of free curcumin and curcumin-loaded nanocapsules in increasing viable cells are possibly due to modulating antioxidant defenses.

In general, in this research, for all parameters evaluated, free curcumin and curcumin coated with p80 polymer did not present toxic effects after 10 days of supplementation. However, it is worth mentioning that curcumin-loaded nanocapsules were effective at much lower concentrations, while free curcumin showed effect at higher concentrations, supporting the idea that nanoencapsulation with P80 may have potentiated the beneficial effects of curcumin. Thus, the use of low concentrations can represent a treatment, avoiding toxic effects, which can derive from longer treatments.

5. Conclusion

Our data provide evidence that with low doses of curcumin-loaded nanocapsules, it is already possible to positively modulate survival, AChE activity, and consequently behavioral development, in addition to activating antioxidant defenses and attenuating oxidative stress in *D. melanogaster*. The effect of curcumin-loaded nanocapsules at low concentrations, observed in the evaluated markers, was possibly associated with nanoencapsulation, as coating appeared to be a strategy to enhance the biological activity and decrease the toxicity of curcumin. This study demonstrated that chronic exposure to curcumin-loaded nanocapsules at these concentrations did not cause toxic effects in *D. melanogaster*, suggesting that curcumin nanoencapsulation can be explored.

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CRediT authorship contribution statement

Eliana Jardim Fernandes: Conceptualization, Methodology, Formal analysis, Investigation, Software, Writing – original draft. **Elize Aparecida Santos Musachio:** Methodology, Formal analysis, Investigation. **Luana Barreto Meichtry:** Methodology, Formal analysis, Investigation. **Dieniffer Espinosa Janner:** Methodology, Formal analysis, Investigation. **Franciéle Romero Machado Balok:** Methodology, Formal analysis, Investigation. **Magna Sotelo Barrientos:** Methodology, Formal analysis, Investigation. **Nathalie Savedra Gomes:** Methodology, Formal analysis, Investigation. **Kétnne Hanna Poleto Pinto:** Methodology, Formal analysis, Investigation. **Mustafa Munir Mustafa Dahleh:** Methodology, Formal analysis, Investigation. **Andrieli Rodrigues:** Methodology, Formal analysis, Investigation. **Sandra Elisa Haas:** Conceptualization, Methodology, Writing – review & editing. **Silvana Peterini Boeira:** Conceptualization, Methodology, Writing – review & editing. **Marina Prigol:** Conceptualization, Methodology, Writing – review & editing. **Gustavo Petri Guerra:** Conceptualization, Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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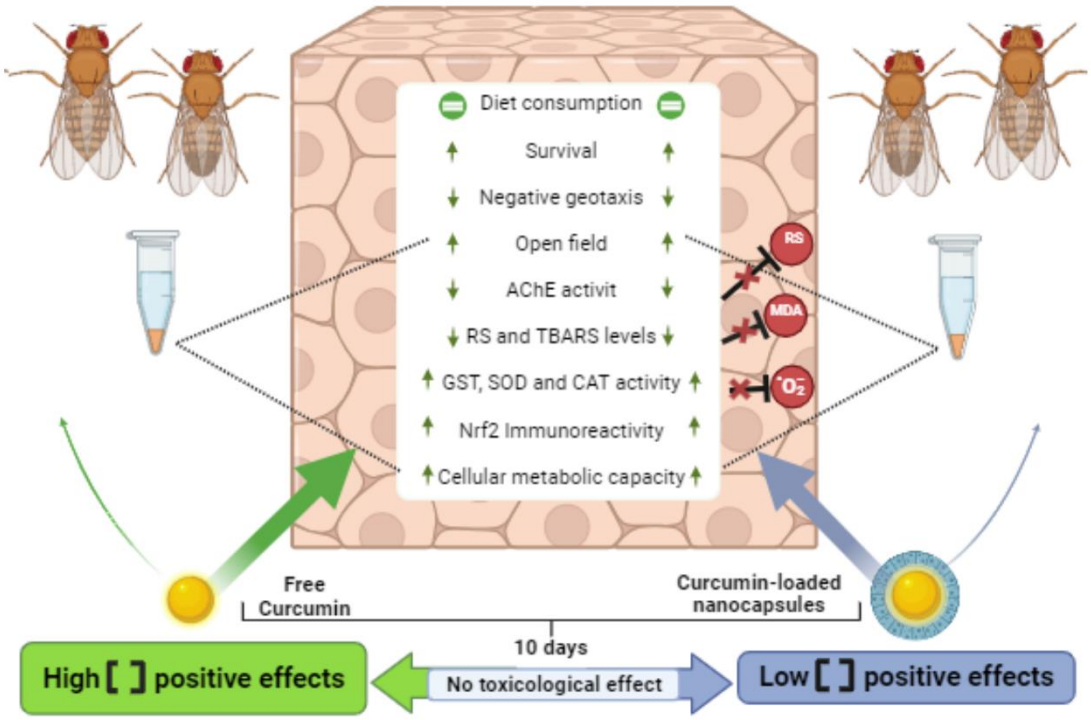
Graphical Abstract



5.2 MANUSCRITO CIENTÍFICO

Curcumin-loaded nanocapsules attenuate LPS-induced toxicity in *Drosophila melanogaster*: through the mechanism of regulation of apoptosis and balance redox

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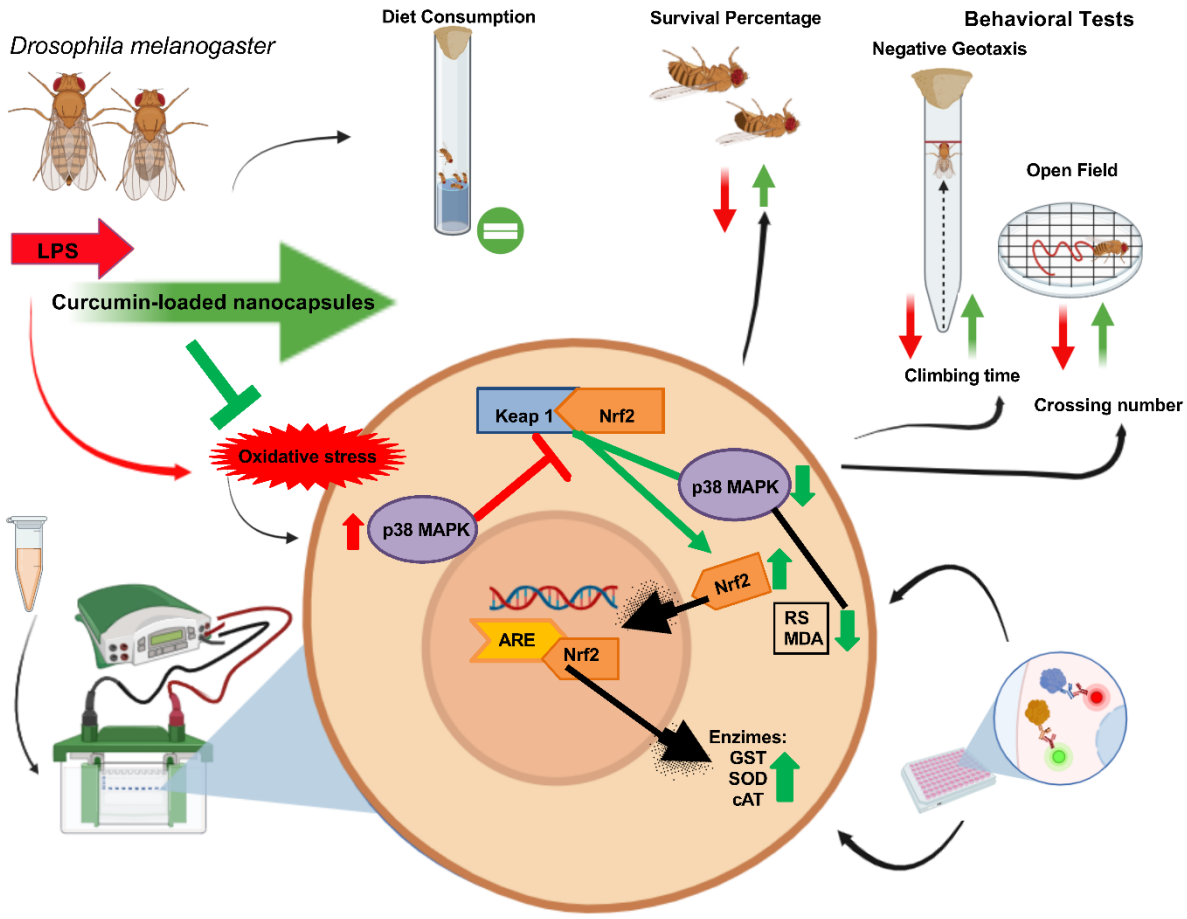
Abstract

The objective was to determine whether curcumin-loaded nanocapsules can protect against damage induced by lipopolysaccharide (LPS), aiming to elucidate their mechanisms of action in *Drosophila melanogaster*. Fifty flies (25 females and 25 males) were added to each treatment flask, and it was divided into the following 4 experimental groups: control (standard diet), LPS (250 µg/kg diet), curcumin-loaded nanocapsules (10 µM), and the co-exposure to LPS and curcumin-loaded nanocapsules. After 7 days of exposure, the LPS group was maintained with a standard diet and the co-exposure group was maintained only with curcumin-loaded nanocapsules, until the end of 10 days. The first analysis to be carried out was the food consumption test, evaluated after 30 minutes of exposing the flies to the treatment. The survival of flies was counted daily until the end of the 10 days of the experiment. At the end of the 10th day of exposure, the flies were subjected to negative geotaxis and open field tests, which were also used for biochemical dosages of oxidative stress indicators, such as levels of reactive species (RS) and quantification of malondialdehyde (MDA); activity of enzyme Glutathione-S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT); immunoreactivity of nuclear factor erythroid-derived 2 (Nrf2) and of apoptotic marker the p38 mitogen-activated protein kinases (MAPK), and cellular metabolic capacity by the resazurin test. No statistically significant in diet consumption between the groups evaluated. Exposure to curcumin-loaded nanocapsules protected against decreased survival, behavioral damage, and changes in indicators of oxidative stress induced by LPS, in addition to increasing the Nrf2 immunoreactivity, and decreased phospho-p38 MAPK immunoreactivity and phospho-p38 MAPK/p38 MAPK ratio, and increased viability cell. These results promote greater knowledge about the protective effect of curcumin-loaded nanocapsules against LPS-induced damage through antiapoptotic activity and antioxidant defenses.

Keywords: Curcumin; lipopolysaccharide; Antioxidant defenses; Antiapoptotic.

Graphic abstract



1. Introduction

Some diseases and toxic agents act through different mechanisms that increase oxidative stress and inflammatory processes (Ansari et al., 2020; Ramos-González et al., 2024; Sul and Ra, 2021). In this context, knowledge of compounds that have potential action on oxidative stress and inflammation can be an promising alternative to treat or alleviate symptoms of already established and untreated diseases, such as osteoarthritis and multiple sclerosis (Ansari et al., 2020; Ramos-González et al., 2024). And still, stand out in the treatment of emerging diseases, as Coronavirus 2019 (COVID-19) (Huang et al., 2020; Polonikov, 2020), Zika virus (ZIKV), an infectious agent that has also been was associated with inflammatory processes (França et al., 2023; Morais et al., 2020).

In this sense, curcimn stands out as a notable exogenous antioxidant, the main active component of the *Curcuma longa* rhizome, a bioactive compound, with a large presence of polyphenolic substances that give it strong antioxidant and anti-inflammatory properties, which comes being described as exogenous source due to its ability to avoid free radical overload and protect biological systems (Aggarwal et al., 2014; Pizzo et al., 2010). However, the high rate of metabolism and consequently limited absorption (Kocaadam and Şanlier, 2017), as well as dose-dependent toxicity (Alafiatayo et al., 2019; Balaji and Chempakam, 2010), significantly limit the use and therapeutic effects of this compound. Thus, the use of nanoparticles has been an alternative method proposed to control these limitations (Kamaly et al., 2016; Nazila et al., 2016).

Nanoparticles can enhance circulation, and permeability and control metabolic processes and interactions induced by curcumin (Mohamed et al., 2017). In recent study, chronic treatment with high concentrations of curcumin nanoencapsulated in

poly(Ɛ-caprolactone) (PCL) as polymer and polysorbate 80 as anionic surfactant, did not cause toxicity, at the same time low concentrations showed an antioxidant effect, and it was suggested that nanoencapsulation of curcumin could be explored (Fernandes et al., 2023). These results demonstrated that it would be interesting to evaluate the antioxidant potential of curcumin-loaded nanocapsules after exposure, and the ability to protect *D. melanogaster* from toxicological damage. In view of this, studies use lipopolysaccharide (LPS), an endotoxin present in the outer wall of gram-negative bacteria to induce oxidative stress, inflammation, and apoptosis (Hou et al., 2019; John et al., 2022), an endotoxin that can activate innate immune system and Toll pathways and cause immunological reactions also of invertebrates (Okun et al., 2011; Rao and Yu, 2010).

Associated with this, nuclear factor erythroid-derived 2 (Nrf2), a protein that acts as a key regulator of the antioxidant system, is linked to Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm. When exacerbated reactive species (RS) production occurs, Nrf2 dissociates from Keap1 and translocates to the nucleus, where it transactivates genes responsible for the antioxidant response (Ji et al., 2013). Apoptotic pathways that negatively regulate Nrf2 have been described, including p38 mitogen-activated protein kinases (MAPK) (Yamamoto et al., 2018). Therefore, proteins that regulate Nrf2 should also receive special attention and be investigated. Furthermore, the decrease in Nrf2 has been considered, therefore strategies that protect or restore the Nrf2 pathway are a promising alternatives.

In this sense, we determined indicators of oxidative stress and apoptosis with the objective of identifying whether curcumin-loaded nanocapsules protect against damage induced by LPS, aiming to explore the action potential of curcumin-loaded nanocapsules and possible future applications.

2. Materials and methods

2.1 Chemicals

Free curcumin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and nanoencapsulated with the polymer P80, purchased from Sigma-Aldrich (St. Louis, MO, USA). LPS from *Escherichia coli* 0111:B4, Sigma-Aldrich), at a concentration (250 µg/kg of diet) was prepared by dissolving in distilled water and used in the treatment (JOHN *et al.*, 2022). The other reagents utilized were of analytical grade.

2.2 Production and characterization of curcumin-loaded nanocapsules

For the production of curcumin-loaded nanocapsules, the poly (ε-caprolactone (PCL) was utilized as polymer and coated with polysorbate 80 (P80) as nonionic surfactant, through the interfacial polymer deposition method, according described by (Santos et al., 2021), provided by the Nanobiotechnology Laboratory of the Federal University of the Pampa, UNIPAMPA, Uruguaiana campus, RS, Brazil. Following this method, the organic phase was composed of Curcumin, PCL (1% w/v), medium chain triglycerides (TCM) (3.3% w/v), and Span 60® (0.78% w/v), dissolved in acetone, at 40 ± 2 °C, was injected into the aqueous phase composed of distilled water and P80 (0.78% w/v), which were maintained under magnetic stirring for 10 min. Subsequently, a rotary evaporator was used to evaporate the acetone and obtain the desired final concentration (Curcumin 0.6 mg. mL^{-1}). Nanocapsules were characterized in terms of diameter, polydispersity index (SPAN), pH, drug content, and encapsulation efficiency (Santos et al., 2021). Analyzes were performed in triplicate. The diameter of the nanocapsules and the polydispersity were analyzed by laser diffraction at room

temperature and dilution of the sample in deionized water, using the Mastesizer 2000 equipment (Malvern®), and by dynamic light scattering in which the suspensions were diluted in NaCl 0.9% (w/v) (previously filtered) (NanoBrook 90Plus Zeta, BrookHaven®). The pH of the formulations was determined through measurements of the formulations, using a potentiometer (HANNA®) previously calibrated with pH 7.01 and 4.01. To determine the drug content, the formulations were dissolved in methanol, filtered, and quantified by high-performance liquid chromatography with a diode array detector (HPLC-PDA), using a method previously validated by the research group de Oliveira Pacheco et al. (2022). The encapsulation efficiency was determined by the ultrafiltration/centrifugation technique (Ultrafree-Millipore®), in which the suspension was centrifuged at 1960 g for 10 min at 4°C, and the ultrafiltrate was quantified by HPLC-PDA, determining the concentration of curcumin. All analyses were performed in triplicate.

2.3 *Drosophila melanogaster* stock and culture

D. melanogaster (Harwich strain) flies obtained from the Laboratory of Pharmacological and Toxicological Evaluations Applied to Bioactive Molecules, of the Federal University of Pampa (Campus Itaqui, Rio Grande do Sul State, Brazil). The *flies* were reared and maintained under 12 h dark/light cycle conditions, temperature, and relative humidity (25 ± 1°C; 60-70%). The flies were kept in glass flasks, standard diet containing (76.59% corn flour, 8.51% wheat germ, 7.23% sugar, 7.23% milk powder, 0.43% salt, 0.08% methylparaben).

2.4 Experimental methodology

The experiment schedule was carried to determine the effect of curcumin-loaded nanocapsules on LPS-induced damage. For that our study used *D. melanogaster* (1-3 day old). Fifty flies were added to each treatment flask (25 females and 25 males). Curcumin-loaded nanocapsules and LPS (dissolved in water) were homogeneously mixed in 10 mL of standard diet and divided into the following experimental groups: control (a standard diet), a standard diet with LPS (250 µg/kg diet), curcumin-loaded nanocapsules (10 µM) and the co-exposure to LPS and curcumin-loaded nanocapsules, maintained for 10 days in the treatment flask. Flies were exposed to LPS for 7 days, after the LPS group was maintained on a standard diet. Flies were exposed to curcumin-loaded nanocapsules for 10 days, to the curcumin-loaded nanocapsules (10 µM) and the co-exposure (LPS and curcumin-loaded nanocapsules) groups.

The concentration of LPS (250 µg/kg diet) and experimental period were selected based on the study of John et al. (2022), which showed that 75% of flies died during the experimental period and increased oxidative stress when compared to control. The concentration of curcumin-loaded nanocapsules was chosen based in our previous study (Fernandes et al., 2023), which showed that curcumin-loaded nanocapsules in concentration of 10 µM did not alter oxidative stress markers and increased the survival percentage of the flies. In this sense, we tested whether this concentration 10 µM of curcumin-loaded nanocapsules protects the damage induced by LPS to understand its effectiveness.

The first analysis to be carried out was the food consumption test. The survival of flies from each experimental group was counted daily until the end of the 10 days of the experiment. At the end of the 10th day of treatment, the flies were subjected to negative geotaxis and open field tests to evaluate locomotor and exploratory activity,

as well as were euthanized on ice for the determination of oxidative stress indicators, such as RS levels and MDA content; activity of enzyme Glutathione-S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT), immunoreactivity of Nrf2 and apoptotic marker p38 MAPK; and cellular metabolic capacity by the resazurin test. These assessments are demonstrated in the experimental methodologies (Figure 1).

2.5 Food consumption

The food consumption was assessed according to Sun et al. (2013). Five flies per group were transferred to each flask containing the treatment medium with the addition of 0.5% blue dye (FD&C Blue dye #1, Sigma Aldrich). The flies were fed in this medium for 30 minutes, after exposure time, flies were rapidly transferred to a microcentrifuge tube and euthanized on ice, and the body was separated from the head, and only the body was used. The Five bodies from each group were homogenized in 200 µL of distilled water and centrifuged at 12,000 g per 2 min. The absorbance of the supernatant was measured on a microplate reader at 625 nm. The absorbance of the fly supernatant who consumed the diet without dyes was used as a standard curve. A total of 40 flies were used per group. For this experiment, eight independent experiments were carried out (n = 8). The absorbance results of eight independent experiments were expressed as percentage compared with the control group.

2.6 Survival percentage

The percentage of survival was evaluated during 7 days of exposure, counting the number of live flies every 24 hours, counting was carried out until the end of the 10-day experimental period. Each group contained 50 flies. A total of 300 flies per

group were utilized. For this experiment, 6 independent experiments were carried out (n = 6). The data were compared using the Log-rank test (Mantel-Cox), and results were expressed as the percentage of surviving flies (Fernandes et al., 2021).

2.7 Behavioral tests

2.7.1 Negative geotaxis

Assessment of locomotor and climbing performance, in the negative geotaxis test was evaluated according to the methodology described by Feany and Bender (2000), with some modifications. At the end of 10 days of exposure to the treatment, five flies from each group were used for the test. The flies were individually transferred to clear tubes. After 10 min of recovery, the tubes containing the flies (one at a time) were tapped lightly on a bench so that the flies remained at the bottom of the tube, with the aid of a stopwatch, the time spent by each fly to reach the 8 cm, measured from the base of the tube. Each fly had a time maximum of 120 seconds to scroll the apparatus until reaching 8 cm. The test was repeated five times for each fly, and the time interval between one repetition and another for each fly was 3 min. A total of 250 flies per group were utilized. For this experiment, 5 independent experiments were carried out (n = 5). The data were analyzed according to individual time. Data are expressed as the time mean in seconds of five independent experiments.

2.7.2 Open field

To evaluate the exploratory activity, the open field test was performed as described by Connolly (1966) with modifications by Musachio et al. (2020). The flies were briefly anesthetized on ice, were separated five flies from each group, and

individually added to a transparent polycarbonate Petri dish (9 mm in diameter), divided by squares measuring 1 cm² each, slightly marked with a thin line. After the 2-minute recovery period, the test was started. This time is indicated to restore the flies' anesthesia and acclimatize them to the plate. The number of crossings covered by each fly was observed and visually counted, and determined during 60 seconds, timed using a stopwatch. The activity was obtained by counting the number of squares that each fly traveled in 60 seconds. A total of 250 flies per group were utilized. For this experiment, 5 independent experiments were carried out (n = 5). The test was performed in duplicate and the mean values were calculated. Data represent the mean of the number of squares of five independent experiments.

2.8 Samples preparation for biochemical analysis

Samples were prepared to perform biochemical analysis at the end of the 10th day of exposure, the flies were euthanized on ice, homogenized, and centrifuged according with the protocol for each analysis. The supernatant for each sample was used in the analysis.

2.9 Determination of protein concentration

The determination of protein concentration in the samples was carried out as described by Bradford (1976), and bovine serum albumin (BSA) was used as a standard. Samples were read at a wavelength of 595 nm and read in duplicate.

2.10 Quantification of reactive species (RS)

The levels of RS were quantified according to the technique of Pérez-Severiano et al. (2004). The technique is based on the oxidation of 2,7-dichlorofluorescein

diacetate (DCFHDA) in the presence of RS, forming dichlorofluorescein (DCFH). Initially, ten flies were homogenized in N-(2-hydroxyethyl) piperazine-N-(2-ethane sulfonic acid) buffer (20 M HEPES, pH 7.0), and centrifuged at 1000 g for 5 min at 4°C. Sequentially, 100 µL of sample supernatant tissue was transferred to a test tube, together with 2890 µL of HEPES buffer and finally 10 µL of DCFHDA (1 mM). The mixture was incubated for 1 hour in the dark at 36°C. After this incubation time, the mixture was transferred to a quartz cuvette, and the fluorescence emitted by the mixture reaction was read in a fluorimeter, using a 2.5 mm light beam, at an excitation wavelength of 488 nm and 520 nm of emission. Seven independent experiments were performed and the results were expressed as percentage compared with the control group.

2.11 Quantification of lipid peroxidation

The evaluation of the lipid peroxidation was performed using the thiobarbituric acid reactive species (TBARS), which measures the MDA content in the sample, according to the method of Ohkawa et al. (1979), followed by some adaptations for *D. melanogaster*. First, 12 flies were homogenized in 600 µL of HEPES buffer (20 M, pH 7.0), and the homogenate was centrifuged at 78 g for 10 min. Sequentially, 250 µL of thiobarbituric acid (TBA, 0.8%, pH 3.2), 250 µL of acetic acid (0,45 M, pH 3.4), 50 µL of distilled water, 100 µL of sodium dodecyl sulfate (1.2%), and 100 µL of sample supernatant tissue, in that order they were added to a test tube. After 2 hours of incubation in a water bath at 95 °C, the samples were cooled to room temperature and transferred to microplates. The samples were read in a microplate reader at a wavelength of 532 nm. The results were corrected to the protein value of the sample,

and the results of seven independent experiments were expressed as nmol MDA/mg protein.

2.12 Determination of the activity of detoxifying enzyme Glutathione-S-transferase (GST)

The activity of the GST enzyme was performed according to the method described by Habig and Jakoby (1981). To prepare the samples, 12 flies were homogenized in 600 µL of a HEPES buffer (20 M, pH 7.0), and centrifuged at 78 g for 10 min. After, the sample supernatant tissue was removed and used for analysis. Was added, 10 µL of the supernatant was transferred to a microplate, and 185 µL of the mixture was added, consisting of 0.25 M Kpi/ EDTA buffer (2.5 mM, pH 7.0), 100 mM GSH, and distilled water. To finish, 5 µL of 50 mM 1-chloro-2-4-dinitrobenzene (CDNB) was added to initiate the reaction. The reading was performed at a 340 nm wavelength for 2 min. The protein value of the sample was used to correct the absorbance results, and the results of seven independent experiments were expressed as mU/mg protein.

2.13 Determination of the activity of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT)

The evaluation of SOD activity was performed according to the protocol described previously by Misra and Fridovich (1972), with modifications by Sun and Zigman (1978). In preparation of samples, 12 flies were homogenized in 600 µL of a HEPES buffer (20 M, pH 7.0), and centrifuged at 78 g for 10 min. The analysis was performed in microplates, where 6, 12, and 18 µL of the sample were pipetted, as well as 254, 248, and 242 µL of Na_2CO_3 sodium carbonate buffer (57.7 mM, pH 11.40, kept

at a temperature of 30°C), respectively, and 30 µL of epinephrine (6 mM), dissolved in 0.1M HCl (200 µL of HCl, over 9.8 µL of distilled water, pH 2.0). The blank was prepared with 260 µL of Na_2CO_3 buffer was used, plus 30 µL of the epinephrine solution. The 3 different sample volumes are applied due to the need to achieve a delta capable of observing that SOD can inhibit the auto-oxidation capacity of epinephrine in adrenochrome by 50%. Therefore, each unit of the enzyme corresponds to the amount of enzyme necessary for this to happen (50% inhibition). This method consists on the inhibition of the superoxide anion reaction in the autoxidation of epinephrine. The absorbance of different sample volumes was determined by evaluating the formation of a pink adrenochrome product at a wavelength of 480 nm, and a kinetic cycle with 12 readings of 10 seconds. The protein value of the sample was used to correct the results, and the results of seven independent experiments of each group were expressed as U/mg of protein.

The evaluation of CAT activity was assessed according to the methodology used by Aebi (1984). For this, 5 µL of the sample and 195 µL of the mixture consisting of 0.25 M Kpi buffer/2.5 mM EDTA pH 7.0, distilled water, 30% hydrogen peroxide (H_2O_2), and Triton X100 (0.012%) were added to the microplate. The reading of the decomposition of H_2O_2 was evaluated at a wavelength of 240 nm, for 120 seconds. The protein value of the sample was used to correct the results. The results of seven independent experiments of each group were expressed as U/mg of protein, where one CAT unit (U) refers to the enzymatic activity necessary to decompose 1 µmol of H_2O_2/min.

2.14 Western Blot Analysis

The Western blotting analysis in samples of *D. melanogaster* was conducted according with the methodology of Guerra et al. (2012) with some modifications by Maitra et al. (2019). For the analysis, 30 flies were used, which were immediately transferred to a microcentrifuge tube, euthanized in ice, and homogenized in 300 µL of ice-cold buffer. The buffer constituting of 10 Mm KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM NaF, 10 µg/mL aprotinin, 10 mM β-glycerolphosphate, 1 mM PMSF, 1 mM DTT, and 2 mM sodium orthovanadate in 10 mM HEPES (pH 7.9). The samples were then incubated on ice for 15 min and centrifuged at 16,000 g for 45 min at 4 °C, and the sample supernatant was used, and the protein concentration was evaluated using the protocol of Bradford (1976). The amounts of 80 µg protein were mixed with a concentrated loading buffer composed of 200 mM Tris, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 2.75 mM β-mercaptoethanol, and 0.04% bromophenol blue in a 0.2:1 ratio, and boiled for 10 min.

The proteins were subjected to electrophoresis and were then separated by 12% SDS–PAGE, and the gels were transferred onto Amersham™ Protran® Premium Western blotting nitrocellulose membranes, using Transfer-Blot® Turbo™ Transfer System (1.0 mA; 30 min). β-actin staining was utilized as a positive control. First, the membrane was blocked with 1% BSA in TBS-T (0.05% Tween 20 in Tris-borate saline), and overnight was incubated with a specific primary polyclonal antibody anti-Nrf2 (1:1000, anti-mouse, sc-365949; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and anti-mouse p38 MAPK (1:1000; Cell Signaling Technology, Beverly, MA, USA), anti-mouse phospho-p38 MAPK (1:1000; Cell Signaling Technology, Beverly, MA, USA). After three washes in TBST for 10 min each, the membranes were incubated for 2 hours with Horseradish peroxidase-conjugated secondary antibody (1:5000, anti-mouse IgG-HRP; sc-516102; Santa Cruz Biotechnology, Inc.).

Protein bands were visualized with 3,3′,5,5′-Tetramethylbenzidine (TMB; Sigma-Aldrich), the TMB was left at room temperature, added enough to the membranes to cover them, and allowed to react for 15 min in the dark. Manufacturer's recommendation (Sigma Aldrich). To complete the analysis, the membranes were dried, scanned, and quantified using the ImageJ PC version (NIH, Bethesda, MD, USA). The results were normalized by setting the densitometry of the control group as 100%.

2.15 Evaluation of Cellular Metabolic Capacity

For the evaluation of cellular metabolic capacity, the method described by Franco et al. (2009), was used. The method is based on the ability of viable cells present in the sample, through dehydrogenases, to reduce resazurin to resorufin, which presents fluorescence and can be quantified. Ten flies were homogenized in 500 µL of Tris buffer (20 mM, pH 7.0) and centrifuged at 1000 g for 10 min. An aliquot of 20 µL of supernatant was used, it was added to a microplate, combined with 180 µL of Tris (20 mM, pH 7.0) and 10 µL of resazurin, and sequentially quantified at a wavelength of 573 nm. The result of eight independent experiments was expressed as percentage in relation to the control group.

2.16 Statistical analysis

Statistical analyses of the data were carried out using the GraphPad Prism software version 8 (San Diego, CA, USA). The analyses of the data were performed by two-way analysis of variance (ANOVA). Bonferroni’s post hoc test was used. Analyses of data for survival were carried out using the log-rank test (Mantel-Cox). In all the groups, differences were considered statistically significant among groups with

values of $P < 0.05$. All data values were expressed as mean and standard error of the mean (SEM).

3. Results

3.1 Curcumin-loaded nanocapsules development and characterization

Nanocapsules were developed by interfacial polymer deposition methodology. The characterization of curcumin-loaded nanocapsules is shown in Table 1. The curcumin-loaded nanocapsules presented nanometric size, population monodisperse, negative zeta potential, acidic pH, and drug content was 100%.

Table 1. Characterization of curcumin-loaded nanocapsules

Parameter	D1
Diameter $_{(4,3)}$ (nm) (LD)	199.33 ± 1.3
SPAN (LD)	1.744 ± 0.019
Diameter (nm) (DLS)	228.76 ± 1.20
Polydispersity index (DLS)	0.095 ± 0.013
Zeta potential (mV)	−15.13 ± 0.35
pH	5.83 ± 0.02
Absolute Drug Content (%)	100.23 ± 0.67

3.2 Food consumption and survival percentage.

The effects of exposure to curcumin-loaded nanocapsules and LPS in food consumption and survival percentage of *D. melanogaster* are shown in Figure 2 (A and B). Statistical analysis revealed that there was no significant difference for the interaction factor (curcumin-loaded nanocapsules vs LPS) on the food consumption [$F_{(1,16)} = 29.37$; $P < 0.0001$] (Figure 2A). The Log-rank test (Mantel-Cox) showed that exposure to LPS (250 µg/kg), significantly decreased the survival of flies over experimental period compared to the control group [$P < 0.0001$]. Post hoc comparisons revealed that exposure to LPS decreased the survival (Figure 2B) of flies. The co-exposure with curcumin-loaded nanocapsules protected against the survival decrease induced by LPS.

3.3 Performance in behavioral tests

Negative geotaxis and open field behavioral tests are shown in Figures 3 (A and B). Statistical analysis revealed a significant difference for the interaction factor (curcumin-loaded nanocapsules vs LPS) on the negative geotaxis test [$F_{(1,16)} = 64.59$; $P < 0.0001$] and open field test [$F_{(1,16)} = 29.37$; $P < 0.0001$]. Post hoc comparisons revealed that the exposure to LPS increased the climbing time (Figure 3A) and decreased the crossing number (Figure 3B) of flies. The co-exposure with curcumin-loaded nanocapsules protected against the damage locomotor induced by LPS.

3.4 Protective effect of curcumin-loaded nanocapsules on oxidative stress and antioxidant defenses after exposure to LPS

3.4.1 Reactive species (RS) and lipid peroxidation

The protective effect of curcumin-loaded nanocapsules on RS e e lipid peroxidation in *D. melanogaster* exposed to LPS are shown in Figure 4 (A and B). Statistical analysis showed a significant difference for the interaction factor (curcumin-loaded nanocapsules vs LPS) on the RS levels [$F_{(1,24)}$ = 19.52; *P* = 0.0002] and MDA content [$F_{(1,24)}$ = 21.65; *P* = 0.0001]. Post hoc comparisons showed that the exposure to LPS increased RS levels (Figure 4A) and MDA content (Figure 4B) of flies. The co-exposure with curcumin-loaded nanocapsules protected against the increase RS levels and MDA content induced by LPS.

3.4.2 Activity of the GST detoxifying enzyme, and antioxidant enzymes SOD and CAT

The activity of GST, SOD and CAT enzymes are shown in Figure 5 (A, B and C). Statistical analysis showed a significant difference for the interaction factor (curcumin-loaded nanocapsules vs LPS) on the GST activity [$F_{(1,24)}$ = 12.60; *P* = 0.0016], SOD activity [$F_{(1,24)}$ = 23.03; *P* < 0.0001] and CAT activity [$F_{(1,24)}$ = 28.64; *P* < 0.0001]. Post hoc comparisons revealed that the exposure to LPS decreased the GST activity (Figure 5A), SOD activity (Figure 5B) and CAT activity (Figure 5C) of flies. The co-exposure with curcumin-loaded nanocapsules protected against the decrease in the GST, SOD, and CAT activity induced by LPS.

3.4.3 Western Blotting

3.4.3.1 Regulation of Nrf2: Balance redox

The evaluation of Nrf2 immunoreactivity is shown in Figure 6. In statistical analysis was possible to observe that there was a significant difference for the interaction factor (curcumin-loaded nanocapsules vs LPS) on Nrf2 immunoreactivity

[$F_{(1,12)}$ = 80.55; P < 0.0001]. Post hoc comparisons revealed that the exposure to LPS decreased Nrf2 immunoreactivity. The co-exposure with curcumin-loaded nanocapsules protected against the decrease in the Nrf2 immunoreactivity induced by LPS.

3.4.3.2 Regulation of apoptotic markers

The evaluation of the total p38, phospho-p38 and phospho-p38 MAPK/p38 MAPK ratio immunoreactivity, are shown in Figure 7 (A, B and C). Statistical analysis showed that there was no significant difference for the interaction factor (curcumin-loaded nanocapsules vs LPS) on the total p38 [$F_{(1,12)}$ = 49.75; P = 0.4941]. Still, the statistical analysis showed a significant difference for the interaction factor (curcumin-loaded nanocapsules vs LPS) on the phospho-p38 [$F_{(1,12)}$ = 56.13; P < 0.0001] and phospho-p38 MAPK/p38 MAPK ratio immunoreactivity [$F_{(1,12)}$ = 17.32; P = 0.0013]. Post hoc comparisons revealed that exposure to LPS increased the phospho-p38 MAPK immunoreactivity (Figure 7B) and phospho-p38 MAPK/p38 MAPK ratio immunoreactivity (Figure 7C). The co-exposure with curcumin-loaded nanocapsules protected against the damage apoptotic induced by LPS.

3.4.4 Cellular Metabolic Capacity

The evaluation of cellular metabolic capacity is shown in Figure 8. Statistical analysis showed a significant difference for the interaction factor (curcumin-loaded nanocapsules vs LPS) on cellular metabolic capacity [$F_{(1,28)}$ = 39.89; P < 0.0001]. Post hoc comparisons revealed that exposure to LPS decreased cellular metabolic capacity. The co-exposure with curcumin-loaded nanocapsules protected against the decrease in the cellular metabolic capacity induced by LPS.

4. Discussion

It is important to highlight that this is the first study to determine the potential protective effect of curcumin-loaded nanocapsules on changes in apoptosis and redox balance induced by LPS in *D. melanogaster*. In the present study, we obtained promising results regarding the protective effect of nanocapsules containing curcumin in *D. melanogaster* exposed to LPS. It was observed that curcumin-loaded nanocapsules at a concentration of 10µM were able to protect against LPS-induced damage, through the regulation of oxidative stress indicators, the apoptosis and redox balance pathways, mediated by p38 MAPK/Nrf2.

In relation to the characterization, curcumin-loaded nanocapsules presented adequate size, with nanometric values, and formation of monodisperse systems, indicating uniformity of the nanoparticles. Furthermore, was obtained a negative value for the zeta potential, attributing lower surface tension between the nanoparticles and the cells and 100% encapsulation efficiency. These results show that nanocapsules can be safely used as a carrier for the bioactive compound curcumin.

Additionally to the characterization of nanocapsules, the food consumption of the four treatment groups was then evaluated, as a form of control, seeking to prevent any result obtained from being attributed to the properties of the curcumin-loaded nanoparticles. It was observed that there was no significant difference in food consumption between the groups evaluated. It was found that the flies from the different groups were consuming the food equally. From this result, we can state that any subsequent significant differences observed in the study cannot be attributed to greater or lesser consumption of the diet by the flies. When evaluating survival, our results show that curcumin-loaded nanocapsules protected against LPS-induced

damage in survival percentage. Corroborating our study, it was described that curcumin prevented mortality after exposure to the toxic compound copper sulfate in *D. melanogaster* (Abolaji et al., 2020), and increased survival in *D. melanogaster* under heat stress conditions (Chen et al., 2018).

Moreover, we observed that exposure to LPS compromised locomotor and exploratory performance in negative geotaxis and open field tests. It has been described that exposure to LPS promotes greater vulnerability to degeneration of dopaminergic neurons and locomotor damage (Frank-Cannon et al., 2008). The toxic effect of LPS has been attributed to oxidative damage, which damages all major cellular constituents and activates toll-like receptor complexes that lead to neuronal cell death (Okun et al. 2011), and giving inflammatory and immunological responses (Hsieh et al., 2021).

It was observed with great significant difference that curcumin-loaded nanocapsules protected against LPS-induced damage in locomotor performance of *D. melanogaster*. These data provide us with evidence of the protective effect of nanocapsules containing curcumin at a concentration of 10 µM against LPS toxicity in *D. melanogaster*. The effect was possibly obtained by a mechanism related to the modulation of the redox balance. Corroborating our study, it was described that curcumin prevented mortality after exposure to the toxic compound copper sulfate (Abolaji et al., 2020), and increased survival in *D. melanogaster* under heat stress conditions (Chen et al., 2018). Just as curcumin prevented behavioral damage induced by LPS in rats (Reis et al., 2022).

To confirm the protective mechanism of curcumin-loaded nanocapsule curcumin against oxidative stress, we evaluated oxidative stress markers RS and peroxidation lipidic. We observed that exposure to LPS increased RS levels and MDA

content. Exposure LPS also increased RS levels and increased MDA content in *D. melanogaster,* induceded oxidative stress (Liu et al., 2023). Regarding the protective effect of curcumin, favorable, we observed that curcumin-loaded nanocapsules protected against the increase RS levels and MDA content induced by LPS. Corroborating our study, it was proven that curcumin exerts antioxidant activity, eliminating RS and inducing an antioxidant response (Dai et al., 2017). Likewise, it was described that curcumin decreased MDA levels in *Drosophila* under heat stress conditions (Chen et al., 2018), and decreased RS and MDA levels in rats treated with Rotenone (Cui et al., 2016). In contrast, curcumin in low and medium doses reduced the levels of MDA and RS, but in high doses, it increased the production of MDA and RS (Lin et al., 2019), indicating that high doses can intensify oxidative stress. In this sense, nanoencapsulation can be a strategy. Our previous study showed that curcumin-loaded nanocapsules decreased RS and MDA levels, without causing a toxic effect on *D. melanogaster* (Fernandes et al., 2023). In our current research, according to the results obtained, we can suggest that curcumin-loaded nanocapsules protected against oxidative stress.

It is known that oxidative stress is characterized as the accumulation of reactive species (RS) or free radicals, which override antioxidant protection, decompensating redox homeostasis (Guan and Lan, 2018; Halliwell, 2011), causing oxidative damage to the cell and subsequently activating apoptosis signaling pathways (Guan and Lan, 2018; Redza-Dutordoir and Averill-Bates, 2016). The organism has antioxidant systems that protect it against the increase in free radicals. The first line of defense is the endogenous enzyme pathways, constituted primarily by enzymes antioxidants, and detoxification, and through exogenous pathways, basically consisting of antioxidants from food (Pisoschi and Pop, 2015; Soares et al., 2015).

In our study, it was also observed that curcumin-loaded nanocapsules protect the detoxifying and antioxidant defenses of *D. melanogaster*. The statistical results demonstrated that LPS decreased the activity of the detoxifying enzyme GST, and decreased the activity of the antioxidant enzymes SOD and CAT. It is known that LPS induces oxidative stress and those enzymes that are modulated negatively under conditions of oxidative stress (Hou et al., 2019). Exposure to curcumin-loaded nanocapsules were able to significantly protect against LPS-induced decrease the activity of these enzymes. The SOD enzyme catalyzes the superoxide anion (O^{2-}) to form H_2O_2, which is catalyzed by CAT to form H_2O, a non-toxic producer (McDermott, 2000). Based on our results, we can suggest that the effect of curcumin-loaded nanocapsules on SOD and CAT activity, result in less H_2O_2 and O^{2-}, protecting against LPS-induced damage. Curcumin-loaded nanocapsules neutralized free radicals and modulated the activity of SOD, CAT and GSH enzymes. To further understand the antioxidant effect of curcumin-loaded nanocapsules, we assessed Nrf2 immunoreactivity. We observed that LPS decreased Nrf2 immunoreactivity and that curcumin significantly restored the immunoreactivity of this protein. These data confirm the results obtained, where curcumin activates the Nrf2 signaling pathway and protects cells against oxidative damage.

According to Balogun et al. (2003), curcumin stimulates the activity of the hemoxygenase-1 (HO-1) gene, responsible for the inactivation of the Nrf2-Keap1 complex, which results in the release of Nrf2 from its suppressor Keap-1 allowing its translocation to the nucleus, leading to increased binding of the Nrf2 transcription factor to DNA in the antioxidant response element (ARE), a signaling pathway that regulates the expression of a variety of enzymes. The antioxidant potential of curcumin has been attributed to its structural conformation that contains two methoxyphenol

rings joined by a β-diketone, groups that favor the elimination of free radicals, restoring redox homeostasis, activating Nrf2 and consequently the antioxidant and detoxifying enzymes (Balogun et al., 2003; Singh et al., 2011). Here, we suggest that curcumin-loaded nanocapsules could neutralize free radicals, protect against oxidative stress caused by LPS, and activate the Nrf2 signaling pathway and consequently SOD, CAT, and GST enzymes, return to their normal activity.

Additionally, knowing that oxidative stress can induce apoptosis (Samarghandian et al., 2014), and that the p38 MAPK protein that phosphorylates Nrf2 on three Ser residues (Ser215, Ser408, and Ser577), improving its interaction with Keap1 and reducing accumulation in the nucleus (Keum et al., 2006; Yamamoto et al., 2018). However, Nrf2 does not accumulate in the cytosol, as Keap1 only maintains it in the cytosol to generate a complex with the E3-ubiquitin ligase based on Cullin 3 (Cul3)/Rbx1, inducing Nrf2 ubiquitination and its degradation by the proteasome (Cores et al., 2020). Moreover, considering that p38 MAPK also stimulates the oxidation of fatty acids (Shen et al., 2004). In this study, we evaluated the apoptosis marker p38 MAPK and cellular metabolic capacity. We observed that LPS increased phospho-p38 MAPK immunoreactivity and phospho-p38 MAPK/p38 MAPK ratio, decreased cellular metabolic capacity and that curcumin-loaded nanocapsules was able to restore these alterations. As in our research, other studies also demonstrate the anti-apoptotic effects of curcumin (Fang et al., 2018; Uzun-Goren and Uz, 2022). On the other hand, it has been described that free curcumin in higher doses caused apoptotic, through increasing p38 MAPK activation (Hsiao et al., 2020), the effect that may be related to the metabolism of free curcumin, but the metabolization mechanism was not analyzed in these studies. It is known that free curcumin can have a high metabolism rate (Kocaadam and Şanlier, 2017).

The p38 MAPK proteins can phosphorylate and negatively regulate Nrf2, so the decreased signaling of this pathway observed in our results may suggest that there was no phosphorylation of Nrf2, which may have contributed to the increase in Nrf2 immunoreactivity and increased activity of GST, SOD and CAT enzymes in *D. melanogaster*. Suppression of p38 MAPK signaling negatively regulates RS homeostasis and increases fatty acid oxidation, and consequently cell survival (Zhao et al., 2017). Based on our results, co-exposure to curcumin-loaded nanocapsules reduced signaling in the p38 MAPK pathway, observed in the decrease of phospho-p38 MAPK immunoreactivity and phospho-p38 MAPK/p38 MAPK ratio, which may have contributed to the decreased RS and MDA levels. The curcumin-loaded nanocapsules protected against increased p38 MAPK signaling, preserving cellular integrity, protecting against apoptosis, and regulating redox balance, which could potentially have promoted increased fly survival.

In this sense, markers of oxidative stress and redox balance, as well as markers of apoptosis, provide us with evidence of the mechanism of action of curcumin-loaded nanocapsules on the toxicological damage of LPS at the cellular level in *D. melanogaster*.

5. Conclusion

The innovative results show that curcumin-loaded nanocapsules protected against alteration in survival, locomotor performance, Nrf2 immunoreactivity, activity of the enzymes GST, SOD, and CAT, phospho-p38 MAPK immunoreactivity and phospho-p38 MAPK/p38 MAPK ratio, RS and MDA levels and cell viability LPS-induced. Regulated the redox balance and presented antiapoptotic effects in *D. melanogaster* exposed to the toxic agent LPS. These results demonstrate the potential

of curcumin-loaded nanocapsules in combating oxidative stress and apoptosis, suggesting that this mechanism of action can in the future be used in the search for the treatment of many diseases and toxic agents that act through a similar mechanism.

Declaration of competing interest

The authors declare that the research for this article was conducted in the absence of any known competing financial interests or personal relationships that could be construed as a conflict of interest.

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Legends

Figure 1. Graphical summary of experimental methodologies used.

Figure 2. Effect of exposure to LPS (250 µg/kg diet), curcumin-loaded nanocapsules (10 µM), and the co-exposure to LPS and curcumin-loaded nanocapsules on (A) food consumption and (B) survival percentage. Results are expressed as mean and standard error of the mean (SEM), for n = 6-8 in each group. * Indicates statistically significant differences ($P < 0.05$) compared to the control group. # Indicates statistically significant differences ($P < 0.05$) compared to the LPS group.

Figure 3. Effect of exposure to LPS (250 µg/kg diet) for 7 days, curcumin-loaded nanocapsules (10 µM) for 10 days, and the co-exposure to LPS and curcumin-loaded nanocapsules for (7 and 10 days respectively) on (A) Negative geotaxis test and (B) Open field test. Results are expressed as mean and standard error of the mean (SEM), for n = 5 in each group. * Indicates statistically significant differences ($P < 0.05$) compared to the control group. # Indicates statistically significant differences ($P < 0.05$) compared to the LPS group.

Figure 4. Effect of exposure to LPS (250 µg/kg diet) for 7 days, curcumin-loaded nanocapsules (10 µM) for 10 days, and the co-exposure to LPS and curcumin-loaded nanocapsules for (7 and 10 days respectively) on (A) RS and (B) MDA content. Results are expressed as mean and standard error of the mean (SEM), for n = 7 in each group. * Indicates statistically significant differences ($P < 0.05$) compared to the control group. # Indicates statistically significant differences ($P < 0.05$) compared to the LPS group.

Figure 5. Effect of exposure to LPS (250 µg/kg diet) for 7 days, curcumin-loaded nanocapsules (10 µM) for 10 days, and the co-exposure to LPS and curcumin-loaded nanocapsules for (7 and 10 days respectively) on the activity of the enzymes (A) GST, (B) SOD, and (C) CAT. Results are expressed as mean and standard error of the mean (SEM), for n = 7 in each group. * Indicates statistically significant differences ($P < 0.05$) compared to the control group. # Indicates statistically significant differences ($P < 0.05$) compared to the LPS group.

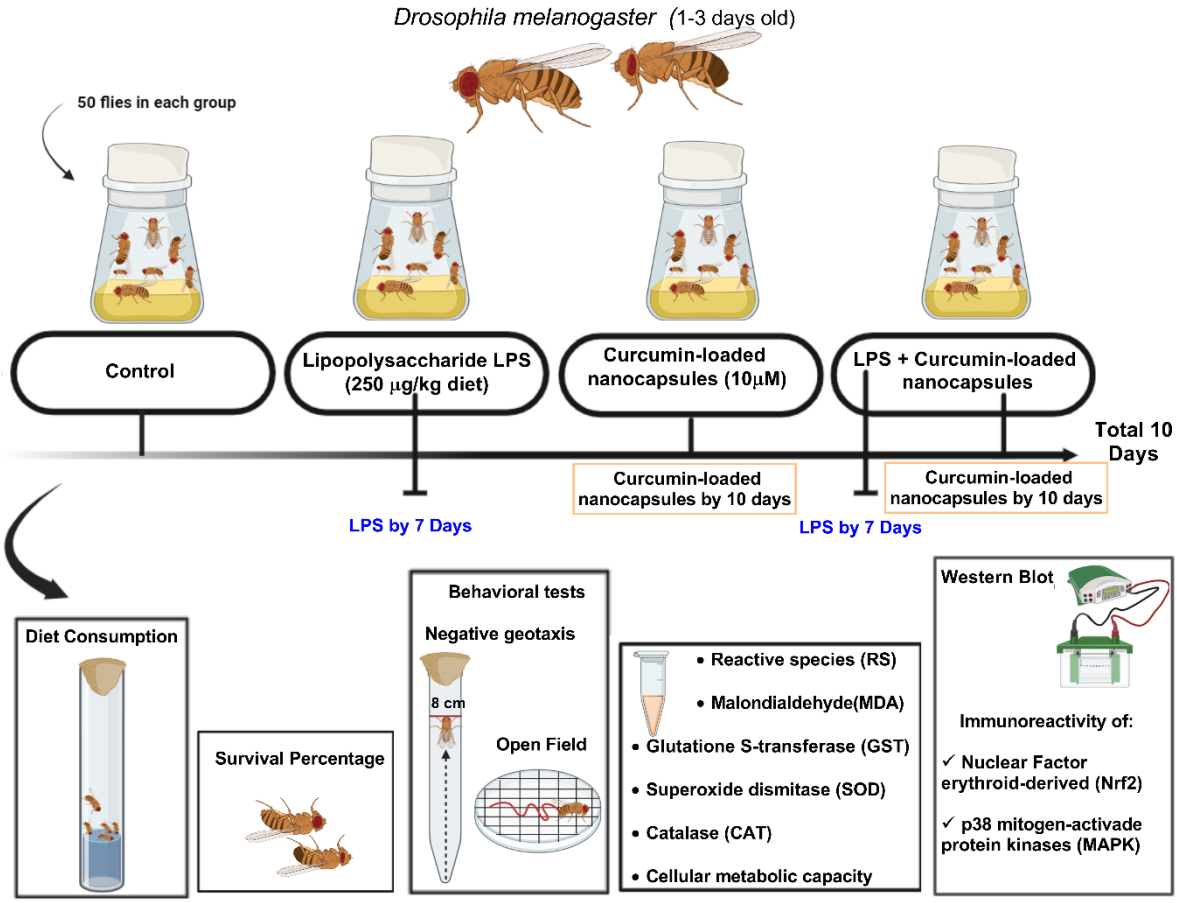
Figure 6. Effect of exposure to LPS (250 µg/kg diet) for 7 days, curcumin-loaded nanocapsules (10 µM) for 10 days, and the co-exposure to LPS and curcumin-loaded nanocapsules for (7 and 10 days respectively) on Nrf2 immunoreactivity. Results are expressed as mean and standard error of the mean (SEM), for n = 4 in each group. * Indicates statistically significant differences ($P < 0.05$) compared to the control group. # Indicates statistically significant differences ($P < 0.05$) compared to the LPS group.

Figure 7. Effect of exposure to LPS (250 µg/kg diet) for 7 days, curcumin-loaded nanocapsules (10 µM) for 10 days, and the co-exposure to LPS and curcumin-loaded nanocapsules for (7 and 10 days respectively) on apoptotic markers (8A) p38 MAPK immunoreactivity, (8B) phospho-p38 MAPK immunoreactivity and (8C) phospho-p38 MAPK/p38 MAPK ratio immunoreactivity. Results are expressed as mean and standard error of the mean (SEM), for n = 4 in each group. * Indicates statistically significant differences ($P < 0.05$) compared to the control group. # Indicates statistically significant differences ($P < 0.05$) compared to the LPS group.

Figure 8. Effect of exposure to LPS (250 µg/kg diet) for 7 days, curcumin-loaded nanocapsules (10 µM) for 10 days, and the co-exposure to LPS and curcumin-loaded nanocapsules for (7 and 10 days respectively) on cellular metabolic capacity. Results are expressed as mean and standard error of the mean (SEM), for n = 8 in each group. * Indicates statistically significant differences ($P < 0.05$) compared to the control group. # Indicates statistically significant differences ($P < 0.05$) compared to the LPS group.

Figures:

Figure 1:



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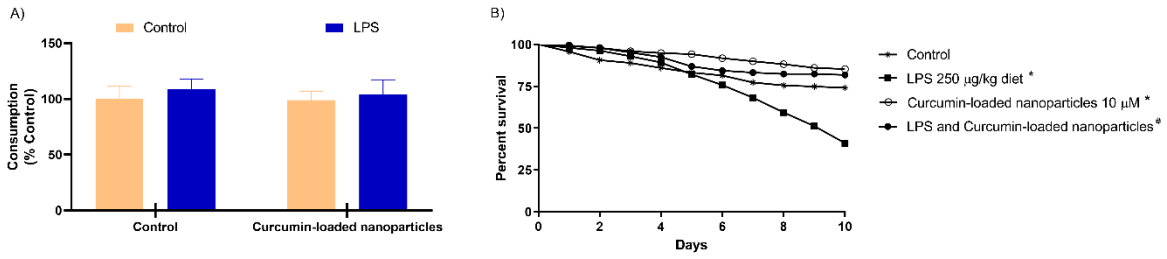

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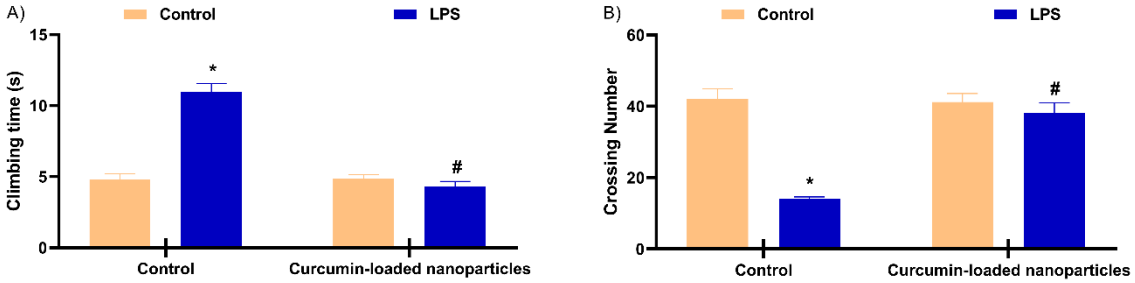

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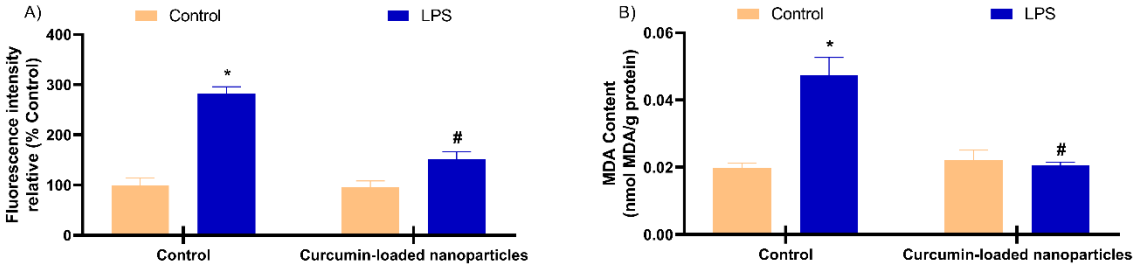

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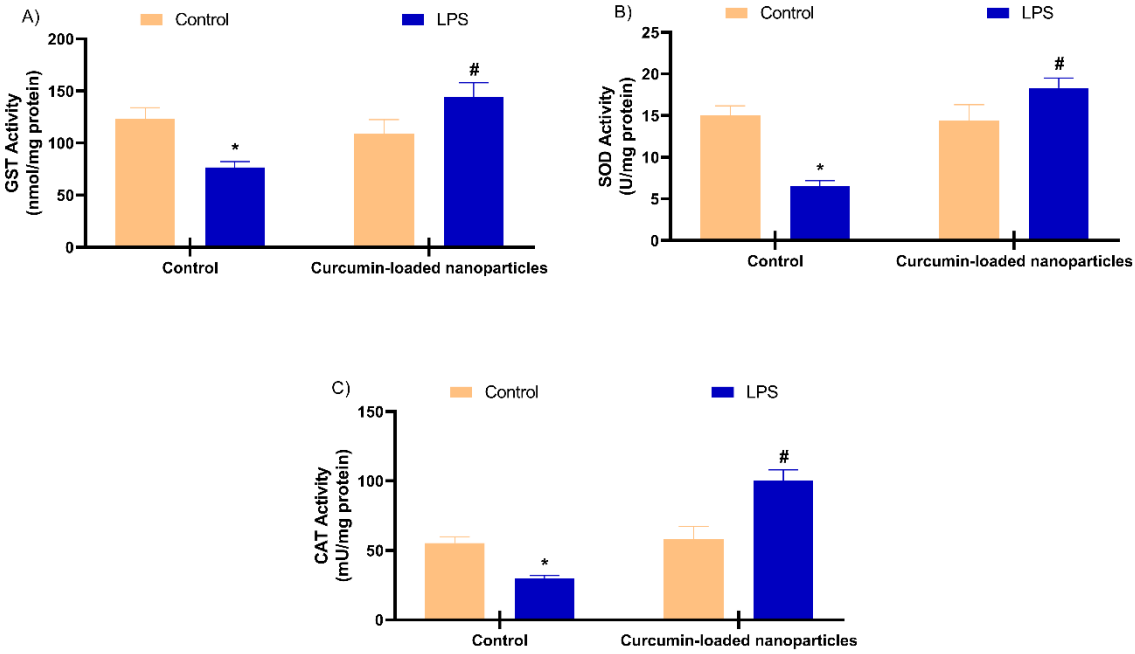

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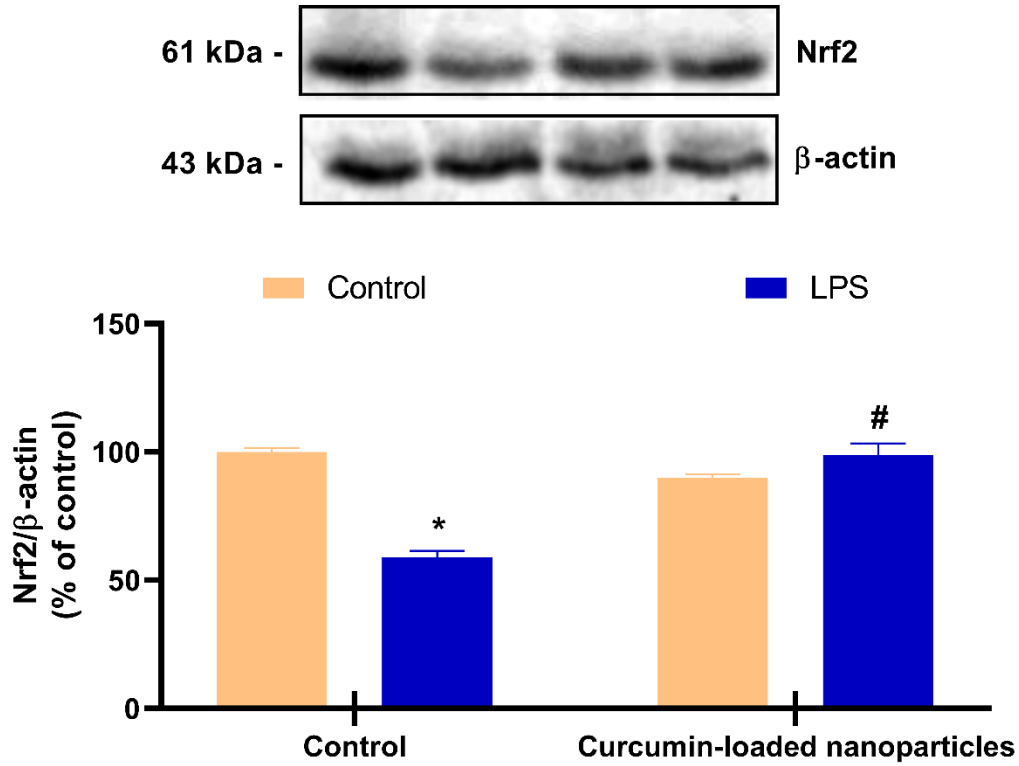

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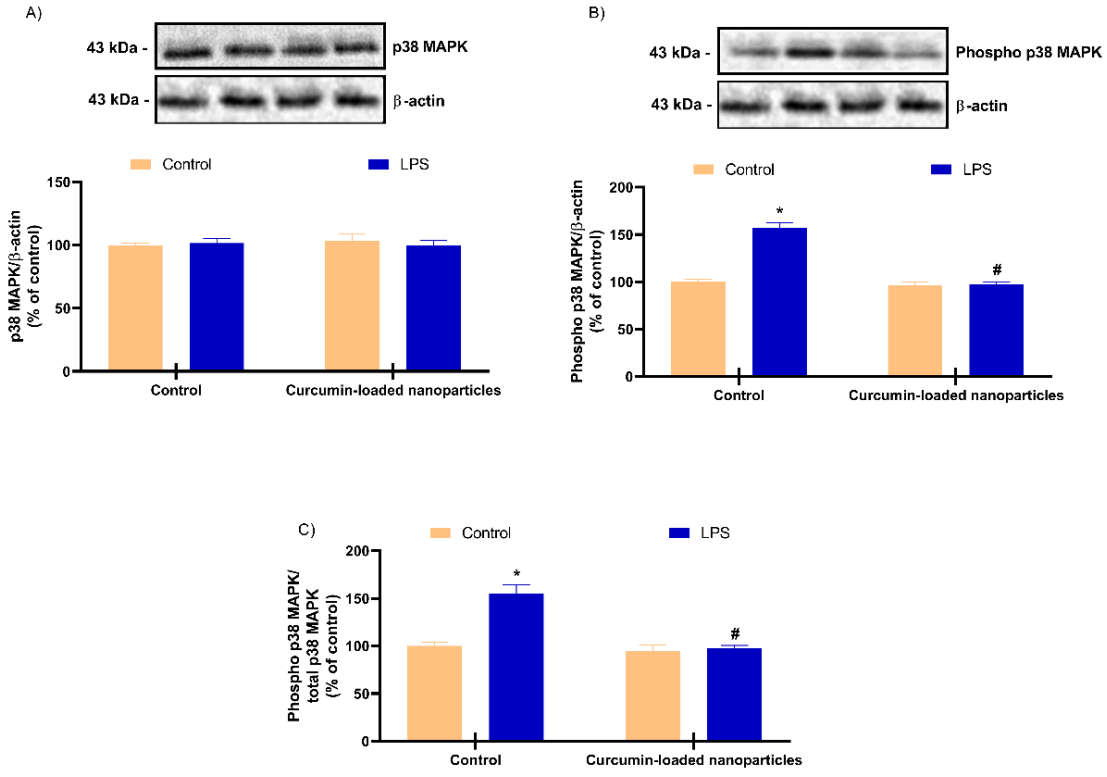

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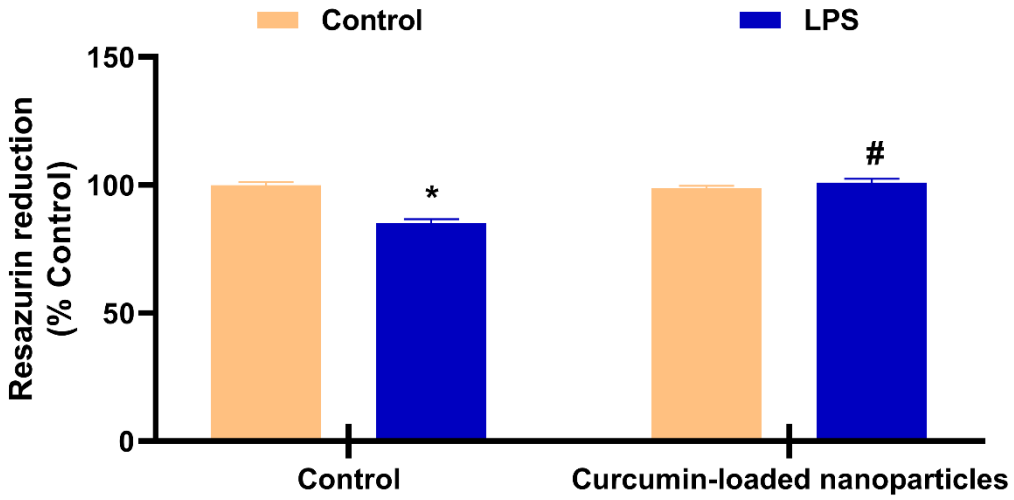
Control
LPS
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(% Control)
150
100
50
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#
Control
Curcumin-loaded nanoparticles

PARTE III

6. DISCUSSÃO

Com os resultados obtidos na presente tese, obtivemos um conjunto de informações, que nos fornecem evidências de que a exposição crônica a nanocápsulas carregadas com curcumina revestidas com P80, nas concentrações avaliadas, não causou efeitos tóxicos em *D. melanogaster*, e que com baixas doses já é possível modular positivamente os marcadores avaliados, sugerindo que a nanoencapsulação da curcumina pode ser explorada. Nesse contexto, dando seguimento ao estudo, determinamos se nanocápsulas carregadas com curcumina na menor concentração avaliada, poderia proteger *D. melanogaster* contra um agente tóxico, nesse sentido, utilizamos a endotoxina LPS. Foi evidenciado informações importantes, quanto ao efeito protetor de nanocápsulas carregadas com curcumina na concentração de 10µM contra as alterações no perfil comportamental e moleculares induzidas pela endotoxina LPS em *D. melanogaster*, através da regulação das vias p38 MAPK/Nrf2. Vale destacar que este estudo é pioneiro em determinar o potencial efeito protetor das nanocápsulas carregadas com curcumina nas alterações de apoptose através da via p38 MAPK e no equilíbrio redox pela via Nrf2, induzidos por LPS em *D. melanogaster*

Inicialmente, tende em vista que a curcumina livre pode ser tóxica, as nanopartículas surgem como alternativa. Em vista disso, o primeiro artigo foi direcionado a avaliar o possível efeito tóxico induzido pela exposição crônica à curcumina livre e nanocápsulas carregadas com curcumina revestidas com P80 em modelos *in vivo* de *D. melanogaster,* através dos parâmetros de toxicidade.

Foi observado que com baixas doses de nanocápsulas carregadas com curcumina já é possível aumentar a sobrevivência, diminuir a atividade da AChE e logo aumentar o desempenho locomotor e exploratório, diminuir o estresse oxidativo, ativar as defesas antioxidantes, e aumentar as células viáveis. Os resultados sugerem que os presentes efeitos antioxidantes de nanocápsulas carregadas com curcumina em baixas concentrações, observado nos marcadores avaliados, foi possivelmente associado à nanoencapsulação, pois o revestimento parecia ser uma estratégia para aumentar a atividade biológica e diminuir a toxicidade da curcumina. E o mecanismo

pelo qual a curcumina agiu, pode estar atribuído ao aumento de Nrf2, que ativou as enzimas GST, SOD e CAT, favorecendo com a diminuição dos níveis de RS e TBARS, cooperando com a diminuição da atividade da enzima AChE e posteriormente aumentando o desempenho comportamental, podemos ver, um conjunto de efeitos protetores, que ativou as defesas antioxidantes e diminuiu o estresse oxidativo em *D. melanogaster*.

Estes resultados estão de acordo com o estudo, que mostrou que a curcumina teve a capacidade de neutralizar o estresse oxidativo causado pela Zearalenona em células da granulosa suína em um estudo *in vitro* (QIN *et al.*, 2015), além de ativar a via de sinalização Nrf2 e proteger as células contra os danos oxidativos (ASHRAFIZADEH *et al.*, 2019), e também foi capaz modular as enzimas antioxidantes em ratos (POAPOLATHEP *et al.*, 2015).

No entanto, apesar das propriedades benéficas da curcumina, ela pode apresentar toxicidade, dependendo muito da duração do tratamento e das doses utilizadas. Altas doses de curcumina produzem efeitos pró-oxidantes e tóxicos (LÓPEZ-LÁZARO, 2008), danos no fígado de roedores (BALAJI; CHEMPAKAM, 2010), aumentam a mortalidade e deformidades em embriões de peixe-zebra (ALAFIATAYO *et al.*, 2019). Nesse contexto, as nanocápsulas são consideradas uma estratégia para aumentar a biodisponibilidade dos compostos, obtendo efeitos terapêuticos a partir de doses menores e, apresentando menor toxicidade e evitando os efeitos indesejáveis causados pela curcumina livre (NAZILA *et al.*, 2016).

Dessa forma, os dados do presente estudo evidenciam que a exposição crônica de nanocápsulas carregadas com curcumina nas concentrações avaliadas, não causou efeitos tóxicos, e são alternativas seguras, e que com baixas doses já é possível regular positivamente os marcadores avaliados. Podemos observar, que os efeitos da curcumina livre, só foi observado em concentrações maiores (300 e 900 µM), enquanto que os efeitos de nanocápsulas carregadas com curcumina, foi observado nas concentrações menores (10, 30 e 100 µM). Nos sugerindo, que nanocápsulas surgem como alternativa para obter efeitos terapêuticos a partir de doses menores, e que são capazes de melhorar a atividade biológica da curcumina.

Quanto ao efeito protetor de nanocápsulas carregadas com curcumina em *D. melanogaster* expostas ao LPS, no presente estudo, constatamos que a exposição à nanocápsulas carregadas com curcumina foi capaz de regular a via p38 MAPK/Nrf2,

combater a geração de compostos oxidantes, ativou o mecanismo de defesa antioxidantes e controlou a ocorrência de apoptose na célula.

Dessa forma, nossos achados permitem dizer, que tais efeitos observados, principalmente antiapoptótica e antioxidantes, estariam relacionados com a diminuição da imunoreatividade da proteína phospho-p38 MAPK (p-P38 MAPK), consequentemente, combatendo o estresse oxidativo causado pelo LPS, ao limitar os níveis de metabólitos da oxidação RS e malondialdeído (MDA), contribuindo para o aumento da capacidade metabólica celular das *D. melanogaster*. Assim como, a diminuição de p-p38 MAPK, regulou a via do equilíbrio redox, por meio do aumento da imunoreatividade de Nrf2, contribuindo para a ativação da atividade da enzima detoxificante e das enzimas antioxidantes, corroborando com o aumento da sobrevivência e do desempenho locomotor e exploratório de *D. melanogaster* expostas ao LPS. Nesse contexto, nanocápsulas carregadas com curcumina eliminou o excesso de radicais livres e aumentou as defesas antioxidantes.

Estes dados confirmam os resultados obtido por (FANG *et al.*, 2018), onde a curcumina reduziu a proteína p38 MAPK em fibroblastos cardíacos humanos. Assim como, a curcumina atenuou a nefrotoxicidade induzida pela gentamicina em ratos, diminuiu p-P38 MAPK e aumentou a imunorreatividade de Nrf2 no tecido renal em ratos (UZUN-GOREN; UZ, 2022). Ademais, esses resultados são corroborados com os dados do estudo de REIS *et al.* (2022b), que mostrou que a curcumina preveniu contra déficits comportamentais de ratos expostos ao LPS, e aumentou a translocação de Nrf2 para o núcleo celular, e propôs que a prevenção observada, pode ter sido, possivelmente por um mecanismo ligado à modulação de Nrf2. Ainda, a curcumina ativou a via Nrf2-Keap1 e aumentou atividade das enzimas SOD, CAT e GPx, e em doses baixas e médias diminuiu os níveis de MDA e RS, protegendo contra o estresse oxidativo induzido por H_2O_2 em células RAW264.7 (LIN *et al.*, 2019).

Com base nas evidências atualmente relatadas, sabe-se que o estresse oxidativo decorre de um desequilíbrio entre a manifestação sistêmica de radicais livres e a capacidade do sistema de defesa antioxidante, em inativar as espécies reativas e reparar o dano celular (SCHIEBER; CHANDEL, 2014). As células, possuem a presença de sistemas antioxidantes, que catalisam a remoção ou impedem a formação de EROs (POLJSAK, 2011). Entre os sistemas antioxidantes e de resposta celular ao estresse oxidativo, podemos destacar o Nrf2, o qual participa da ativação

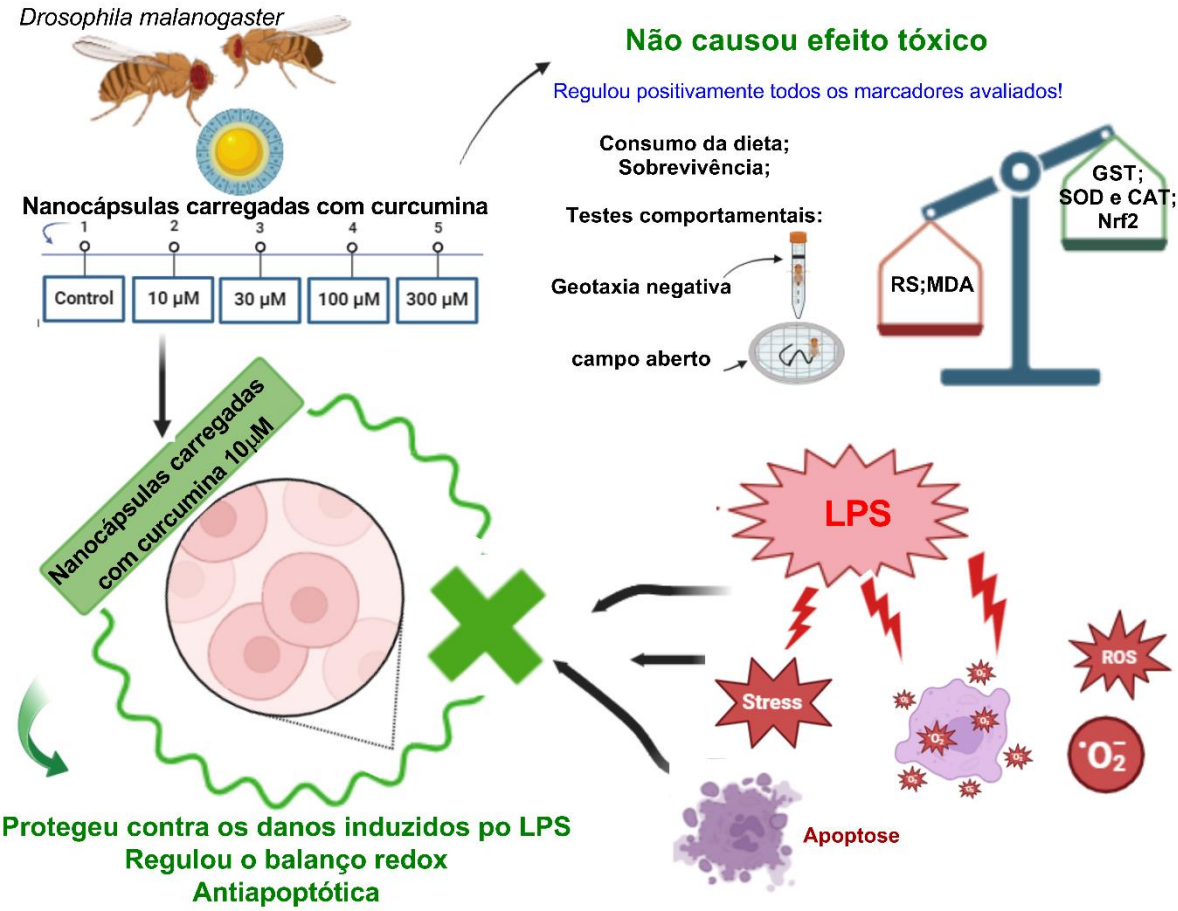
de uma variedade de enzimas mediante ligação ao ERA (CHEN *et al.*, 2015). A partir dessa ligação são expressas enzimas antioxidantes SOD e CAT, e de detoxificação (GST e GPX, entre outras (LEE; PARK, 2021), que atuam eliminando EROs.

Sabendo da importância desses sistemas, e que quando ocorre um desequilíbrio, tendo uma produção excessiva de EROs ou deficiência nos sistemas de defesas antioxidantes, manifesta-se o estresse oxidativo, que pode levar a disfunções e morte celular (ZIŃCZUK *et al.*, 2020). Sendo assim, podemos dizer que nanocápsulas carregadas com curcumina avaliadas nesse estudo, atuaram nesses sistemas de defesa, regularam positivamente as vias p38 MAPK/Nrf2, ativando enzimas de defesa, catalisando a remoção de metabólitos tóxicos.

7. CONCLUSÕES

Nosso estudo mostra que a exposição crônica à curcumina livre e nanocápsulas carregadas com curcumina não causou efeitos tóxicos. Outro achado importante é que baixas doses de nanocápsulas carregadas com curcumina modularam positivamente todos os marcadores de toxicidade avaliados, essa vantagem foi associada possivelmente à nanoencapsulação, que é proposta para aumentar a atividade biológica e diminuir a toxicidade da curcumina. A partir da ausência de toxicidade, foi possível avaliar o efeito protetor de nanocápsulas carregadas com curcumina. Onde podemos identificar, que nanocápsulas carregadas com curcumina protegem contra danos induzidos por LPS. Regulou positivamente as vias p38 MAPK/ Nrf2, mostrando ter um efeito antiapoptótico, e antioxidantes ao regular o equilíbrio redox em *D. melanogaster* exposta ao agente tóxico LPS. Podemos sugerir que nanoencapsulação de curcumina em P80 potencializou positivamente seus efeitos e pode ser explorada de forma segura em possíveis aplicações futuras.

Figura 7: Resumo dos resultados



Fonte: Arquivo pessoal.

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