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KÉTNNE HANNA POLETO PINTO

ÁCIDO *p*-CUMÁRICO ATENUA O DANO NEUROMOTOR E OXIDATIVO NO MODELO EXPERIMENTAL DE DOENÇA DE PARKINSON EM *Drosophila melanogaster* INDUZIDO POR ROTENONA

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Dissertação apresentada ao programa de Pós-graduação *Stricto Sensu* em Bioquímica da Universidade Federal do Pampa, como requisito parcial para obtenção do título de Mestre em Bioquímica.

Orientador: Prof. Dr. Gustavo Petri Guerra.

Coorientadora: Profª. Drª. Marina Prigol.

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RESUMO

Doença de Parkinson é uma das doenças neurodegenerativas mais comuns mas ainda complexa, caracterizada por um grande número de alterações motoras e não motoras que podem afetar sua função em grau variável. Neste estudo, investigamos o efeito do ácido *p*-cumárico nas alterações comportamentais e neuroquímicas induzidas pela exposição à rotenona em *Drosophila melanogaster* em um modelo semelhante à doença de Parkinson (DP). O ácido *p*-cumárico é um importante composto fenólico e antioxidante, que pode ser uma alternativa na recuperação da neurotoxicidade observada nas doenças neurodegenerativas. Para realização do estudo as moscas foram divididas: dois grupos com dieta padrão (controle) e dois grupos com exposição a rotenona (500 µM), após o período de 72 horas um grupo controle e um grupo rotenona passaram a receber a dieta com ácido *p*-cumárico (0,3 µM) por mais 48 horas. Finalizando o estudo de 120horas (7 dias) com 4 grupos: Dieta padrão (controle), ácido *p*-cumárico (0,3 µM), rotenona (500 µM), e rotenona (500 µM) mais ácido *p*-cumárico (0,3 µM). A exposição ao ácido *p*-cumárico reduziu a mortalidade induzida por rotenona, além disso, os indicadores de estresse oxidativo foram normalizados espécies reativas (RS), Espécies Reativas ao Ácido Tiobarbitúrico (TBARS), superóxido dismutase (SOD), atividade da catalase (CAT), glutationa-S-transferase (GST) e tiol não proteico (NPSH) prevenindo dano oxidativo, e houve a restauração dos níveis de dopamina. Os resultados demonstram que o ácido *p*-cumárico reduziu efetivamente os danos causados pelo modelo de doença de Parkinson. O ácido *p*-cumárico é um composto fenólico não flavonóide que apresenta segurança e eficácia muito explorada na ciência dos alimentos devido suas excelentes propriedades antioxidante e anti-inflamatória, além de tudo, apresentando baixa toxicidade oral, sendo propriedades ainda pouco exploradas em produtos medicamentosos. Estudos complementares são necessários para elucidar o ácido *p*-cumárico como possível alvo terapêutico para novos fármacos no tratamento da doença de Parkinson.

Palavras-chave: estresse oxidativo; cadeia respiratória; dopamina; atividade da acetilcolinesterase; ácido fenólico.

ABSTRACT

Parkinson's disease is one of the most common but still complex neurodegenerative diseases, characterized by a large number of the motor and non-motor alterations that can affect its function to a variable degree. In this study, we investigated the effect of p-coumaric acid on behavioral and neurochemical changes induced by exposure to rotenone in Drosophila melanogaster in a model similar to Parkinson's disease (PD). P-coumaric acid is an important phenolic and antioxidant compound, which may be an alternative in the recovery of neurotoxicity observed in neurodegenerative diseases. To carry out the study, the flies were divided: two groups with a standard diet (control) and two groups with exposure to rotenone (500 μM), after a period of 72 hours a control group and a rotenone group started to receive the diet with p acid. -coumaric (0.3 μM) for another 48 hours. At the end of the 120-hour study (7 days) with 4 groups: Standard diet (control), p-coumaric acid (0.3 μM), rotenone (500 μM), and rotenone (500 μM) plus p-coumaric acid (0. 3 μM). Exposure to p-coumaric acid reduced rotenone-induced mortality, in addition, oxidative stress indicators were normalized reactive species (RS), Thiobarbituric Acid Reactive Species (TBARS), superoxide dismutase (SOD), catalase activity (CAT), glutathione-S-transferase (GST) and non-protein thiol (NPSH) preventing oxidative damage, and there the was the restoration of dopamine levels. The results demonstrate that p-coumaric acid effectively reduced the damage caused by the Parkinson's disease model. P-coumaric acid is a non-flavonoid phenolic compound that has safety and efficacy that has been widely explored in food science due to its excellent antioxidant and anti-inflammatory properties, in addition to having low oral toxicity, and properties still little explored in drug products. Complementary studies are needed to elucidate p-coumaric acid as a possible therapeutic target for new drugs in the treatment of Parkinson's disease.

Keywords: oxidative stress; respiratory chain; dopamine; acetylcholinesterase activity; phenolic acid.

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LISTA DE ABREVIATURAS E SIGLAS

AChE – Acetilcolinesterase

BDNF - fator neurotrófico derivado do cérebro

CAT- Catalase

DA- Dopamina

DP- Doença de Parkinson

GST- Glutationa-S-transferase

HPLC-DAD- Cromatografia Líquida de Alta Performance com Detector de Matriz de Diodo

DP- Doença de Parkinson

ER- Espécies reativas

MDA- Malondialdeído

NADPH – Nicotinamida Adenina Dinucleotídeo Fosfato reduzido

ROS – Espécies Reativas de Oxigênio

SOD – Superóxido Dismutase

TBARS – Espécies Reativas ao Ácido Tiobarbitúrico

SUMÁRIO

1. INTRODUÇÃO

A doença de Parkinson (DP) é um distúrbio neurodegenerativo progressivo de início tardio caracterizado por degeneração dopaminérgica nigroestriatal e a formação da α-sinucleína (REICH & SAVITT, 2019). Estudos epidemiológicos sugerem que a exposição a agentes ambientais, como pesticidas, pode aumentar o risco de DP (ADEDARA et al., 2022).

A disfunção mitocondrial também tem sido associada à DP, especificamente, ocorre uma deficiência na atividade do complexo I da cadeia de transferência de elétrons mitocondrial, principalmente no cérebro e músculos em pacientes com DP (SIMON et al., 2020). A rotenona (ROT) é uma toxina natural que inibe o complexo I da cadeia de transporte de elétrons mitocondrial (SUDATI et al., 2013), e já estão bem descritos os modelos utilizando a Rotenona por reproduzir as características da DP em modelos animais(MOTAWI et al., 2020).

A *Drosophila melanogaster* tem sido utilizada como modelo para testar a eficácia de compostos naturais no contexto de doenças neurodegenerativas, incluindo o modelo de doença de Parkinson (SIIMA et al., 2020). Este modelo apresenta inúmeras vantagens como baixas limitações éticas, ciclo de vida curto, alta fecundidade e baixo custo de manutenção (ILIADI et al., 2016).

Os ácidos cumáricos são derivados do ácido cinâmico que são mono-hidroxilados no grupo fenil (KIM et al., 2017). O ácido *p*-cumárico possui propriedades antioxidantes, antibacterianas, anti-inflamatórias, neuroprotetoras e muito utilizado na indústria alimentícia (HUANG et al., 2020).

A rotenona é uma das neurotoxinas ambientais utilizada na etiopatogenia da DP, reproduzindo características comportamentais,

bioquímicas e patológicas da DP, modelo experimental é usado para investigar os mecanismos subjacentes que levam à DP e avaliar as novas terapias potenciais para a doença (Chia et al., 2020b).

A administração de rotenona nas *Drosophila melanogaster* demonstrou causar degeneração de neurônios dopaminérgicos, neuroinflamação e anormalidade locomotora (SIIMA et al., 2020). Portanto, neste estudo, utilizamos o ácido *p*-cumárico para investigar os seus efeitos e sua capacidade protetora diante da toxicidade induzida por rotenona, bem como seus possíveis mecanismos de ação em *Drosophila melanogaster.*

2. REVISÃO BIBLIOGRÁFICA

2.1 Epidemiologia

A doença de Parkinson, é a segunda doença neurodegenerativa mais comum depois do Alzheimer, acometendo principalmente idosos acima dos 65 anos (GARCIA et al., 2021). Segundo a OMS, estima-se que 1% da população mundial é acometida pela doença de Parkinson (DP), uma doença crônica e progressiva, com maior incidência em adultos homens, sendo que somente 15% dos diagnosticados possuem a DP por causas genéticas (MOTAWI et al., 2020).

Embora seja considerada uma doença idiopática, muitos estudos ajudaram a decifrar as causas da doença descrevendo os fatores podem estar envolvidos e combinados, como a geração de espécies reativas induzidas pelo estresse oxidativo (Subramaniam & Chesselet, 2013).

Anormalidades mitocondriais levando ao declínio da atividade do complexo I da cadeia respiratória mitocondrial, interrompendo todo processo da cadeia transportadora de elétrons e a síntese de ATP (Dionísio et al., 2021). O principal neurotransmissor excitatório do sistema nervoso central é p glutamato, a recaptação de glutamato irregular, leva a excitotoxicidade neuronal, associada aos distúrbios neurológicos (Kim & Baik, 2019)

Fatores ambientais como a exposição ocupacional aos pesticidas e herbicidas, fungicidas e inseticidas agrícolas aumentam o risco de DP (Narayan et al., 2017). Em média apenas 10% dos acometidos por DP a possuem por fatores hereditários, pesquisas mostram que pacientes mesmo com parentes de primeiro grau com DP, têm o risco elevado de desenvolver a doença por

fatores genéticos em relação à população comum. No entanto, este número continua considerado baixo, uma vez que o risco geral gira em torno de 1 a 1,5% (RADAD et al., 2019).

2.2 Doença de Parkinson (DP)

Descrita há um pouco mais de 200 anos atrás pelo Dr. James Parkinson, “um ensaio sobre a paralisia dos tremores”, onde ele foi essencialmente breve e sucinto ao descrever os sintomas de apenas seis pacientes, relatando principalmente o agravamento dos tremores de apenas um dos membros superiores e a postura curvada (BERRIOS, 2016). Tendo como característica mais notórias da doença, a lentidão nos movimentos, rigidez muscular, tremores em repouso e até mesmo a instabilidade postural (MACHADO et al., 2020).

A DP afeta muitas áreas do sistema nervoso central e diferentes tipos de neurônios, afetando principalmente regiões do cérebro associadas aos sintomas motores, como a substância negra na região do mesencéfalo (Fig. 1), sendo que essa região faz parte da principal via do cérebro ligada aos movimentos (DORSEY et al., 2018). Os neurônios dopaminérgicos localizados nesta região, possuem pigmento escuro característico chamado neuromelanina. Portanto quanto mais escura a região, mais neurônios dopaminérgicos se encontram ali localizados (KHAN et al., 2019).

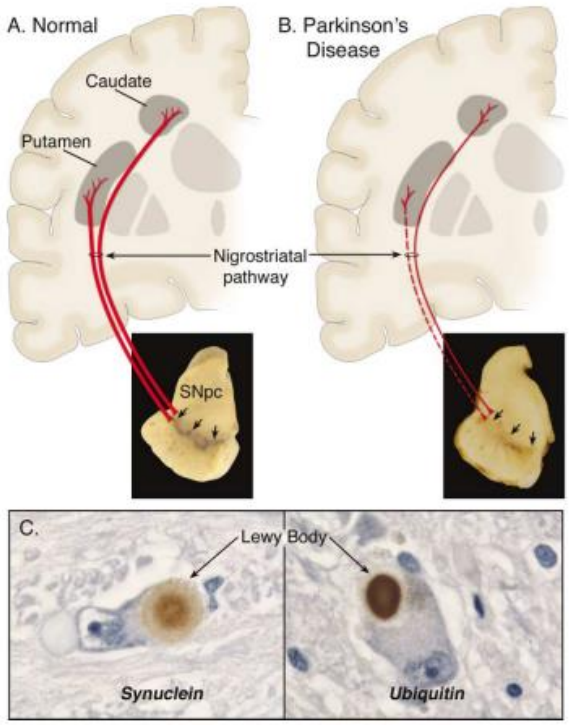
Na DP, os neurônios dopaminérgicos na substância negra morrem gradualmente, levando ao mau funcionamento desta via, ocasionando os problemas motores que caracterizam a doença (SIMON et al., 2020). Drogas que elevam a biossíntese da dopamina para aumentar os seus níveis, e são

frequentemente usadas para tratar os déficits motores, mas pela decorrência do uso diário e repentino tornam-se resistentes, tornando cada vez menos eficazes, sendo que nenhum tratamento atual retarda a neurodegeneração (CALIGIORE et al., 2016).

A AChE é a enzima que catalisa a hidrólise do neurotransmissor acetilcolina (ACh) nas sinapses colinérgicas, regulando a função motora e a locomoção (KIM et al., 2017). A exposição a pesticidas dos tipos organofosforados, como a rotenona, alteram a atividade da enzimamAChE, levando a toxicidade. Portanto, em doenças neurodegenerativas como DP se faz necessário utilizar inibidores de AChE, estes elevam os níveis de acetilcolina presentes nas sinapses entre neurônios colinérgicos (ARAUJO et al., 2015).

Em alguns casos de DP, há a presença de aglomerados de proteínas mal dobradas dentro dos neurônios, chamados de corpos de Lewy (RAZA et al., 2019). A α-sinucleína, é uma proteína mal dobrada tendo como característica, a formação de oligômeros ou fibrilas longas, tóxicos às células neuronais, desempenhando papel fundamental para a surgimento da DP. As proteínas indesejadas são normalmente eliminadas pelos diferentes tipos de degradação de proteínas celulares. Algumas evidências apontam para que esses sistemas sejam sobrecarregados por α-sinucleína, ocasionando a neurodegeneração (HIJAZ & VOLPICELLI-DALEY, 2020).

Figura 1: Via nigroestriatal em condição normal (A) e na DP (B). Em (C), marcação imuno-histoquímica demonstrando corpos de Lewy, contendo α-sinucleina e ubiquitina.



Fonte: DAUER & PRZEDBORSKI, 2003.

A DP tem uma progressão multifatorial, onde as células da glia, que circundam os neurônios, também desempenham um papel para o desenvolvimento da doença (RAO et al., 2016) . À medida que os neurônios dopaminérgicos são perdidos, a célula glial chamada microglia, é ativada para absorver os restos celulares, desencadeando uma resposta imunológica do SNC (GARCIA et al., 2021)

Uma vez ativados, eles liberam ocitocinas inflamatórias, ativando as microglias vizinhas e os astrócitos. A ativação crônica e desregulada dessas vias da micróglia, levam a produção de moléculas pró inflamatórias, ocasionando a morte dos neurônios (PICILLO et al., 2021).

A DP também está relacionada com problemas nas mitocôndrias, as

quais fornecem às células, energia para realização de funções vitais. Na DP os processos mitocôndrias são prejudicados, ocasionando a incapacidade de sustentar funções neuronais adequadas e normais ao sistema (Cen et al., 2021). À medida que envelhece ou são danificadas, as mitocôndrias são removidas e substituídas, podendo esse processo de renovação ser interrompido, levando ao acúmulo de mitocôndrias danificadas e degastadas ocasionando o aumento de estresse oxidativo e a redução da produção de energia celular (CHEN et al., 2019).

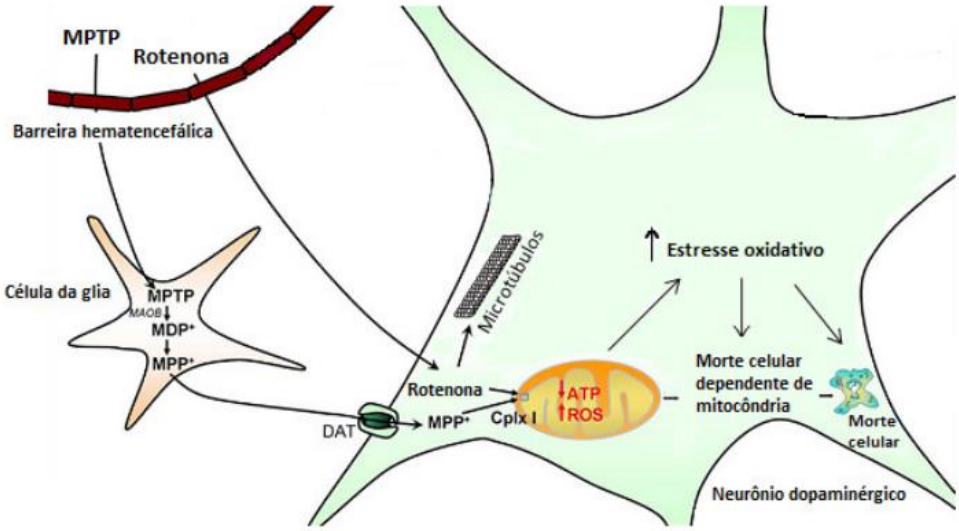
2.3 Relação entre pesticidas e doenças neurodegenerativas

As doenças neurodegenerativas são definidas como doenças nas quais as células nervosas são perdidas e causam disfunção do sistema nervoso em muitas regiões do córtex, incluindo os gânglios da base cerebelo, tronco cerebral e sistema motor (BELLOU et al., 2016).

A rotenona é um composto lipofílico que pode atravessar a barreira hematoencefálica e outras membranas biológicas. Acumula-se nas mitocôndrias, onde inibe o complexo I da cadeia respiratória mitocondrial, esta inibição acarreta à diminuição nos níveis de ATP, aumento na produção de espécies reativas e à ativação da via de morte celular dependente de mitocôndria (Fig. 2). A rotenona também pode induzir a despolimerização de microtúbulos celulares, o que pode contribuir no processo de morte celular (BOVÉ & PERIER, 2012).

A rotenona tem se destacado no uso como indutor da DP em modelos científicos, por induzir as principais disfunções que causam a DP em humanos (MOTAWI et al., 2020;).

Figura 2: Mecanismo de ação da rotenona.



Fonte: BOVÉ & PERIER, 2012.

2.4 Tratamentos

Para que o tratamento farmacológico seja realmente eficaz, são aplicadas terapias medicamentosas colinérgicas que atravessaram a barreira hematoencefálica (GARCIA et al., 2021). A levodopa é o medicamento tido como base no tratamento dos sintomas, no sistema nervoso é convertida em dopamina pela enzima dopa-descarboxilase (REICH & SAVITT, 2019b). A absorção de levodopa pode ficar comprometida quando há combinação com outros fármacos na terapia medicamentosa do paciente, podendo impedir seu metabolismo fora do encéfalo, como exemplo a fluoxetina, sertralina e também o pramipexol que é um agonista da dopamina (OPARA et al., 2017).

Tratamentos alternativos e tratamentos complementares para DP além de ajudarem a aliviar os sintomas, também reduzem o tempo de internações hospitalares pela doença (RAO et al., 2016). Entre essas abordagens alternativas, estudos discutem o uso terapêutico do ácido *p*-cumárico que se

mostrou promissor como substância neuroprotetora com menos efeitos colaterais em modelos i*n vivo* com expressão de neurotoxicidade e inibição mitocondrial do complexo respiratório I (OPARA et al., 2017).

Sempre considerando a importância da busca por novos tratamentos, e considerando que a DP é uma doença crônica, degenerativa e progressiva do sistema nervoso central, o uso de substâncias neuroprotetoras pode realmente ser uma alternativa relevante para redução dos sintomas (CHIA et al., 2020).

2.5 Ácido *p*-cumárico

Compostos fenólicos possuem o poder antioxidante podendo ser úteis na prevenção ou alívio de muitas doenças crônicas causadas pelo estresse oxidativo, visto seu potencial de eliminação de radicais livres (BOEING et al., 2021). Os ácidos cumáricos são derivados do ácido cinâmico mono-hidroxilado no grupo fenil, sendo o ácido *p*-cumárico a isoforma mais abundante (GUVEN et al., 2015). O ácido *p*-cumárico (Fig.3) é encontrado em níveis significativos em muitas frutas, vegetais e cereais como o milho, aveia, trigo, feijão, pera, maçã, uva, laranja, tomate, batata, espinafre e cebola (ABDEL-MONEIM et al., 2017).

A atividade antioxidante do ácido *p*-cumárico está associada ao grupamento fenol, sabe-se que a proteção se dá através da grande concentração de polifenóis, no entanto, estudos ainda são necessários para delimitar seus efeitos terapêuticos e entendimento do seu mecanismo de ação (VAUZOUR et al., 2010).

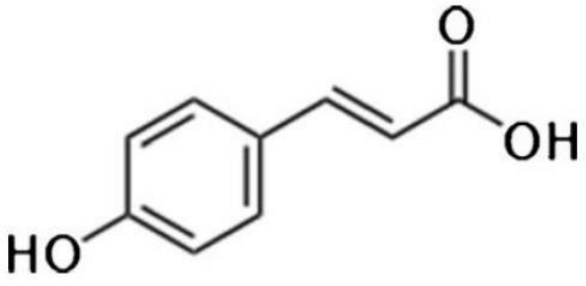
Entre os potenciais efeitos benéficos do ácido *p*-cumárico, um estudo demonstrou que alterou fatores que contribuíram para o crescimento e

progressão tumoral como, angiogênese, mitoses irregulares e atividade moduladora de células inflamatórias, incluindo macrófagos e neutrófilos (GASTALDELLO et al., 2021). Em estudos realizados, *in vitro* e *in vivo,* para ser utilizado em dermocosméticos o ácido *p*-cumárico atenuou a citotoxicidade e inflamação induzida por raios UV (BOO, 2019).

Lee e colaboradores (2008), observaram o efeito hepatoprotetor em um estudo onde examinou-se o efeito do ácido *p*-cumárico em camundongos intoxicados com etanol. O ácido *p*-cumárico, mostrou sua eficácia, onde os dados sugerem que ele possa ser útil para a prevenção de doenças hepáticas causadas pelo abuso de álcool (LEE et al., 2008).

Os efeitos do ácido *p*-cumárico também foram avaliados em um modelo de camundongo depressivo crônico induzido por corticosterona, apresentar baixa toxicidade em ratos (DL_{50} = 2.850 mg/kg de peso corporal) O tratamento com ácido *p*-cumárico acabou aliviando os comportamentos relacionados à depressão e o comprometimento da memória através da diminuição na geração de citocinas pró-inflamatórias no hipocampo e regulou positivamente a expressão de BDNF de ratos isquêmicos, resultando em melhor aprendizado espacial e memória (YU et al., 2022).

Figura 3: Estrutura do ácido trans-4-cinâmico ou ácido *p*-cumárico.



Fonte: (PRAGASAM et al., 2012).

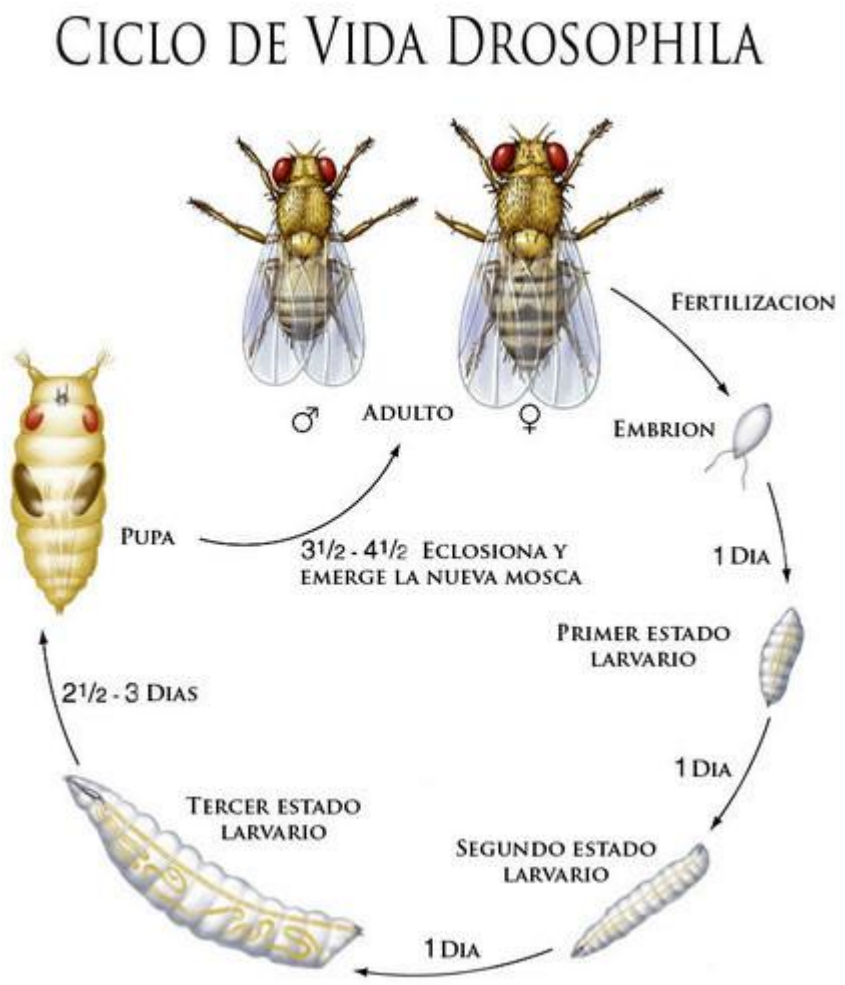
2.6 Drosophila melanogaster

A *Drosophila melanogaster*, também conhecida como a mosca da fruta, têm sido empregadas na elucidação de vários mecanismos patológicos de distúrbios neurodegenerativos de humanos (ROCHA et al., 2013). É considerada um modelo alternativo, considerado de substituição adequado para roedores e outros animais superiores utilizados na pesquisa para a Doença de Parkinson (HIRTH, 2010).

De fácil de cultivo e baixo custo, com a vida útil convenientemente curta (Fig.4), possui o genoma totalmente sequenciado e genética bem caracterizada, e ao mesmo tempo é um sistema complexo o suficiente para ser relevante no desenvolvimento de modelos de doenças humanas (ILIADI et al., 2016).

Estudos genômicos comparativos estimam que até 75% dos genes humanos implicados em doenças são conservados nas *Drosophilas r*essaltando que também podemos acessar esse grande número de ferramentas genéticas em *Drosophila* para identificar genes e/ou vias para melhor vantagem e viabilização nos estudos (RAO et al., 2016).

Figura 4: Ciclo de vida da *Drosophila melanogaster*.



Fonte: https://dendroterra.jimdofree.com/art%C3%ADculos/droshophila-alimento-vivo

3. OBJETIVOS

3.1 Objetivo geral

Avaliar a ação do ácido *p*-cumárico nas alterações comportamentais, oxidativas e neuroquímicas no modelo experimental tipo Doença de Parkinson em *Drosophila melanogaster*, causadas pela exposição à rotenona.

3.2 Objetivos específicos

- Avaliar o efeito do ácido *p*-cumárico sobre o comportamento motor e taxa de sobrevivências em *Drosophila melanogaster*;

- Avaliar o efeito da co-exposição de rotenona e ácido *p*-cumárico sobre o comportamento motor e taxa de sobrevivências em *Drosophila melanogaster*;

- Avaliar o consumo alimentar nas diferentes dietas do estudo;

- Avaliar o efeito da co-exposição de rotenona e ácido *p*-cumárico sobre os níveis de dopamina, atividade da AChE e viabilidade celular em *Drosophila melanogaster*;

- Avaliar o efeito da co-exposição de rotenona e ácido *p*-cumárico sobre os indicadores de estresse oxidativo em *Drosophila melanogaster*;

4. MANUSCRITO CIENTÍFICO

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de ***artigo científico***. Os itens ***Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas***, encontram-se no próprio artigo.

A ***conclusão,*** encontra-se no final desta dissertação, apresentam. As ***referências bibliográficas*** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO** e **REVISÃO BIBLIOGRÁFICA** desta dissertação.

Artigo:

“Neuroprotective effects of p-coumaric acid in a model of rotenone-induced parkinson's disease in *Drosophila melanogaster***.”**

Neuroprotective effects of p-coumaric acid in a model of rotenone-induced parkinson's disease in *Drosophila melanogaster*

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Abstract

Parkinson's disease (PD) is a chronic degenerative disease of the central nervous system that mainly affects motor coordination. The *p*-coumaric acid is an important phenolic compound with potent antioxidant activity that can be an alternative to repair the neurotoxicity developed by neurodegenerative diseases. Thus, this study investigated the action of *p*-coumaric acid on the behavioral and neurochemical changes induced by exposure of *Drosophila melanogaster* to rotenone in a Parkinson's disease (PD)-like a model. To carry out the study, the flies were divided: two groups with a standard diet (control) and two groups with exposure to rotenone (500 μM), after a period of 72 hours a control group and a rotenone group started to receive the diet with p acid. -coumaric (0.3 μM) for another 48 hours. At the end of the 120-hour study (7 days) with 4 groups: Standard diet (control), p-coumaric acid (0.3 μM), rotenone (500 μM), and rotenone (500 μM) plus p-coumaric acid (0. 3 μM). The exposure to *p*-coumaric acid protected against locomotor damage and attenuated mortality induced by rotenone. Additionally, *p*-coumaric acid normalized oxidative stress indicators (ROS, TBARS, SOD, CAT, GST, and NPSH) avoided the oxidative damage, which was reflected in the restoration of dopamine levels, AChE activity, and cellular viability, after exposure to rotenone. The results showed that *p*-coumaric acid effectively attenuates PD-like model-induced damage, confirming its antioxidant activity and the potential neuroprotective effect.

Keywords: phenolic compound; neurodegeneration; antioxidant; neuroprotective; rotenone.

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder, in which nerve cells in the brain slowly die over time (Raza et al., 2019). It is estimated that for every 100 people over the age of 60, at least one is affected by PD (Reich & Savitt, 2019a). Characteristic behavioral changes such as muscular rigidity, bradykinesia (slowness or absence of movement), imbalance, and postural instability are developed and considered motor symptoms of PD (Opara et al., 2017). Although the pathophysiology of PD is not fully known, the presence of the aggregates of α-synuclein that originate from the Lewy bodies is one of the most important hallmarks of the disease. The accumulation of these prevent neurotransmission, compromising the functionality of synapses between neurons, and progressing to the degeneration of dopaminergic neurons. The damage generated in the dopaminergic neurons associated with dopamine autoxidation, mitochondrial dysfunction, increased oxidative stress, alteration of acetylcholine (ACh) levels, and acetylcholinesterase (AChE) activity, are factors that contribute to the development of the disease and compromise the patients' locomotor performance (Dorsey et al., 2018).

Current drugs used for PD aim to combat symptoms and improve patient’s quality of life. However, most treatments have an initial effect, but their effectiveness decreases over time or between doses (Simon et al., 2020). In addition, in many cases, side effects occur that can affect the continuity of treatment (Khan et al., 2019).

Thus, it is important to investigate new therapeutic options trying to avoid the progressive neuronal degeneration of the disease. As oxidative stress plays an important role in the pathogenesis of PD, compounds that have a

neuroprotective potential against the formation of free radicals and, in addition, can prevent the decrease or restore dopamine levels should be evaluated as possible treatments for PD (Musachio et al., 2020). Though several synthetic antioxidants are available, an increasing trend has been targeting bioactive compounds present in plants and foods as antioxidants for the development of treatments able to avoid disease evolution and its symptoms (Picillo et al., 2021).

The *p*-coumaric acid, a derivative of cinnamic acid, is one of the found phenolic compounds most in plants and foods (Abdel-Moneim et al., 2017). With broad antioxidant and anti-inflammatory activity, the potential neuroprotective effects of *p*-coumaric acid have been shown in different models (Boeing et al., 2021). In this sense, the coumaric acid restored the deficit of movement and climbing capability induced by paraquat in *Drosophila melanogaster* (Jimenez-Del-Rio et al., 2010). In addition, *p*-coumaric acid presents a positive response to oxidative stress in *Caenorhabditis Elegans* (C. Elegans) as well as increasing longevity (Yue et al., 2019a). The *p*-coumaric acid enhanced long-term potentiation and facilitated the recovery of scopolamine-induced memory impairment (H. B. Kim et al., 2017b).

Furthermore, a polyphenolic extract containing *p*-coumaric acid improves scopolamine-induced memory deficit and restores AChE activity (Souza et al., 2020). However, the effect of *p*-coumaric acid on neurochemical changes induced by PD, such as oxidative stress dopamine levels and AChE activity, still needs to be explored to provide insights into more effective antioxidant therapeutic approaches to PD. Therefore, the present study aims to investigate the action of *p*-coumaric acid on the behavioral and neurochemical changes

caused by exposure of *Drosophila melanogaster* to rotenone in a Parkinson's disease-like model.

2. Materials and methods

2.1 Chemicals

The *p*-coumaric acid end rotenone was purchased from Sigma - Aldrich (St Louis, MO, USA) and dissolved in 98% ethanol immediately before use. All the other reagents used were of analytical grade.

2.2 *Drosophila melanogaster* stock

Fruit flies (*Drosophila melanogaster* - Harwich strain) of both genders (1-3 days old) were used, kept in flasks in the incubator, under controlled light conditions (12-hour light/dark cycle) temperature (25 ± 1°C), 60% humidity and fed with 10 mL of standard food composed of cornflour (76.59%), wheat germ (8.51%), sugar (7.23%), milk powder (7.23%), salt (0.43%) and antifungal methylparaben.

2.3 Rotenone exposure-induced PD model

Rotenone causes the death of dopaminergic neurons in the substantia nigra, causing an imbalance of neurotransmitters. This model contributes to elucidating pathogenic mechanisms of neurodegeneration in PD (Bové & Perier, 2012b). Rotenone was added to the flies' diet at a final concentration of 500 µM, responsible for causing 50% death of the flies after 7 days of exposure. The

total food for treatments is composed of 2% sucrose, 1% milk powder, 1% agar, 1% yeast, and 0.08% nipagin.

2.4 Experimental protocol

2.4.1 Experiment 1: Assessment of locomotor, exploratory activity and percentage of survival after 72 hours of exposure to *p*-coumaric acid

Flies of both sexes aged 1-3 days were selected, divided into 6 groups, containing 50 flies each, and exposed to a standard diet (control group), a diet containing either ethanol (at a final concentration of 0.3%) or *p*-coumaric acid (0.03; 0.1; 0.3 or 1 µM; dissolved in ethanol) for 72 hours. After exposure to the diet, locomotor and exploratory activity were evaluated. Furthermore, the survival percentage was recorded every 24 hours, and evaluated after three days of exposure. The treatment schedule is depicted in (Fig. 1A).

2.4.2 Experiment 2: Rotenone exposure and *p*-coumaric acid treatment

The effect of *p*-coumaric acid in the rotenone-induced experimental model of Parkinson's disease was evaluated. Flies were exposed to a standard diet (control), a standard diet containing rotenone (500 µM; 4 days), *p*-coumaric acid (0.3 µM; 3 days) or rotenone (500 µM; 4 days) plus *p*-acid-coumaric (0.3 µM; 3 days). The exposure of the groups with rotenone occurs for 4 days, after this period, the exposure to *p*-coumaric acid occurs, from the fourth to the seventh day, evaluating the reversal effect. After exposure to diet, the flies were submitted to negative geotaxis, open field, and motor coordination tests to

evaluate locomotor and exploratory activity. Furthermore, the survival percentage during 7 days of exposure and food consumption were assessed. After behavioral analyses, biochemical analyses were performed, including oxidative stress indicators (ROS, TBARS, SOD, CAT, GST, PSH, and NPSH), dopamine levels, AChE activity, and resazurin. The treatment schedule is depicted in (Fig. 2A).

2.5 *In vivo* **assays**

2.5.1 Survival percentage

Daily counting of the number of live flies was performed at the end of each day for the 7 days of treatment. Where 250 flies per group were included in the survival data, the total number of flies represents five independent trials. Each group contains 50 adult flies of both sexes randomly added to the treatment. The data were expressed as the percentage of surviving flies.

2.5.2 Negative geotaxis assay

The climbing capacity of the flies was evaluated using the negative geotaxis described by Jimenez-Del-Rio et al. (2009) with some adaptations (Araujo et al., 2015). Flies from each group were individually placed in a test tube of 1.5 cm diameter recording the time spent by each fly to reach a height of 8 cm, in a maximum time of 2 min. The data were analyzed according to the average time of the five-fly repetition for each fly-in in which five independent experiments were performed.

2.5.3 Open field test

To evaluate locomotor and exploratory activity, the open field test was performed with 5 flies in each group, according to Connolly (1966), adapted by Musachio et al.,2020. Each fly was kept in a Petri dish divided into quadrants (1 cm x 1 cm), where the crossing number during 2 min was determined. The total (25 per group) represents the sum of five independent experiments.

2.5.4 Motor coordination test

The test was performed based according to the method described by Iliadi et al (2016) with adaptations by Musachio (2020). On the fourth day of treatment, three days before the test was performed, the flies were anesthetized on the ice where they had their wings clipped. The base for performing the test consists of an acrylic box, measuring (28 cm long, 11.8 cm wide, and 2.4 cm high), where a transparent nylon line (0.6 mm) was adapted as a platform 5 mm from the water level (23-27 °C). Flies were added individually to the line and timed the time it took them to cross 13 cm of the platform a maximum time of 60 seconds. Each fly performed the test three times (triplicate), so the time performance of crossing the platform was averaged.

2.5.5 Food consumption

The test was performed according to Sun et al. (2013) with some modifications, as a marker of food consumption. The method consists of adding 0.5% blue dye (FD & C Blue Dye no. 1) to the diet preparation of respective treatments. The flies were allowed to feed for 30 min. Five independent

experiments were conducted, using a total of 20 flies per group. After the feeding period, the flies were cold anesthetized. The analysis was performed with the entire bodies, where they were homogenized in 200 mL of distilled water and then centrifugation was performed at 12,000 xg for 2 min. Their respective absorbances were analyzed in the spectrophotometer and measured at 625 nm in the supernatant.

***2.6** Ex vivo* **assays**

2.6.1 Sample preparation

Immediately after the behavioral evaluations, the flies were sacrificed on ice, the body and the heads of the flies were separated, where only the heads of the flies were used to carry out the analyses, except for the analysis of consumption which was carried out with the whole fly. For the determination of enzymatic and non-enzymatic indicators of oxidative stress, AChE, and resazurin activity, the fly heads were homogenized in HEPES buffer (20 mM, pH 7.0), 10:1 (flights/volume µL) for 2 min, the resulting homogenate was then centrifuged at 10,600 xg for 10 min at 4°C using the supernatant fraction (S1). Protein content was measured colorimetrically using the Bradford method (Bradford, 1976) and bovine serum albumin (1 mg/mL) was used as a standard.

2.6.2 Determination of dopamine levels (HPLC-DAD)

The procedures were performed as proposed by (Bianchini et al., 2019). Initially, sample preparation consisted of weighing 30 individual fly heads

into 2 mL microtubes. We added 400 µL of saline solution (0.9% NaCl and 0.5 M HCl 96:4 v/v) cold (4 °C). The samples were homogenized for 30 seconds and then centrifuged (10000 RPM for 10 minutes at 4°C). Finally, the supernatant was collected and filtered through a 0.22 µm hydrophobic PTFE syringe filter with a diameter of 13 mm. The filtered sample was transferred to the vial and kept at 4 °C (for a maximum of 6 hours) until analysis by HPLC-DAD. To quantify dopamine by HPLC-DAD, we used a chromatograph composed of an A-10 Altus^{TM} sample and solvent module and an A-10 Altus^{TM} PDA detector (both PerkinElmer), equipped with an Inertsil ODS-3 5 µm column (4,6 × 250 mm) and a Security Guard^{TM} cartridge guard (Phenomenex KJ0-42B2). The compounds were separated using the mobile phases (FM) composed of water acidified with H_3PO_4 (phosphoric acid) to pH 2,55 (A) and methanol (B). An injection volume of 20 µL was used, and the compounds were identified by comparing their retention time and DAD spectrum with the reference standards of the analytical curve. The analytical curve was constructed with dopamine (198 nm) and consisted of 6 points at concentrations of 0.05; 0.1; 1.0; 2.0; 5.0 and 10.0 mg.L^{-1}. Calibration curve parameters for dopamine, 0.05 – 10.0 linear range (mg.L^{-1}), y=311548x + 36287 (y=ax=b), 0.9977 (R^2). Dopamine levels expressed in milligrams of compound per gram of sample [mg.g^{-1}].

2.6.3 Determination of acetylcholinesterase (AChE) activity

To determine the activity of acetylcholinesterase which is the enzyme responsible for the breakdown of the neurotransmitter acetylcholine, the method used was described by Ellmann (1961) with some adaptations. Only the heads

of the flies were used, in a total of 20 per group, the samples form a homogenized mixture of 200 µL of 20 mM HEPES buffer (pH 7.0) and centrifuged at 78-x g for 10 min. Then a mixture of 0.25 M KPI buffer (pH 8.0) and 5,5'-dit ibis (2-nitrobenzoic acid) (DTNB; 5 mM) was performed, where 935 µL was added to the head supernatant sample (15 µL) and added after 50 µL of acetylthiocholine (ACh) (7.25 µM). AChE activity was expressed as (µmol/min/mg protein) and was determined spectrophotometrically at 412 nm for 2 min. Five independent experiments were performed.

2.6.4 Evaluation of cellular metabolic activity by the Resazurin reduction

Viability cellular was measured using a method based on the ability of viable cells to reduce resazurin to resorufin, a fluorescent molecule (Franco et al., 2009). Twenty flies per group had their heads separated and homogenized in 100 µL of 20 mM Tris buffer (pH 7.0) and 400 µL of 20 mM Tris buffer (pH 7.0), respectively, and centrifuged at 999 xg for 10 min at 4°C. Subsequently, the samples were incubated on ELISA plates with 180 µL of 20 mM Tris buffer (pH 7.0) and 10 µL of resazurin for 1 hour. The absorbance was recorded using a microplate reader at a wavelength of 573 nm. Five independent experiments were performed (20 fly heads per group). Data were expressed as resazurin reduction (% of control).

2.6.5 *Determination of reactive species levels*

The levels of reactive species (RS) were determined according to Pérez-Severiano et al. (Ellman et al., 1961; Pérez-Severiano et al., 2004). Twenty fly heads per group were homogenized in 1 ml of Tris buffer (10 mM, pH 7.0) and centrifuged at 1000 xg for 5 min. From the sample supernatant, 34 µL, was

added to 964 µL of HEPES buffer (pH 7.0) and 10 µL of 2,7-dichlorofluorescein 2,7-diacetate (DCFDA). After 1 h of incubation, the fluorescence emission of DCFH resulting from the oxidation of DCFDA was analyzed at an excitation wavelength of 488 nm and an emission wavelength of 520nm using a fluorescence spectrometer. The results obtained of relative fluorescence intensity were expressed as a percentage relative to the control. An average of five independent experiments was used.

2.6.6 Levels of thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation of thiobarbituric acid was performed according to (Ohkawa et al., 1979), which expresses the content of the biomarker malondialdehyde (MDA). The MDA reacts with thiobarbituric acid (TBA) to form a colored complex. Performed with 20 fly heads from each group, they were homogenized in 120 µL HEPES buffer and centrifuged at 78 xg for 10 min (4°C). TBARS content was measured in a medium containing 50 µL of head tissue homogenate, 50 µL of 1.2% sodium dodecyl sulfate (SDS), 125 µL of acetic acid buffer (0.45 M, pH 3.4), and 125 µL of 0.28% TBA. The mixture was then heated to 95°C for 120 min in a water bath. After cooling to room temperature, the absorbance of the supernatant was measured at 532 nm. The results were expressed as µmol MDA/ mg protein.

2.6.7 Superoxide dismutase (SOD) activity

The activity of superoxide dismutase (SOD) in the head of the flies was tested according to Kostyuk and Potapovich (Kostyuk & Potapovich, 1989). A

reaction mixture containing sodium phosphate buffer (0.025 M/0.1 mM EDTA, pH 10) and N, N, N tetramethylethylenediamine (TEMED) was added (1 mL) along with 10 µL of the head tissue sample. Added in 50µL of quercetin dissolved in dimethylformamide and performed spectrophotometer reading at 406 nm for 2 min to assess its auto-oxidation. The mean of five independent experiments represents the results of each group. Enzyme activity is expressed as U/mg protein (one unit is defined as the amount of enzyme required to inhibit the auto-oxidation rate of quercetin by 50% at 26 °C).

2.6.8 Catalase (CAT) activity

The analysis was performed using the methodology of Aebi (Aebi, 1984) and the modifications made by Zemolin (Zemolin et al., 2012). CAT activity was assessed by adding 30 µL of the head sample to a mixture of phosphate buffer (0.25 M / EDTA 2.5 mM, pH 7.0), hydrogen peroxide (10 mM) (H_2O_2), and Triton X-100 (0.012%). The ability of the enzyme to undergo H_2O_2 decomposition was monitored at 240nm for 2 min, and the activity was expressed as U/mg protein (1 U decomposes 1 µmol H_2O_2/ min at pH 7.0 and 25 ^oC). It was determined from the average of five independent experiments.

2.6.9 Glutathione-S-transferase (GST) activity

GST activity was evaluated spectrophotometrically at 340 nm for 2 min (Pérez-Severiano et al., 2004). This method is used to evaluate the activity of this defense enzyme antioxidant, which is responsible for the metabolization of xenobiotics. The reaction mixture contained an aliquot of supernatant, 0.1 M

potassium phosphate buffer pH 7.4, 100 mM reduced glutathione (GSH), and 100 mM 1-chloro-2,4-dinitrobenzene (CDNB), which was used as substrate. Enzyme activity was expressed as nmol CDNB conjugate/ min/ mg protein and was performed in five independent assays.

2.6.10 Total thiols (PSH) and non-protein thiols (NPSH) content

Evaluation of non-enzymatic antioxidant defenses, determination of protein (PSH) and non-protein thiol (NPSH) described by Ellman (Ellman et al., 1961; Macdonald et al., 1985). The supernatant was mixed with 10% chloroacetic triacid (TCA). The tubes were centrifuged at 10,000 xg for 5 min at 4 °C, the protein pellet was discarded and the free thiol groups were determined in the clear supernatant. An aliquot of the supernatant was added to 1M potassium phosphate buffer pH 7.4 and 5,5′-di-this- (2-nitrobenzoic acid) (DTNB). The color reaction was measured at 412 nm in a spectrophotometer. The results obtained were expressed as a percentage relative to the control of PSH or NPSH content and represent the mean of five dependent experiments (10 flies in each group).

2.6.11 Statistical Analysis

The GraphPad Prism 8 software was used for statistical analysis and plotting graphs. In experiment 1, data were analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni's post-hoc test to identify which of the pairs of groups differed. In experiment 2, data were analyzed by two-way Analysis of variance (ANOVA), followed by Bonferroni's Post hoc test. The

survival rate of flies during the 3 or 7 days of treatment (experiment 1 or 2, respectively) was determined by a log-rank test (Mantel-Cox). Differences between groups were considered significant when $P < 0.05$. All data are expressed as the mean and S.E.M.

3. Results

3.1 Experiment 1: Assessment of locomotor and exploratory activity and survival percentage after 72 hours of exposure to *p*-coumaric acid

Figure 1 (B, C, and D) shows the effect of the exposure to *p*-coumaric acid (0.03 - 1 µM), for 3 days, on climbing time and crossing number in the geotaxis and open-field task respectively, and the survival percentage of *Drosophila melanogaster*. Statistical analysis (one-way ANOVA) revealed that exposure to *p*-coumaric acid (1 µM), significantly decreased the climbing time [$F_{(5,55)} = 3.13$; $P < 0.05$] however, it showed no difference between groups for a number of crosses. Post hoc comparisons demonstrated that p-coumaric acid, at a concentration of 1 µM, improved locomotor and exploratory activity in geotaxis (Fig. 1C) compared to the control group. But p-coumaric acid, at the same concentration (1 µM) presented a mortality rate when compared to other concentrations.

3.2 Experiment 2: Rotenone exposure and *p*-coumaric acid treatment

3.2.1 In vivo assays

Figure 2 (B-F) shows the effect of the exposure to rotenone (500 µM), *p*-coumaric acid (0.3 µM), and the co-exposure to rotenone (7 days) and *p*-coumaric acid (3 days), on the survival percentage, climbing time (geotaxis test), crossing number (open field test), walking speed (motor coordination test) and Food consumption of *Drosophila melanogaster*. Statistical analysis revealed that exposure to rotenone significantly decreased the survival percentage of flies over the experimental period compared to the control group. However, *p*-coumaric acid co-exposure protected the flies against rotenone-induced mortality (Fig. 2B). Statistical analysis (two-way ANOVA) also revealed the reversal of observed damage from rotenone exposure after *p*-coumaric acid was added to the diet, a significant effect for the interaction factor (*p*-coumaric acid versus rotenone) on the climbing time [$F_{(1,28)}$= 20.89; $P < 0.05$], the crossing number [$F_{(1,28)}$= 10.34; $P < 0.05$] and the walking speed [$F_{(1,16)}$= 30.91; $P < 0.05$]. *Post hoc* comparisons demonstrated that *p*-coumaric acid co-exposure protected the flies against locomotor and exploratory damage in the geotaxis (Fig. 2C), open field test (Fig. 2D), and motor coordination deficit induced by rotenone (Fig. 2E). Statistical analysis (two way ANOVA) did not demonstrate a significant difference between groups for food consumption of the flies (Fig. 2F), suggesting that both compounds added to the diet rotenone and *p*-coumaric acid did not alter the diet intake.

3.2.2 Restoration of Dopamine levels, AChE activity, and resazurin

Figure 3 (A-C) shows the effect of the exposure to rotenone (500 µM), *p*-coumaric acid (0.3 µM), and the co-exposure to rotenone and *p*-coumaric acid, on Dopamine levels, AChE and activities and resazurin in the head of *Drosophila melanogaster*. Statistical analysis (two-way ANOVA) revealed a significant effect for the interaction factor (*p*-coumaric acid versus rotenone) on dopamine levels [$F_{(1,16)} = 11.71$; $P < 0.05$], AChE activity [$F_{(1,16)} = 7.61$; $P < 0.05$] and resazurin [$F_{(1,20)} = 336.6$; $P < 0.05$]. Post hoc comparisons demonstrated that the exposure to rotenone decreased dopamine levels (Fig. 3A) and resazurin (Fig. 3C), as well as increased AChE activity (Fig. 3B). The co-exposure with p-coumaric acid reversed rotenone-induced changes in flies.

3.2.3 Determination of Oxidative Stress indicators

Figure 4 (A-E) shows the effect of the exposure to rotenone (500 µM), *p*-coumaric acid (0.3 µM), and the co-exposure to rotenone and *p*-coumaric acid, on oxidative stress indicators (ROS, TBARS, SOD, CAT, GST, NPSH, and PSH) in the head of *Drosophila melanogaster*. Statistical analysis (two-way ANOVA) revealed a significant effect for the interaction factor (*p*-coumaric acid versus rotenone) on oxidative stress indicators: ROS [$F_{(1,15)} = 6.76$; $P < 0.05$], TBARS [$F_{(1,20)} = 49.96$; $P < 0.05$], SOD [$F_{(1,16)} = 35.30$; $P < 0.05$], CAT [$F_{(1,16)} = 4.86$; $P < 0.05$], GST [$F_{(1,16)} = 24.98$; $P < 0.05$], NPSH [$F_{(1,16)} = 13.06$; $P < 0.05$]. *Post hoc* comparisons demonstrated that *p*-coumaric acid co-exposure protected the flies against ROS production (Fig. 4A), lipid peroxidation increases (Fig. 4B), increased antioxidant enzymes activity SOD (Fig. 4C), CAT

(Fig. 4D) induced by rotenone. Furthermore, p*ost hoc* comparisons demonstrated that *p*-coumaric acid co-exposure protected the flies against decreased activity GST (Fig. 4E) and NPSH content (Fig. 4F) induced by rotenone. Statistical analysis (two-way ANOVA) also revealed that the rotenone exposure decreased PSH content compared to the control group, however, the *p*-coumaric acid co-exposure was not able to protect against this damage.

4. Discussion

In the present study, we investigated the effect of *p*-coumaric acid and its possible mechanism involved in the rotenone-induced PD model in *Drosophila melanogaster*. Initially, the effect of *p*-coumaric acid per se was assessed to avoid any toxic effect and to define the concentration to be used in experiments involving the PD model. Our results showed that *p*-coumaric acid (1 μM) decreased the climbing time in the negative geotaxis behavioral test (Fig. 1C), compared to the control group, however, did not affect the number of crossings in the open field test (Fig. 1D). In addition, the exposure to *p*-coumaric acid did not affect the survival percentage (Fig. 1B). Thus, the highest concentration of *p*-coumaric acid (0.3 μM) that did not present effects on the locomotor and exploratory activity was chosen for the experiments of co-exposure to rotenone. Aiming to evaluate the interaction effect of *p*-coumaric acid with rotenone and avoid a summation effect of the two treatments.

Rotenone is a substance that occurs naturally in some species, in the roots of plants. In Brazil, we find the species of the genus *Derris*, they are popularly called timbó (Chia et al., 2020b). It is known to induce clinical and pathologic features similar to PD in humans (Radad et al., 2019) and animal models (Chia et al., 2020a). This compound interrupts the electron transport chain by complex I inhibition, causing mitochondrial dysfunction, and leading to the production of reactive species. Due to high sensitivity to oxidative insults, rotenone affects dopaminergic neurons in the substantia nigra and induces α-synuclein accumulation in the nervous system (Araujo et al., 2015). Furthermore, the dopaminergic neuron degradation associated with a decrease in dopamine levels causes an imbalance between the neurotransmitters

dopamine and acetylcholine and alters AChE activity (Jamwal & Kumar, 2019). Our confirmation corroborate this neurotoxic event induced by rotenone. The rotenone exposure was able to change dopamine levels, AChE activity, cellular viability, and oxidative stress indicators, such as ROS, TBARS, SOD, CAT, and GST, as well as NPSH and PSH content. These neurochemical changes are probably responsible for the PD-like phenotype observed in flies since rotenone exposure decreased the survival rate, caused locomotor damage in the geotaxis and open field tests, as well as, motor coordination deficit. Thus, the behavioral deficits associated with the observed neurochemical changes allow us to support that the PD model was effectively induced in the flies exposed to rotenone.

Several studies have attributed to *p*-coumaric acid a potential protective effect on neurodegenerative diseases and their involved mechanisms, however, these studies generally use compounds in the form of phenolic extracts (Rahman et al., 2022). This consequently limits the conclusion that the observed effect is related to a specific phenolic compound since there is probably a synergistic effect between the different substances present in the extract. Thus, the evaluation of isolated phenolic compounds is important. The present study showed that exposure to isolate *p*-coumaric acid protected against behavioral damage induced by a PD model. This effect is evidenced by the ability of *p*-coumaric acid to promote survival and avoid locomotor and exploratory damage observed through the geotaxis (Fig. 2C), open field test (Fig. 2D) and motor coordination deficit (Fig. 2E) induced by rotenone, which corroborates the results described by Musachio (Musachio et al., 2020). But in addition, interestingly, this is the first report to demonstrate the ex vivo potential

of isolated *p*-coumaric acid to restore dopamine levels, AChE activity, cellular viability (Fig. 3), and oxidative damage (Fig. 4) after exposure of *Drosophila melanogaster* to the rotenone-induced PD-like model.

Our results demonstrated a significant increase in oxidative stress after exposure to rotenone, as evidenced by the increase in ROS production and lipid peroxidation. The decrease in the GST activity (xenobiotic detoxifying enzyme) and the reduction in NPSH content (an indirect oxidative stress biomarker) may contribute to an increase in oxidative stress. Associated with oxidative stress, there was an increase in the antioxidant enzymes activity SOD and CAT, which probably reflects a compensatory mechanism in an attempt to protect the neuronal function against oxidative damage provoked by rotenone. Furthermore, SOD and CAT are considered key enzymes for being the first to act in the defense mechanism against oxidative stress (Avelar et al., 2015). Interestingly, the antioxidant action of *p*-coumaric acid was able to decrease ROS production and lipid peroxidation. Thus, the combat against oxidative stress by *p*-coumaric acid probably allowed for the normalization of the antioxidant defense system, restoring the activity of enzymes SOD, CAT, GST and NPSH content, altered by exposure to rotenone. The antioxidant action of *p*-coumaric acid and its effects found in this study can be explained by the chemical structure of acid, which is characterized by the presence of phenolic carboxylic groups, which are responsible for both increasing the free radical scavenging activity via electron or hydrogen donation and metal chelation (Reina et al., 2021). Additionally, the previous finding shows that *p*-coumaric acid could activate Nrf2, which plays an important role in the regulation of oxidative stress response (Yue et al., 2019b). Besides that, the co-exposure to

p-coumaric acid restored GST activity which may result in a high ability of flies to metabolize xenobiotics, contributing to the neurochemical and behavioral effects of *p*-coumaric acid.

Dopamine and ACh are abundant neurotransmitters in the CNS which play a regulatory role in controlling locomotor activity, being strongly correlated with the motor symptoms of PD (Lester et al., 2010). Previous reports indicate that dopamine deficiency is accompanied by an increase in ACh availability, leading to overactivation of the cholinergic system. The imbalance between ACh and dopamine results in the loss of primary functions of movement control, balance, and postural stability, characteristic symptoms of PD (Sanchez-Catasus et al., 2021). We showed that the rotenone-induced PD-like model decreased dopamine levels and increased AChE activity, however, co-exposure to *p*-coumaric acid restored dopamine levels and AChE activity. We understand that the increase in AChE activity must be an adaptive response to a probable increase in ACh and the attempt to normalize the levels of this neurotransmitter.

In summary, our results support that *p*-coumaric acid protected against locomotor and exploratory damage, motor coordination deficit, and increased mortality, as well as restored oxidative stress indicators, dopamine levels, AChE activity, and cellular viability induced by rotenone. The main hypothesis is that the *p*-coumaric acid antioxidant capacity combats the oxidative stress induced by exposure to rotenone, which should reflect in the restoration of dopamine levels and antioxidant enzymes and AChE activities, reestablishing the condition of balance between neurotransmitters. Modulation of this set of events should mitigate behavioral damage.

5. Conclusion

In conclusion, our results indicate that *p*-coumaric acid reverse the behavioral deficit in the rotenone-induced PD-like model in *Drosophila melanogaster.* Suggesting that this protective effect is associated with restoring neurochemical damage. Improved the percentage of survival, locomotor, exploratory activity and motor coordination of Drosophila melanogaster. There was no significant difference in food diets. Restored dopamine levels, AChE activity, recovered cell viability in the % urine reduction technique to control. It restored the levels of reactive species, the expression of lipid peroxidation, the activity of superoxide dismutase, catalase of glutathione-S-transferase and the content of non-protein thiols. Therefore, *p*-coumaric acid emerges as a putative candidate in the search for treatments for Parkinson's disease and could be studied as adjunctive therapy to reduce the doses and/or improve the effectiveness of treatments already used.

Conflict of interest: The authors declare that there are no conflicts of interest.

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Figures

Figure 1.

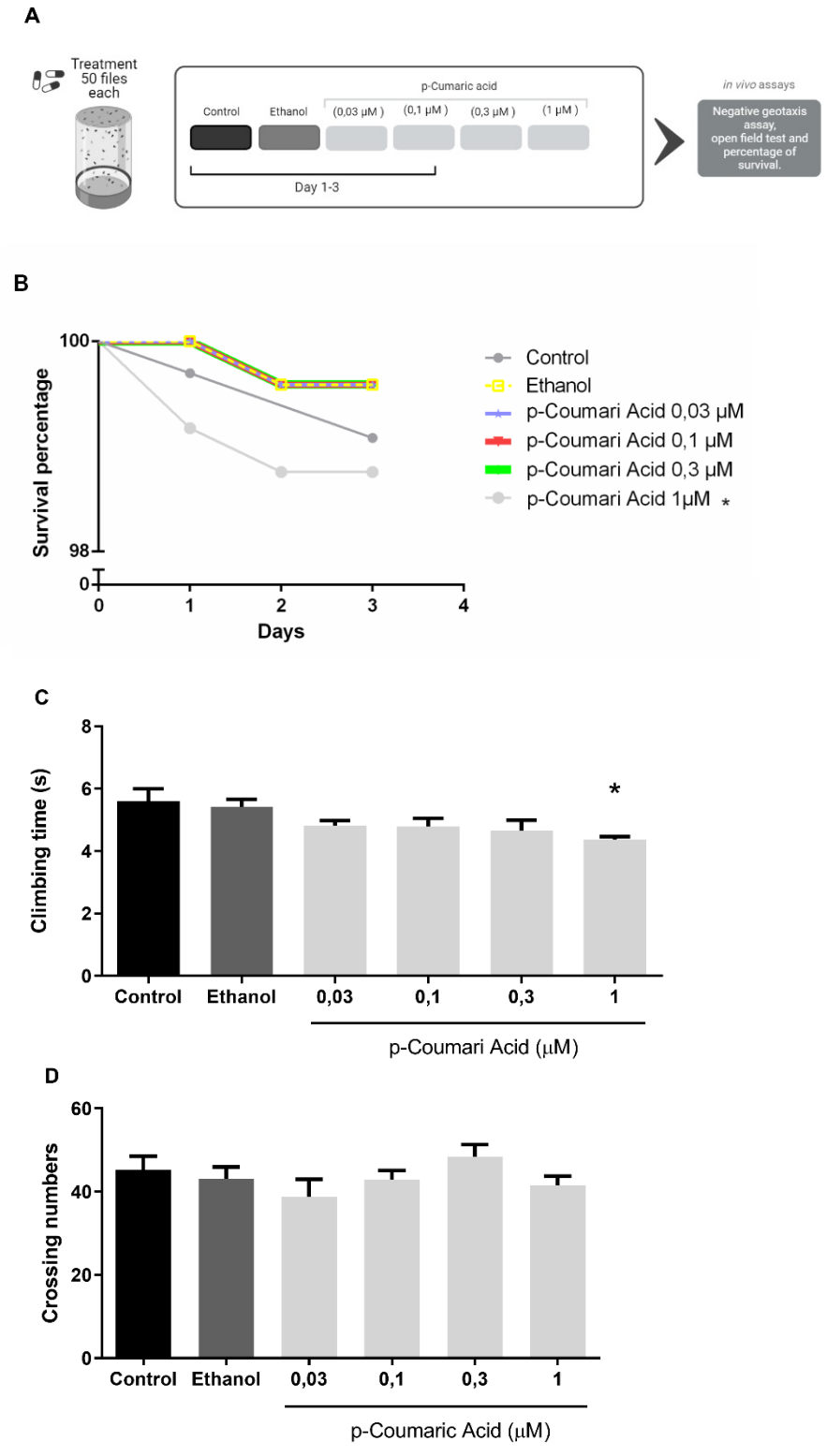


Figure 2.

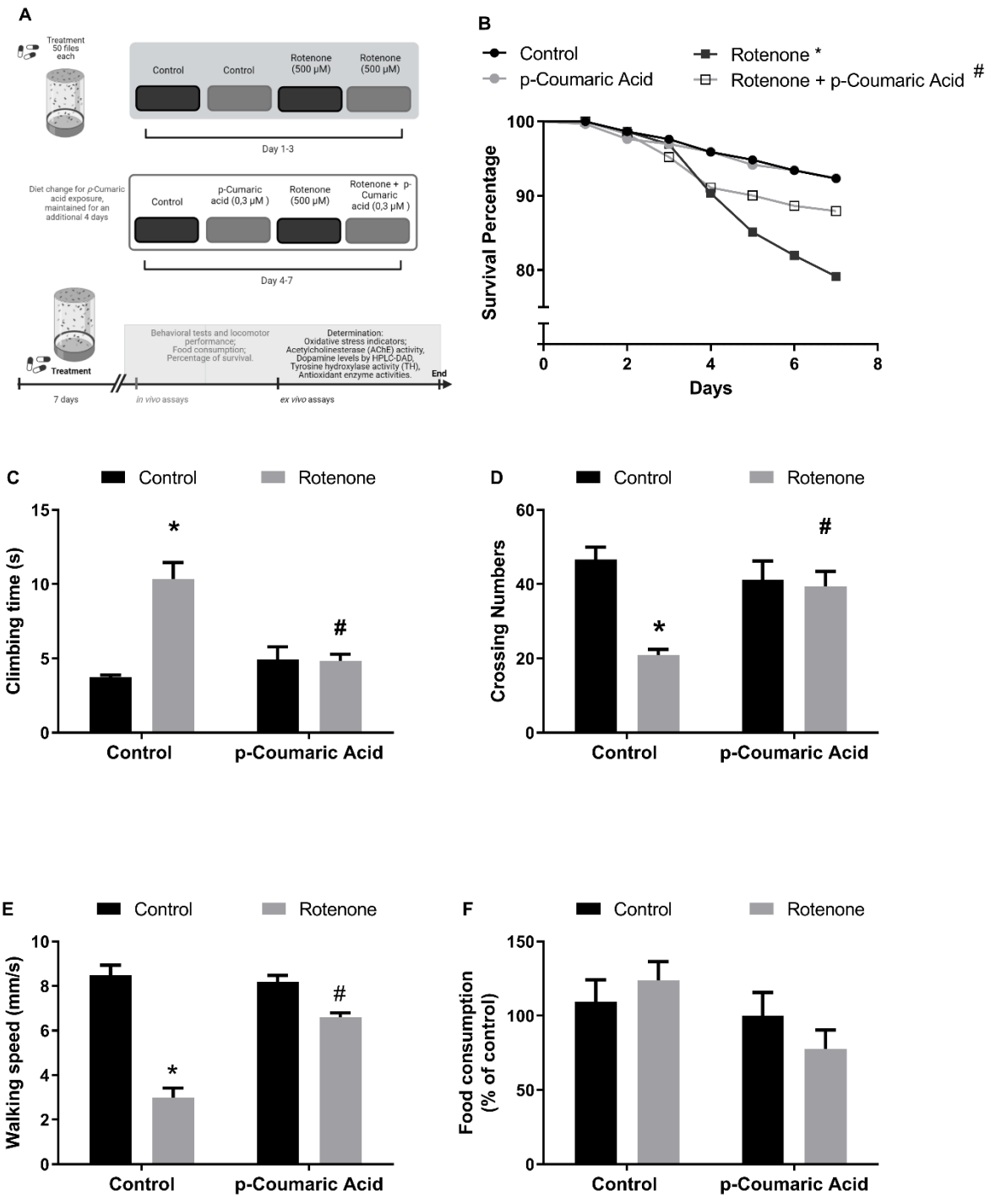

A
Treatment 50 files each
Control
Control
Rotenone (500 µM)
Rotenone (500 µM)
Day 1-3
Diet change for p-Cumaric acid exposure, maintained for an additional 4 days
Control
p-Cumaric acid (0,3 µM)
Rotenone (500 µM)
Rotenone + p-Cumaric acid (0,3 µM)
Day 4-7
Treatment
Behavioral tests and locomotor performance; Food consumption; Percentage of survival.
Determination: Oxidative stress indicators; Acetylcholinesterase (AChE) activity, Dopamine levels by HPLC-DAD, Tyrosine hydroxylase activity (TH), Antioxidant enzyme activities.
End
7 days
in vivo assays
ex vivo assays
B
Control
p-Coumaric Acid
Rotenone *
Rotenone + p-Coumaric Acid #
Survival Percentage
Days
C
Control
Rotenone
Climbing time (s)
Control
p-Coumaric Acid
D
Control
Rotenone
Crossing Numbers
Control
p-Coumaric Acid
E
Control
Rotenone
Walking speed (mm/s)
Control
p-Coumaric Acid
F
Control
Rotenone
Food consumption (% of control)
Control
p-Coumaric Acid

Figure 3.

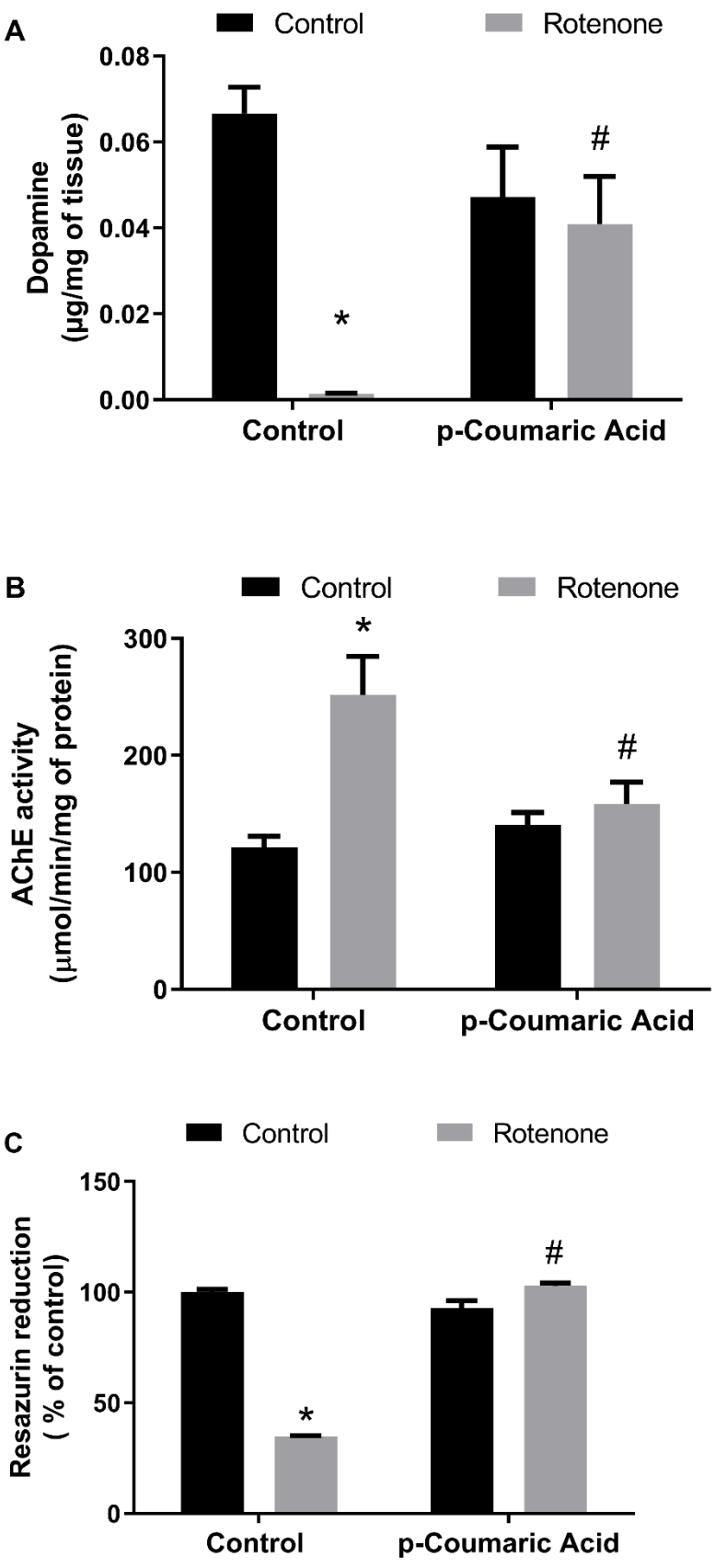
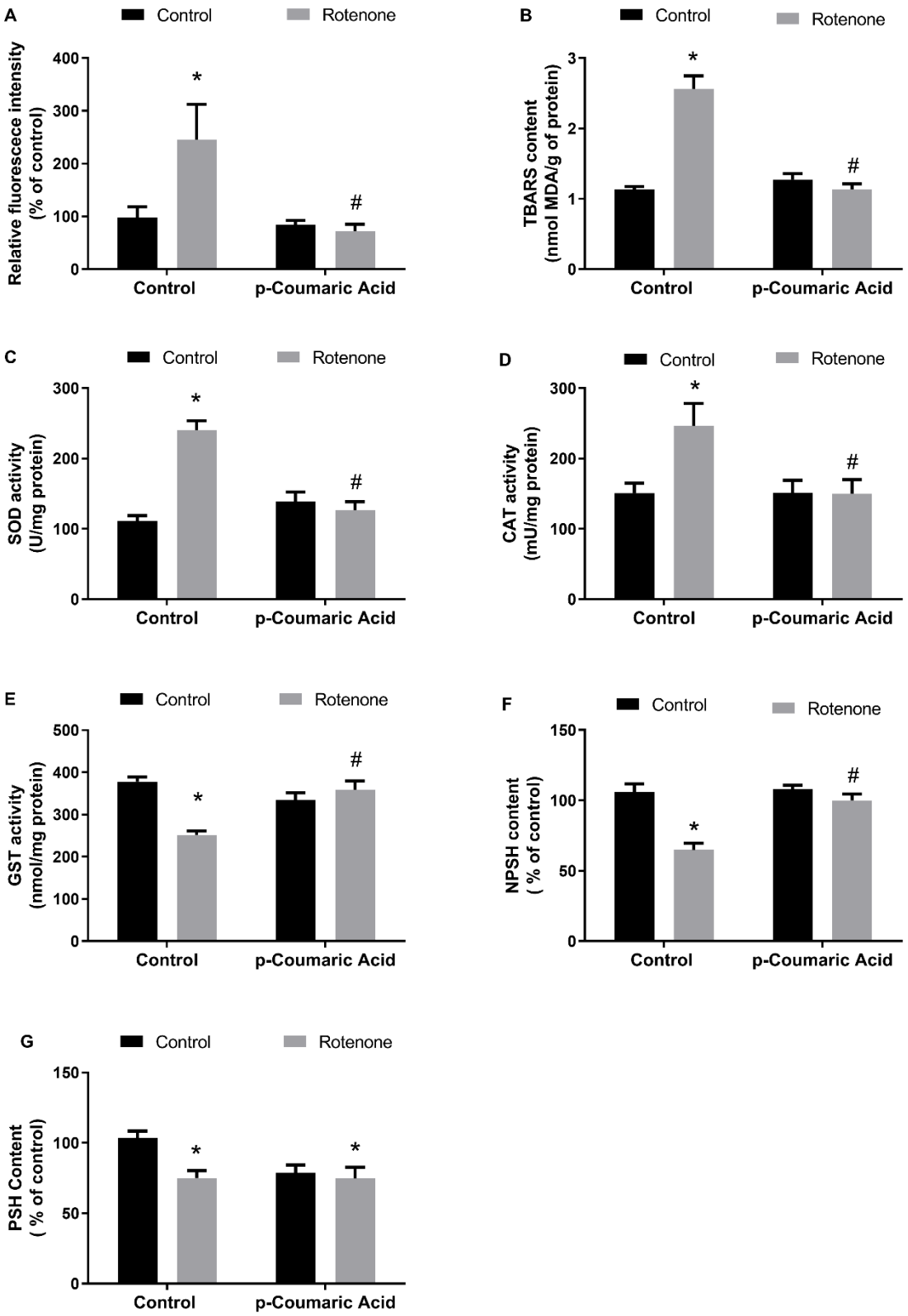

A
Control
Rotenone
Dopamine (µg/mg of tissue)
0.08
0.06
0.04
0.02
0.00
*
#
Control
p-Coumaric Acid
B
Control
Rotenone
AChE activity (µmol/min/mg of protein)
300
200
100
0
*
#
Control
p-Coumaric Acid
C
Control
Rotenone
Resazurin reduction (% of control)
150
100
50
0
*
#
Control
p-Coumaric Acid

Figure 4.


A
Control
Rotenone
Relative fluorescece intensity (% of control)
400
300
200
100
0
*
#
Control
p-Coumaric Acid
B
Control
Rotenone
TBARS content (nmol MDA/g of protein)
3
2
1
0
*
#
Control
p-Coumaric Acid
C
Control
Rotenone
SOD activity (U/mg protein)
300
200
100
0
*
#
Control
p-Coumaric Acid
D
Control
Rotenone
CAT activity (mU/mg protein)
300
200
100
0
*
#
Control
p-Coumaric Acid
E
Control
Rotenone
GST activity (nmol/mg protein)
500
400
300
200
100
0
*
#
Control
p-Coumaric Acid
F
Control
Rotenone
NPSH content (% of control)
150
100
50
0
*
#
Control
p-Coumaric Acid
G
Control
Rotenone
PSH Content (% of control)
150
100
50
0
*
*
Control
p-Coumaric Acid

Legends

Figure 1: Effect of exposure to *p*-coumaric acid (0.03; 0.1; 0.3 or 1 µM) for 3 days, on behavioral assessments in *Drosophila melanogaster*. (B) survival percentage; (C) negative geotaxis, for n = 10-11 in each group; (D) open field for n = 8 in each group. Data are mean and SEM. * Indicates a significant difference ($P < 0.05$) compared to the control group.

Figure 2: (A) Experimental protocol design. Effect of co-exposure of *p*-coumaric acid (0.3 µM) and rotenone (500 µM) for 3 or 7 days respectively, on locomotor and exploratory activity and survival percentage in *Drosophila melanogaster.* (B) Survival percentage, data were collected every 24 h for each group over 7 days. (C) Negative geotaxis and (D) Open field, data are mean and SEM, for n = 8 in each group. (D) Motor coordination test, data are mean and SEM, for n = 5 in each group. (E) Food consumption, data are mean and SEM, for n = 5 in each group. * Indicates a significant difference ($P < 0.05$) compared to the control group. # Indicates a significant difference ($P < 0.05$) compared to the rotenone group.

Figure 3: Effect of co-exposure of *p*-coumaric acid (0.3 µM) and rotenone (500 µM) for 3 or 7 days respectively, on (A) dopamine levels, for n = 5 in each group; (B) AChE activity, for n = 5 in each group and (C) Resazurin, for n = 6 in each group, in the head of *Drosophila melanogaster*. Data are mean and SEM. * Indicates a significant difference ($P < 0.05$) compared to the control group. # Indicates a significant difference ($P < 0.05$) compared to the rotenone group.

Figure 4: Effect of co-exposure of *p*-coumaric acid (0.3 µM) and rotenone (500 µM) for 3 or 7 days respectively, on oxidative stress indicators (A) ROS, n = 4-5; (B) TBARS, n = 6; (C) SOD; (D) CAT; (E) GST; (F) NPSH and (G) PSH, for n = 5 in each group, in the head of *Drosophila melanogaster*. Data are mean and SEM. * Indicates a significant difference ($P < 0.05$) compared to the control group. # Indicates a significant difference ($P < 0.05$) compared to the rotenone group.

5. CONCLUSÃO

Em conjunto, os resultados obtidos neste estudo, podemos concluir que as moscas que foram expostas à rotenona no modelo de DP, sofreram alterações comportamentais e bioquímicas semelhantes às características parkinsonianas.

Através da exposição ao ácido *p*-cumárico, foi possível reverter essas alterações. O ácido *p*-cumárico reverteu o déficit comportamental neuromotor adquiridos pelas moscas expostas a rotenona.

Mostrou-se desempenhar um papel antioxidante às espécies reativas de oxigênio, ao regular o estresse oxidativo, e ainda quanto sua propriedade de modular a expressão de algumas enzimas envolvidas no metabolismo oxidativo das células, restaurando a atividade da AChE.

Assim, o ácido *p*-cumárico surge como um potencial tratamento para a DP, podendo ainda ser explorado como uma terapia e/ou complementar para aumentar a eficácia dos tratamentos existentes.

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