UNIVERSIDADE FEDERAL DO PAMPA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

Ilex paraguariensis e seus componentes majoritários atenuam as alterações comportamentais, mortalidade e nos parâmetros bioquímicos associados a exposição ao malonato ou metilmalonato em *Drosophila melonogaster*

TESE DE DOUTORADO

José Luiz Ribeiro Portela

Uruguaiana, RS, Brasil.
2020

Ilex paraguariensis* e seus componentes majoritários atenuam as alterações comportamentais, mortalidade e nos parâmetros bioquímicos *associados a exposição ao malonato ou metilmalonato em Drosophila melonogaster

por

José Luiz Ribeiro Portela

Tese apresentada como requisito parcial para obtenção do **grau de Doutor em Bioquímica**, pelo programa de Pós-Graduação em Bioquímica, da Universidade Federal do Pampa, UNIPAMPA

Orientador: Prof. Dr. Robson Luiz Puntel

Uruguaiana, RS, Brasil.

2020

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

A comissão Examinadora, abaixo assinada, aprova a Tese de Doutorado

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2020

Dedico a toda minha família e em
especial a minha noiva Jana.

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Agradeço primeiramente aos meus pais, Antônio e Rosane, e a minha irmã Luana, por todo o incentivo e apoio mesmo estando longe. Obrigado pelo carinho, amor e confiança no meu trabalho.

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Epígrafe

"Sempre há limites. Eu não conheço os meus."

Usain Bolt

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Bioquímica
Universidade Federal do Pampa

Ilex paraguariensis* e seus componentes majoritários atenuam as alterações comportamentais, mortalidade e nos parâmetros bioquímicos *associados a exposição ao malonato ou metilmalonato em Drosophila melonogaster

Autor: José Luiz Ribeiro Portela
Orientador: Robson Luiz Puntel
Local e Data da defesa: Uruguaiana, 28 fevereiro de 2020.

A acidemia metilmalônica é um erro inato autossômico recessivo e raro do metabolismo de aminoácidos de cadeia ramificada que causam significativa morbidade e mortalidade na infância e, para os sobreviventes, significativos danos debilitantes na vida adulta. Essa doença é resultado de um defeito no metabolismo mitocondrial uma vez que a diminuição na atividade da enzima metilmalonil-CoA mutase (responsável por degradar L-Metilmalonil-CoA à Succinil-CoA) leva ao acúmulo de ácidos orgânicos no organismo, entre eles os ácidos malônico e metilmalônico. Embora não elucidado(s) o(s) mecanismo(s) pelo(s) qual(is) esses ácidos atuam/agem, o estresse oxidativo parece contribuir para a toxicidade desses ácidos orgânicos. Diante o exposto, e sabendo que o extrato aquoso de *Ilex paraguariensis* (IP) possui atividade antioxidante, este estudo testa a hipótese de que o extrato de IP, o ácido cafeico e a cafeína possam atenuar a toxicidade dos ácidos malônico e metilmalônico em modelo de *D. melanogaster*. Nossos dados mostram que o extrato de IP atenuou o dano comportamental, protegeu contra a mortalidade, bem como foi capaz de proteger dos danos (oxidativos e não oxidativos) associados à exposição aos diferentes ácidos orgânicos. A fim de buscar um melhor entendimento sobre o(s) mecanismo(s) pelo(s) qual(is) o extrato de IP age, demos sequência ao estudo utilizando o ácido cafeico e a cafeína (compostos majoritários encontrados em nosso extrato de IP), sejam isolados

ou combinados, na co-exposição aos ácidos malônico e metilmalônico. Frente a isto os isolados diminuíram a mortalidade e aboliram completamente as alterações comportamentais, além disso foram capazes de proteger contra as alterações bioquímicas, sendo assim responsáveis pelo menos em parte pelo efeito protetor do extrato de *I. paraguariensis* como relatado em estudo anterior.

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Palavras-chave: Acidemia Metilmalônica, Malonato, Metilmalonato, Estresse Oxidativo, *Ilex paraguariensis* e *Drosophila melanogaster.*

ABSTRACT

Doctoral Thesis
Program of Post-Graduation in Biochemistry
Federal University of Pampa

Ilex paraguariensis* and major components attenuate behavioral changes, mortality and biochemical parameters associated with exposure to malonate or methylmalonate in *Drosophila melanogaster

Author: José Luiz Ribeiro Portela

Advisor: Robson Luiz Puntel

Site and Date of defense: Uruguaiana-RS, February 28, 2020.

Methylmalonic acidemia is a rare autosomal recessive inborn error of branched-chain amino acid metabolism that causes significant childhood morbidity and mortality and, for survivors, significant debilitating damage in adulthood. This disease is a result of a defect in mitochondrial metabolism since the decrease in activity of the enzyme methylmalonyl-CoA mutase (responsible for degrading L-Methylmalonyl-CoA to Succinyl-CoA) leads to the accumulation of organic acids in the body, including acids. malonic and methylmalonic. Although unclear the mechanism (s) by which these acids act, oxidative stress seems to contribute to the toxicity of these organic acids. Given the above, and knowing that the aqueous extract of *Ilex paraguariensis* (IP) has antioxidant activity, this study tests the hypothesis that IP extract, caffeic acid and caffeine may attenuate the toxicity of malonic and methylmalonic acids in a model of D. melanogaster. Our preliminary data show that IP extract attenuated behavioral damage, protected against mortality, and was able to protect against damage (oxidative and non-oxidative) associated with exposure to different organic acids. In order to better understand the mechanism (s) by which IP extract acts, we continued the study using caffeic acid and caffeine (major compounds of IP extract), either alone or in combination with co-exposure to malonic and methylmalonic acids. In view of this, the isolates reduced mortality and completely abolished behavioral changes and were able to protect against biochemical changes, thus being responsible at least in part for the protective effect of I. *paraguariensis* extract as reported in a previous study.

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Keywords: Methylmalonic Acidemia, Malonate, Methylmalonate, Oxidative Stress*, Ilex paraguariensis* and *Drosophila melanogaster*.

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Lista de Abreviaturas

AChE – Enzima acetilcolinesterase

AMM – Acidemia Metilmalônica
ATP – Adenosina Trifosfato

CA – Ácido Caféico

CAF - Cafeína

cblA – Vitamina B12, cobalamina

CK - Ciclo de Krebs

CR - Cadeia respiratória

DM - *Drosophila melanogaster*

EO – estress oxidativo

ERO – Espécies Reativas de Oxigênio

MA – Ácido Malônico, malonato

MCM – Metilmalonil-CoA mutase

MCD-D - Malonil CoA-descarboxilase

MMA – Ácido Metilmalônico, metilmalonato

MUT – Gene 609058 que codifica a enzima metilmalonil-CoA mutase

IP – *Ilex Paraguariensis*

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1. Introdução

A acidemia metilmalônica (AMM) é um dos mais frequentes erros do metabolismo dos ácidos orgânicos. O defeito bioquímico está localizado no metabolismo do propionato, na etapa de conversão de ácido metilmalônico a ácido succínico. A grande maioria dos pacientes tem um defeito na conversão de metilmalonil-CoA a succinil-CoA pelo defeito da enzima metilmalonil-mutase (E.C 5.4.99.2) (MUT) (Trindade *et al*., 2009). Como consequência, metilmalonil-CoA acumula-se e é subsequentemente hidrolisado em CoA e ácido metilmalônico (MMA) (Chandler e Venditti, 2005; Melo *et al*., 2011). Esta acidemia é caracterizada pelo acúmulo do ácido metilmalônico (MMA) nos tecidos e fluidos corpóreos (Fenton *et al*, 2001). O MMA, por sua vez, é um dos causadores da disfunção mitocondrial, que resulta na perturbação energética, ou seja, alterando a produção de ATP (Mirandola *et al*., 2008; Melo *et al*., 2011; Wajner e Goodman, 2011). No entanto, o MMA não é único metabólito que se acumulam na AMM, outros metabólitos estão acumulados e fazem parte da fisiopatologia desta doença. (Melo *et al*., 2011). Pacientes com MMA apresentam aumento considerável de ácido malônico (MA), um inibidor reversível da enzima succinato desidrogenase (SDH)(E.C 1.3.99.1). Relatos demonstram que o MA foi capaz de induzir disfunção mitocondrial, que por sua vez pode desencadear a geração de radicais superóxido, excitotoxicidade secundária mediada pelo influxo de Ca^{2+}, e apoptose devido à geração de espécies reativas (ER) (Kalonia *et al*., 2010; Kumar *et al*., 2013). Importante ressaltar, que o acúmulo desses metabólitos acarretam atraso do desenvolvimento, cardiomiopatia, baixa estatura, displasia renal, hipotonia, neurodegeneração e até mesmo a morte neonatais (Brown *et al*., 1984; Kolker e Okun, 2005; Salomons *et al*., 2007; Melo *et al*., 2011).

Neste contexto, existe uma busca constante por uma alternativa farmacológica, para reverter estes danos causados por estes ácidos. A erva-mate (*Ilex paraguariensis, Saint Hilaire*) é uma planta amplamente consumida na América do Sul. É naturalmente cultivado no Brasil, Argentina e Paraguai. As folhas são usadas para preparar diferentes bebidas, incluindo “chimarrão” (Miranda *et al*, 2008). Alguns estudos científicos relatam que a *I. paraguariensis* demonstra efeito hipocolesterolêmico, hepatoprotetor, antioxidante e

estimulante do sistema nervoso central (Filip e Ferraro, 2003; Portela *et al.*, 2017). Entre os efeitos benéficos de *I. paraguariensis*, o potencial antioxidante poderia contribuir para reduzir o risco de desenvolver doenças neurodegenerativas relacionadas ao processo oxidativo. Estes efeitos foram atribuídos aos compostos fenólicos presentes nesta planta, como os ácidos fenólicos (ácido clorogênico e derivados) e flavonóides, especialmente rutina (Heck e De Mejia, 2007). Além disso, os principais metabólitos contidos em nossa amostra de extrato de IP, o ácido cafeico e cafeína, possuem diversos efeitos biológicos. O ácido cafeico (CA) é um composto polifenólico com grande potencial antioxidante, assim como a cafeína, o psicoestimulante mais consumido no mundo, que também possui efeito antioxidante (Devasagayam *et al.*, 1996;Gocer e Gulcin, 2011).

Entretanto, muitos obstáculos práticos e éticos limitam o espaço para experimentos usando seres humanos na ciência biomédica, com isso, a utilização da mosca da fruta *Drosophila melanogaster* apresenta-se como um modelo alternativo e versátil que tem sido usado na pesquisa biomédica há mais de um século. Existem muitas vantagens para utilizar a mosca da fruta em relação aos modelos de vertebrados, tais como a facilidade no manuseio, a procriação de baixo custo, o curto ciclo de vida, além do que as mesmas poderem ser geneticamente modificadas. Comparações entre os genomas de *D. melanogaster* e humanos, revelaram-se que aproximadamente 75% genes de doenças humanas conhecidas têm uma correspondência reconhecível no genoma das moscas da fruta consolidando sua legitimidade como um modelo alternativo de pesquisa (Reiter *et al.*, 2001). Diante o exposto, esse trabalho teve como objetivo avaliar o potencial terapêutico do extrato aquoso de *I. paraguariensis* e seus compostos majoritários, ácido cafeico e cafeína, frente a toxicidade induzida pelos ácidos malônico ou metilmalônico em *D. melanogaster.*

APRESENTAÇÃO

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigo publicado e um manuscrito em fase final de redação que deverá ser submetido após as considerações da banca. Os mesmos encontram-se no item ARTIGOS CIENTÍFICOS. As seções Introdução, Materiais e Métodos, Discussão, Conclusão e Referências Bibliográficas encontram-se nos ARTIGOS CIENTÍFICOS e representam a íntegra deste estudo. Os itens DISCUSSÃO E CONCLUSÕES, encontram-se no final desta tese e apresentam interpretações e comentários gerais sobre os artigos científicos neste trabalho. As REFERÊNCIAS BIBLIOGRÁFICAS referem-se somente às citações que aparecem itens INTRODUÇÃO, REVISÃO BIBLIOGRÁFICA e DISCUSSÃO desta tese.

2. Revisão Bibliográfica

2.1 Acidemia Metilmalônica

A acidemia metilmalônica (AMM) foi descrita pela primeira vez por Oberholzer et al, em 1967, o qual constatou níveis elevados de ácido metilmalônico (MMA) no sangue e urina de crianças com cetoacidose (Fenton, 1995). A AMM é um erro inato, autossômico recessivo e raro do metabolismo de aminoácidos de cadeia ramificada (isoleucina, metionina, treonina e valina), ácidos graxos de cadeia ímpar e cadeia lateral do colesterol que causam significativa morbidade e mortalidade na infância e, para os sobreviventes, significativos danos debilitantes na vida adulta. O propionato e a proprionil-CoA são produzidos pela oxidação dos aminoácidos (Marks et al, 1996).

No metabolismo normal, metilmalonil-CoA mutase (MCM) degrada L-Metilmalonil-CoA a Succinil-CoA (Figura 1). A diminuição da atividade desta enzima provoca o acúmulo de ácido metilmalônico no organismo, com vários efeitos prejudiciais. De fato, defeitos na metilmalonil-CoA mutase ou a sua coenzima, a cobalamina (vitamina B12), levam ao acúmulo de ácido metilmalônico e um quadro chamado acidúria (ou acidemia) metilmalônica (Matsui *et al.*, 1983).

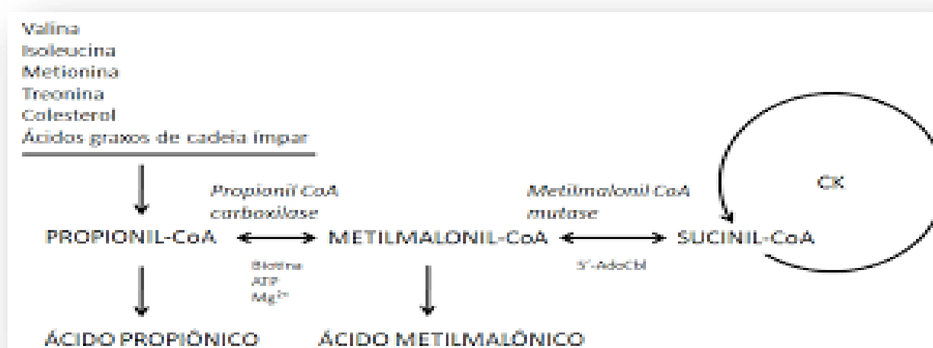

Figura 1: Erro no metabolismo mitocondrial de Aminoácidos. A enzima metilmalonil-CoA mutase responsável pela conversão do L-metilmalonil-CoA a succinil-CoA na rota de degradação de aminoácidos de cadeia ramificada, ácidos graxos de cadeia impar e colesterol. (Google Imagens)

Assim explica-se que AMM está relacionada com a deficiência da enzima MCM, e é codificada pelo gene MUT (Gene 609058), podendo apresentar duas formas: MUT (o), quando a enzima não tem qualquer atividade e não responde a altas doses de cobalamina e MUT (-), onde a enzima tem uma pequena reação quando paciente recebe doses de cobalamina. Nos demais casos, a AMM se deve a defeitos na síntese de ativação das cobalaminas (cblA, cblB, cblC, cblD e cblE) ou no transporte da mesma. A deficiência desta enzima leva ao acúmulo do ácido metilmalônico (MMA) e de metabólitos secundários, como ácido propionico, propionil-CoA e também do ácido malônico (Okun *et al.*, 2002).

Os sinais clínicos podem apresentar-se nas primeiras horas de vida ou nas primeiras semanas até 21 dias, os sinais surgem e progridem: falta apetite, vômitos, perda de peso, distensão abdominal e manifestação neurológica progressiva (Poggi *et al*, 1994). As manifestações neurológicas incluem: movimentos anormais, hipotonia, letargia e convulsões. Caso estes sintomas não sejam tratados, pode-se levar ao coma, déficit cognitivo e os pacientes morrem em poucos dias, ou desenvolvem lesão cerebral permanente (Deodato, B *et al*, 2006). No entanto, os mecanismos subjacentes a esses sintomas são pouco compreendidos, tanto clinicamente quanto patologicamente.

2.1.1 Ácido Metilmalônico

Como apresentado no tópico acima, a acidemia metilmalônica é caracterizada por diversos distúrbios, devido à deficiência da enzima Metilmalonil-CoA mutase (MCM), e isto leva ao acúmulo de ácidos orgânicos, entre eles o ácido metilmalônico (MMA). O MMA é um ácido dicarboxílico (Figura 2) derivado do ácido propiônico (Figura 3) e transformado em ácido succínico sob ação do cofator vitamina B12. O ácido propiônico provém do catabolismo de diversos aminoácidos e ácidos graxos, enquanto o ácido succínico é metabolizado no ciclo de Krebs.

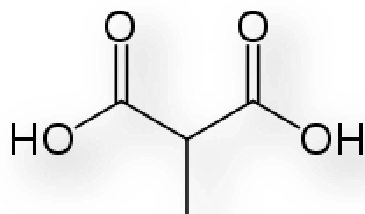

Figura 2: Estrutura Ácido Metilmalônico (Google Imagens)

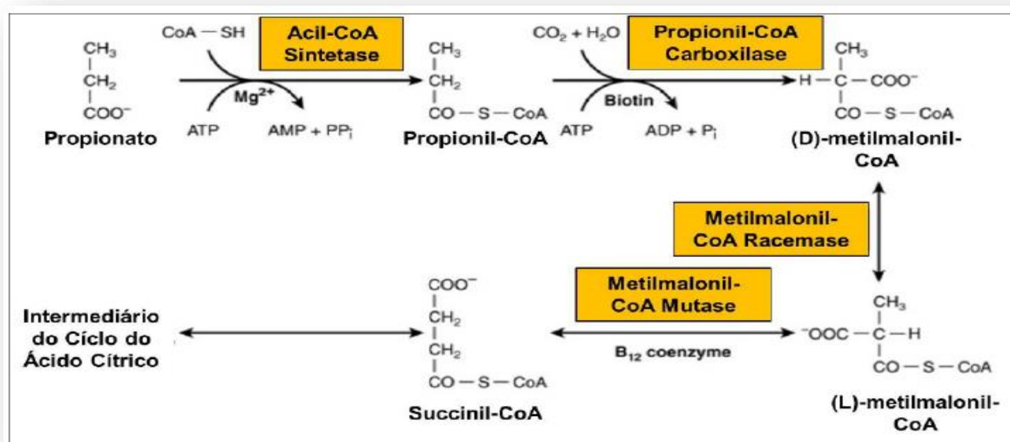

Figura 3. Metabolismo do propionato. Adaptado de Murray et al. (2012).

Estudos mostram que o MMA inibe o complexo II (sinônimo, succinato: ubiquinona oxidoredutase), ciclo de Krebs e o sistema mitocondrial da cadeia respiratória de elétrons (CR) (Wajner e Coelho, 1997). Devido ao mesmo ser estruturalmente muito semelhante ao ácido malônico (MA) um inibidor do complexo II (Greene *et al.*, 1993). Foi observado que o acúmulo de MMA acarreta em um decréscimo da relação ATP/ADP observado em células neuronais tratadas com MMA, que foi atribuído a produção de malonato, um inibidor clássico da CR e do ciclo de Krebs, a partir do MMA (Mcclung e Hirsh, 1998). Há outros estudos que mostram a inibição do metabolismo energético pelo MMA (Brusque *et al.*, 2002) (Marisco *et al*, 2003). Estudo realizado por Fontella, et al.,2000) demonstra que o MMA induz danos oxidativos a proteínas, peroxidação lipídica, e diminui defesa antioxidante enzimático e não enzimático *in vitro* e *in vivo.*

2.1.2 Ácido Malônico

O malonato (Figura 4) é um inibidor reversível da succinato desidrogenase, sendo inibidor da respiração celular, pois une-se ao sítio ativo da succinato desidrogenase durante o ciclo do ácido cítrico, mas não reage, apenas compete com o succinato. Nas reações de fosforilação oxidativa uma das enzimas é a succinato desidrogenase onde o malonato atua comoinibidor do complexo II.

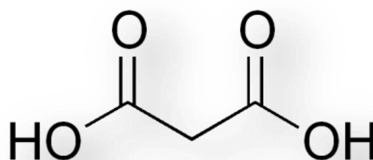

Figura 4: Estrutura Ácido Malônico (Google Imagens)

A Succinato desidrogenase (E.C 1.3.99.1) (SDH), é uma enzima que desempenha um papel central no metabolismo energético neuronal; participa do ciclo de Krebs e na fosforilação oxidativa (Beal *et al*., 1993; Greene *et al*., 1993). O acúmulo de malonato se dá pela deficiência da enzima malonil-CoA descarboxilase (MCD), que catalisa a conversão de malonil-CoA a Acetil-CoA mantendo os níveis homeostáticos desses metabólitos nas mitocôndrias e peroxissomos. A deficiência de MCD, ou acidúria malônica, é um distúrbio metabólico congênito causado por mutações que reduzem ou eliminam a atividade dessa enzima e, portanto, compromete a conversão de malonil-CoA em acetil-CoA (FitzPatrick, D. R, 1999). De fato, o malonato pode desencadear disfunção mitocondrial, que por sua vez pode aumentar a geração de radicais superóxido, excitotoxicidade secundária e apoptose (Dedeoglu et al., 2002). Diversos sintomas são observados entre os pacientes com acidúria malônica, incluindo atraso no desenvolvimento, convulsões, diarréia, vômitos, baixo nível de açúcar no sangue (hipoglicemia) e cardiomiopatia (FitzPatrick, D. R,1999).

2.2 Estresse Oxidativo

Nos seres humanos, o estresse oxidativo está implicado em uma ampla variedade de patologias, incluindo câncer, diabetes tipo II, arteriosclerose, e várias alterações neurodegenerativas (Droge W, 2002). A cadeia respiratória mitocondrial é a principal fonte de geração de EROS intracelular e, ao mesmo tempo, um alvo importante para os efeitos prejudiciais da EROS, onde induz dano ao DNA, oxidação de proteínas, prejudica a respiração mitocondrial e dependendo da dose estimula ou inibe a proliferação celular. (Fig. 5).

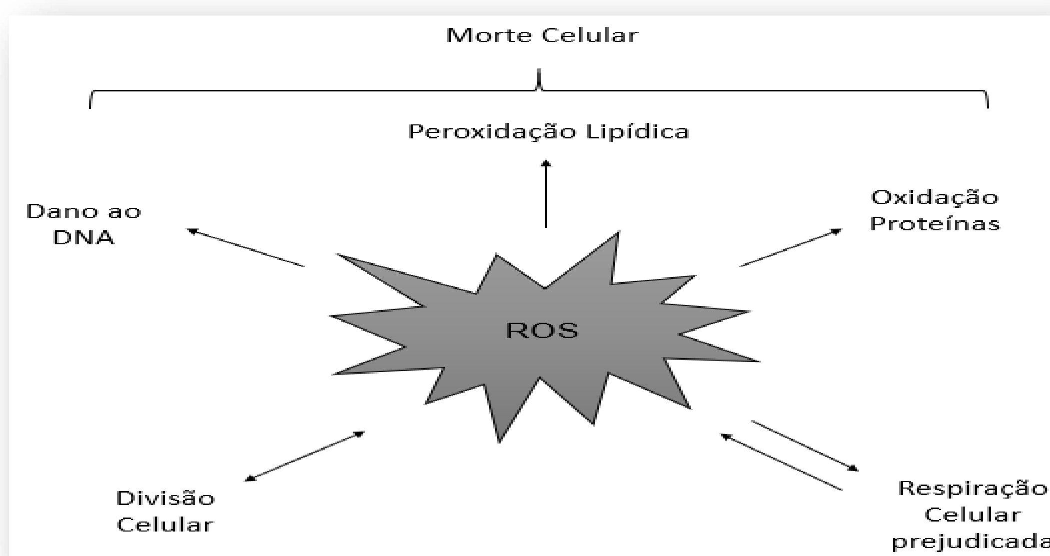

Figura 5. Efeitos das espécies reativas de oxigênio nas funções celulares e na indução da morte celular. (Adaptação Halliwell e Gutteridge, 1999)

O estresse oxidativo é definido como um desequilíbrio que favorece a produção de ERO sobre as defesas antioxidantes (Halliwell e Gutteridge, 1999); no entanto, os mecanismos precisos pelos quais os ERO causam as lesões permanecem não completamente compreendidas. Estudos realizados por Fontella e colaboradores 2000, demonstraram que o ácido metilmalônico (MMA) induz a geração de radicais livres em córtex cerebral de ratos *in vitro*. Paciente portadores de acidemia metilmalônica após descompensação

metabólica, demonstram diminuição nos níveis de glutationa reduzida (Treacy *et al*, 1996).

Defeitos na cadeia respiratória (CR) podem levar ao estresse oxidativo, que pode, por sua vez, danificar os complexos, gerando um ciclo vicioso culminando com a morte celular. Um exemplo a ser demonstrado, a rotenona, um inibidor do complexo I, a azida, inibidor do complexo IV e o malonato, inibidor da succinato desidrogenase, aumentam a produção de radicais livres *in vivo* e *in vitro* (Schulz *et al*, 1996; Cassarino *et al*, 1999). Essas observações sugerem, que os radicais livres, geradas no estresse oxidativo, estejam envolvidos na patofisiologia da acidemia metilmalônica.

Apesar de o organismo humano apresentar mecanismos endógenos para combater o estresse oxidativo, esse sistema de defesa antioxidante nem sempre é suficiente para neutralizar os agentes oxidantes (Yang, *et al* 2012). Dessa forma, a utilização de antioxidantes exógenos derivados da dieta parece ser uma importante estratégia na proteção contra as doenças crônicas.

2.3 *Ilex paraguariensis st hill*

A erva-mate (*Ilex paraguariensis A. St. Hil.*) (Figura 6) é uma espécie arbórea da família Aquifoliácea, originária das regiões subtropicais da América do Sul, presente Brasil, Argentina, Uruguai e Paraguai. Ela é consumida por grande parte da população desses países, apresentando importância cultural, econômica e social para essas regiões (Heck e De Mejia, 2007; Bracesco *et al.*, 2011).

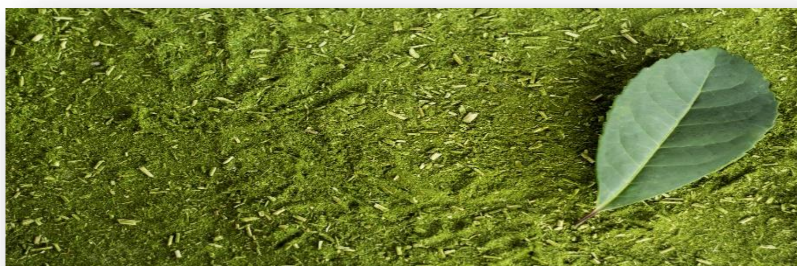

Figura 6. Erva mate: *Ilex paraguariensis* Fonte (Google imagens)

A partir das folhas de erva-mate são produzidos diferentes tipos de bebida, tais como o chimarrão e o tererê, preparados com as folhas verdes, e o chá mate, preparado com as folhas tostadas (Bastos *et al.*, 2007; Bracesco *et al.*, 2011). Estima-se que milhões de pessoas consomem cerca de um a dois litros por dia de infusão de erva mate, constituindo-se na principal alternativa ao consumo do café (Bastos *et al.*, 2007; Heck e De Mejia, 2007).

A erva-mate apresenta na sua composição uma diversidade de constituintes, incluindo nutrientes, minerais e vitaminas solúveis em água. Também é caracterizado pela sua riqueza em fitoquímicos, particularmente polifenóis (ácidos fenólicos, flavonoides), alcalóides (incluindo metilxantinas, cafeína, teobromina, teofilina) e terpenos (carotenóides, saponinas) (Heck e De Mejia, 2007). Os compostos fenólicos são os constituintes mais abundantes e mais estudados da erva-mate. Extrato feito das folhas de IP contém vários fitoquímicos como o ácido cafeico, clorogênico, catequinas, aminoácidos, cafeína, quercetina, kaempferol, ácido ascórbico, vitaminas B1 / B2 e rutina (Carini *et al.* 1998).

Um grande número desses estudos avaliou a capacidade antioxidante do extrato de erva mate com diferentes metodologias, e mostrou que o efeito antioxidante está relacionado com a presença dos polifenóis (Bracesco *et al.*, 2011; Peralta *et al.*, 2013; Portela *et al.*, 2017). Consequentemente, o extrato de IP atua por meio da neutralização das espécies reativas e modula a expressão de genes e enzimas antioxidantes (Bracesco *et al.*, 2011). A IP também tem demonstrado possuir atividades antioxidantes, anti-inflamatórias, antimutagênicas, antiobesidade, antidiabéticas (interferindo na absorção de glicose), diuréticas, antifúngicas, estimulantes e antidepressivas (Gorgen *et al.*, 2005; Filip *et al.*, 2010; Bracesco *et al.*, 2011; Reis Ede *et al.*, 2014; Colpo *et al.*, 2016; Colpo *et al.*, 2017; Portela *et al.*, 2017).

Visto isso, os principais metabólitos contidos em nossas amostras estudadas de extrato de IP, o ácido cafeico e cafeína, possuem diversos efeitos biológicos. O ácido caféico (AC) é um composto polifenólico natural derivado do café e algumas frutas e vegetais (Jeon, Y.D, 2014). O AC (Figura 7) demonstra ser um metabólito biologicamente ativo, que possui atividade de inibir peroxidação lipídica, antiviral, anti-inflamatório e regulação de enzimas antioxidantes (Gocer e Gulcin, 2011; Murtaza *et al.*, 2014). A cafeína (CAF) é

um psicoestimulante conhecida como 1,3,7-trimetilxantina sendo a substância mais consumida no mundo. A CAF (Figura 8) é encontrada naturalmente em grãos de café, folhas de chá, nozes de cola, grãos de cacau e na erva-mate (Heckman MA, 2010; Carini *et al*. 1998). A CAF pode inibir a peroxidação lipídica e reduzir a produção de espécies reativas de oxigênio (EROs) (Devasagayam *et al*., 1996). De fato, a ingestão crônica de forma moderada de cafeína inibe o estresse oxidativo e melhora a função mitocondrial (Mishra e Kumar, 2014), também aumenta a atividade da enzima glutationa S-transferase (E.C 2.5.1.18) e inibe apoptose da membrana celular (Spiff e Uwakwe, 2004; Carelli-Alinovi *et al*., 2016).

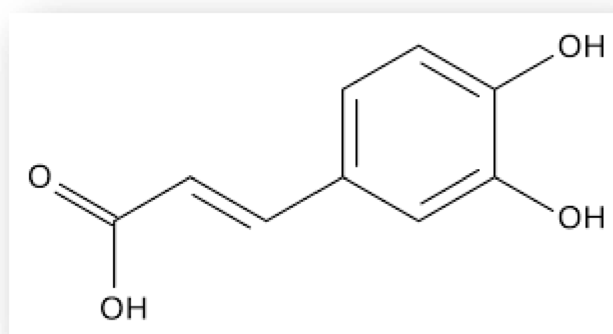

Figura 7. Estrutura molecular do ácido caféico. Fonte (Google Imagens)

Figura 8. Estrutura molecular da cafeína. Fonte (Google Imagens)

2.4 Modelo Experimental Alternativo: *Drosophila Melanogaster*

Drosophila melanogaster (DM), popularmente conhecida como "mosca da fruta", tem uma longa história de seu uso em pesquisa genética (110 anos) (H J, Bellen, 2010). Este organismo modelo foi o centro das atenções de um trabalho pioneiro de Thomas Hunt Morgan, pai da genética, "descoberta de Fenômeno ligado ao X em *Drosophila*". Além disso, em 1933, Morgan ganhou o Prêmio Nobel de Fisiologia ou Medicina por sua importante descoberta de que "o cromossomo desempenha um papel fundamental na hereditariedade" (Roberts, 2006). Aproximadamente 13.600 genes codificados de proteínas estão localizados em apenas quatro cromossomos. O sequenciamento do genoma das DM revelou que elas possuem uma similaridade de 75% com genes relacionados a doenças em humanos, tendo grande potencial para ser utilizada como organismo modelo para estudos de doenças humanas (Panchal & Tiwari, 2017). A lista de vantagens de usar DM é longa: um curto ciclo vida (Figura 9), uma alta taxa de reprodução, baixo custo e fácil manutenção de culturas e sistemas moleculares com função celular e fisiológica conservada, redundâncias menos funcionais em comparação com mamíferos (Adicionar referência).

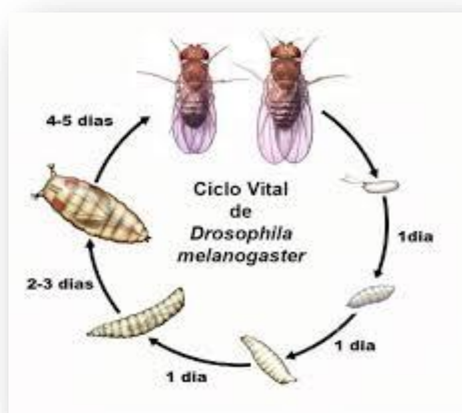

Figura 9: Ciclo de vida: *Drosophila melanogaster.* Fonte (Google imagens)

A *D. melanogaster* pode ser considerada um modelo de múltiplos organismos, cada um com suas próprias vantagens específicas, definidas pelo estágio de desenvolvimento: embrião, larva, pupa e fase adulta. Significativamente, a resposta das moscas a muitas drogas que atuam dentro do SNC é semelhante aos efeitos observados nos sistemas de mamíferos (Mcclung e Hirsh, 1998; Moore *et al.*, 1998; Bainton *et al.*, 2000; Nichols *et al.*, 2002; Rothenfluh e Heberlein, 2002; Satta *et al.*, 2003; Andretic *et al.*, 2008). Ademais, a DM, foi recomendada pelo Centro Europeu para a Validação de Métodos Alternativos (ECVAM) para promover os 3Rs (redução, refinamento e substituição) do uso de animais de laboratório em estudos de toxicidade (Benford *et al*, 2000).Também conhecida por sua alta sensibilidade a substâncias tóxicas e está sendo considerada como um modelo para estudos de toxicidade, bem como para avaliar a ação de agentes farmacológicos (Adedara *et al.*, 2015).

Portanto a finalidade da utilização deste modelo serve como um “screening”, a fim de caracterizar os efeitos tóxicos causados pelos ácidos malônico e metilmalônico, e neste contexto buscar alternativas terapêuticas que minimizem os efeitos causados por estes metabólitos que possam ser úteis em pesquisas futuras.

3. Objetivos

3.1 Objetivos Geral

O objetivo do presente estudo foi avaliar a hipótese de que o extrato de *Ilex paraguariensis*, o ácido cafeico e a cafeína possam atenuar a toxicidade causada pelos ácidos malônico e metilmalônico em modelo de *D. melanogaster*.

3.2 Objetivos Específicos 1

- ✓ Analisar a composição fitoquímica do extrato aquoso de *Ilex paraguariensis* IP através da técnica HPLC-DAD;
- ✓ Avaliar o efeito do tratamento com extrato de IP sobre a taxa de sobrevivência da *Drosophila melanogaster* (DM) expostas ao ácido malônico MA e metilmalônico MMA;
- ✓ Através de diferentes testes comportamentais, investigar os possíveis efeitos de proteção do extrato de IP sobre alterações motoras em DM causadas pela exposição ao MA e MMA;
- ✓ Avaliar o efeito do tratamento do extrato de IP sobre a atividade da enzima acetilcolinesterase (AChE), níveis de peroxidação lipídica (TBARS), ensaio de redução da Resazurina, dosagem das desidrogenases (MTT), atividade da superóxido dismutase (SOD), Determinação de tiol não-proteico (NPSH) em DM expostas ao MA e MMA.

3.3 Objetivos Específicos 2

- ✓ Avaliar o efeito do tratamento com os compostos majoritários Ácido Cafeico, Cafeína ou combinados sobre a taxa de sobrevivência de *Drosophila melanogaster* (DM) expostas ao ácido malônico MA e metilmalônico MMA;

- ✓ Através de diferentes testes comportamentais, investigar os possíveis efeitos de proteção Ácido Cafeico, Cafeína ou combinados sobre alterações motoras em (DM) causadas pela exposição ao MA e MMA;
- ✓ Avaliar o efeito antioxidante do tratamento com Ácido Cafeico, Cafeína ou combinados sobre a atividade da enzima acetilcolinesterase (AChE), níveis de peroxidação lipídica (TBARS), dosagem das desidrogenases (MTT), atividade superóxido dismutase (SOD), Determinação de tiol não-proteico (NPSH), níveis de Glicose e triglicerídeos totais em DM expostas ao MA e MMA.

4. ARTIGOS CIENTÍFICOS

Os resultados inseridos nesta tese apresentam-se sob a forma de artigo científico e um manuscrito, os quais se encontram aqui estruturados. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se nos mesmos e estão dispostos na mesma maneira que foi publicado e/ou será submetido.

4.1 Artigo: *Ilex paraguariensis* atenua mudanças no comportamento, mortalidade e parâmetros bioquímicos associados a exposição ao malonato e metilmalonato em *Drosophila melanogaster*

Artigo aceito para publicação na revista Neurochemical Research: doi.org/10.1007/s11064-019-02862-w

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ORIGINAL PAPER

Ilex paraguariensis Attenuates Changes in Mortality, Behavioral and Biochemical Parameters Associated to Methyl Malonate or Malonate Exposure in *Drosophila melanogaster*

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Abstract

Methylmalonic acidemia is a genetic disease characterized by accumulation of organic acids, such as methylmalonic (MMA) and malonic (MA) acids. Considering that the accumulation of MMA and MA causes several damages due to oxidative stress, antioxidants are thought to play a pivotal role in preventing deleterious effects associated with exposure to such compounds. *Ilex paraguariensis* (IP) was used here to test the hypothesis that supplementation with the aqueous extract of this plant could exert protective effect against MMA or MA induced mortality, behavioral and/or biochemical changes in *Drosophila melanogaster* (DM). Initially, a curve time- and dose–response to MMA (1–10 mM), MA (1–10 mM) and IP (63–500 µM) was performed. Thereafter, flies were concomitantly exposed to MA (5 mM), MMA (5 mM) and/or IP (250 µg/mL) during 15 days for survival assay, and for 48 hs to MA (1 or 5 mM), MMA (1 or 5 mM) and/or IP (250 µg/mL) for subsequent investigations. Both MMA and MA exposure resulted in higher incidence of mortality, a worse performance in the negative geotaxis assay and increased locomotion in open-field test as compared with control group. Furthermore, a marked increase in non-protein thiol (NPSH) and in thiobarbituric acid reactive substances (TBARS) levels, decrease in superoxide dismutase (SOD), catalase and acetylcholinesterase (AChE) activities, and decrease in MTT and resazurin reduction were noted in MMA or MA treated groups. IP treatment offered significant protection against all alterations associated to MMA or MA exposure. This study confirm the hypothesis that supplementation with IP offers protection against changes associated to MMA or MA exposure in DM, due, at least in part, to its antioxidant effect.

Keywords Methylmalonic acid · Malonic acid · *Ilex paraguariensis* · Drosophila · Oxidative stress

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Introduction

Methylmalonic acidemia, a group of autosomal recessive genetic disorders, is generally caused by mutations in the MUT gene (609058). In the process, a series of enzymes and bimolecules are affected and leads to the formation of methylmalonic acid (MMA) [1, 2]. Evidences suggests that methylmalonic acidemia is associated with mitochondrial dysfunction, energy disturbance, hinders in ATP production and diminishes various enzymatic activities like NADH:cytochrome c oxireductase and succinate:cytochrome c oxireductase [2–5].

Similarly, it is important to note that MMA is not the only major toxic metabolite in methylmalonic acidemia. In fact, patients with methylmalonic acidemia also presented elevated levels of malonic acid (MA) [2, 6]. Involvement of MA has

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been reported in mitochondrial dysfunction, superoxide radical generation, secondary excitotoxicity mediated by Ca^{2+} influx and reactive species-induced apoptosis [7, 8].

In the stated context, antioxidants are thought to play a pivotal role in preventing MMA and MA oxidative activity. Accordingly, there is a growing interest in the use of natural antioxidants, including polyphenols found in medicinal and dietary plants that might prevent damage associated with such conditions [9–14].

In view of the above, *Ilex paraguariensis* (IP), a plant from the aquifoliaceae family, possess antioxidant, anti-inflammatory, antimutagenic, antiobesity, antidiabetic, diuretic, antifungal, stimulant and antidepressant-like activities [15–17]. Biochemical characterization of IP, revealed the presence of a diverse range of chemical compounds like polyphenols, methylxanthines, caffeoyl derivatives, saponins and minerals, which may be responsible for its biological efficacy [11, 17]. Mechanistically, IP acts via scavenging of reactive species (RS) and modulating the expression of genes and antioxidant enzymes [17]. However, the modulation of oxidative stress in MMA or MA-induced toxicity by aqueous extract of IP remains to be explored.

Considering the necessity to counteract oxidative stress damage associated to methylmalonic acidemia (a condition in which MMA or MA accumulation occurs), the development of simple animal models is profoundly needed. Accordingly, invertebrate organisms such as *Drosophila melanogaster* (DM) emerged as useful animal model for the study of molecular mechanisms involved in human neuronal dysfunction [18, 19]. It can be attributed to the simplicity of its nervous system, rapid life cycle, easy genetic manipulation and sequenced genome.

The present study was designed to not only explore the involvement of oxidative stress in MMA as well in MA-induced toxicity but also to test the hypothesis that supplementation with the aqueous extract of IP could exert protective effect against changes associated to both agents in *Drosophila melanogaster*. In this context, we designed for the first time a short-term dietary regimen model, where DM was exposed to different concentrations of MMA, MA and/or IP. Thereafter, we evaluated several behavioral and biochemical determinations such as mortality and locomotor deficit, superoxide dismutase (SOD), catalase, acetylcholinesterase (AChE) activities, MTT and resazurin reduction capacity, non protein thiol (NPSH) contents and TBARS levels in head and entire body homogenates. Using HPLC, we also characterized the IP extract, which may give us significant insights about its major phytochemical constituents.

Materials and Methods

Chemicals

Malonic Acid (MA), Methylmalonic acid (MMA), Thiobarbituric acid (TBA), acetylthiocholine iodide, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), quercetin, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 2,4-dinitrophenyl hydrazine (DNPH), gallic acid, catechin, chlorogenic acid, caffeic acid, caffeine, epigallocatechin, rutin, quercetin and kaempferol were acquired from Sigma Chemical Co. (St. Louis, MO, USA). Methanol and acetic acid were purchased from Merck (Darmstadt, Germany). All the other chemicals were commercial products of the highest purity grade available.

Plant Material and Extract Preparation

Ilex paraguariensis marketed and distributed by Ponche Verde Industrial do Mate Ltda. – Arvorezinha/RS/Brazil was obtained from local commercial source. Accordingly, an independent batch (#07) was randomly purchased and used in this study. The aqueous extract was prepared as infusion immediately before use as described previously [11]. Accordingly, 1 g of the IP sample was placed in 10 mL (in a proportion of 10 g/100 mL) of distilled hot water (96 °C) for 10 min and cooled at room temperature (20–25 °C). Thereafter, extracts were diluted in distilled water for subsequent use.

Chromatographic Analyses

High performance liquid chromatography (HPLC–DAD) was performed following the method described previously [20], with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software.

In short, IP aqueous extract at a concentration of 10 mg/mL were injected by means of a model SIL-20A Shimadzu Auto sampler. Separations were carried out using Phenomenex C_{18} column (4.6 mm × 250 mm × 5 μm particle size). The sample and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. The mobile phase was water with 0.5% acetic acid (v/v) (solvent A) and HPLC grade methanol (solvent B) at a flow rate of 0.6 mL/min and injection volume 40 μL. The composition gradient was: 5% solvent B reaching 15% at 10 min; 30% solvent B at 25 min, 65% solvent B at 40 min and 98% solvent B at 45 min, followed by 50 min at

isocratic elution until 55 min. At 60 min the gradient reached the initial conditions again. In order to verify if plant antioxidants and the organic acids (MA or MMA) are making complexes in the media, HPLC fingerprinting of the aqueous extract of IP in the presence of MMA (5 mM) or MA (5 mM) was also carried out (Data not shown).

Stock solutions of standards references were prepared in the methanol at a concentration range of 0.025–0.400 mg/mL. Quantifications were carried out by integration of the peaks using the external standard method, at 254 nm for gallic acid; 280 nm for catechin, epigallocatechin and caffeine; 327 nm for chlorogenic acid and caffeic acid; and 366 for quercetin, kaempferol and rutin. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to 700 nm). All chromatography operations were carried out at ambient temperature and in triplicate.

Drosophila Culture Condition

DM wild-type (Harwich strain) were obtained from the National Center species, Bowling Green, Ohio, USA. The flies were maintained and reared on corn meal medium (1% w/v brewer's yeast, 1% w/v sucrose, 1% w/v powdered milk, 1% w/v agar, and 0.08% v/w methylparaben) at constant temperature and humidity (22 ± 1 °C; 60% relative humidity, respectively) under 12 h dark/light cycle conditions. Flies were maintained in this medium during developmental period until treatment (1–3 days old). Thereafter flies were transferred to "treatment medium" containing agar, sucrose 1% and the respective treatment (see details on 2.5. MMA or MA exposure and treatment with IP section). All the experiments were carried out with the same fly strain.

MMA or MA Exposure and Treatment with IP

Fifty flies (1–3 days old; both genders) were exposed to each different MA or MMA concentrations (1–10 mM) and evaluated each 24 h for the longevity, until day 15. The medium were replaced each 48 h. This protocol was repeated at least ten times, and was carried out at room temperature and in quadruplicate. Of particular importance, 48 h of exposure at 5 mM of both organic acids were chosen for the biochemical tests since in these conditions, mortality was significantly enhanced by both MMA and MA treatments.

Fifty flies (1–3 days old) were also exposed to each different extract IP concentrations (63–500 µg/mL) and evaluated each 24 h for the longevity, until day 15. The medium were replaced each 48 h. This protocol was repeated at least four times, and was carried out at room temperature and in quadruplicate. Of note, IP (250 µg/mL) was chosen to subsequent experiments once that no mortality and no significant behavioral changes were found at this concentration, and also because it was the lowest concentration capable of preventing the behavioral changes associated to both MMA (5 mM) or MA (5 mM) exposure during 48hs (Data not Shown).

Fifty flies (1–3 days old; both genders) were also exposed to MA (5 mM) or MMA (5 mM) and/or IP (250 µg/mL) and evaluated each 24 h for the longevity, until day 15 (the medium were also replaced each 48 h). For subsequent set of experiments, DM (both genders) of 1–3 days old were divided as follow: (1) control (no treatment) (2) IP 250 µg/mL (3) MA (1 or 5 mM) (4) MA (1 or 5 mM) + IP (250 µg/mL) (5) MMA (1 or 5 mM) and (6) MMA (1 or 5 mM) + IP (250 µg/mL). Flies were exposed a diet containing MA, MMA and/or IP for 48 h for mortality, behavioral and biochemical assays. This treatment protocol was independently repeated, at least 4 times and in triplicate, to each assay.

In Vivo Assays

Negative Geotaxis

Locomotor ability of flies was performed with a negative geotaxis assay as described previously [21]. In short, flies (15 per group from each independent experiment) were sorted under a brief ice anesthesia and placed in a vertical glass column (length: 10 cm, diameter: 1.5 cm/ 10 flies each). After the recovery from cold exposure (approximately 15 min) the flies were gently tapped to the bottom of the column. The flies that reached the top of the column and the flies that remained at the bottom were counted separately during 6 s. The scores represent the mean of the numbers of flies at the top (ntop) as percentage of the total number of flies (ntot). The values represent the mean of five independent experiments. The results are expressed as percentage of flies that escaped beyond a minimum distance of 6 cm in 6 s during four independent experiments. Data are expressed as a % of flies that reach the top in 6 s.

Open-Field Test

Open-field test was performed according to the method described previously [22]. Accordingly, three flies from each group were kept in an arena divided by squares (1 cm × 1 cm) measuring 9 cm of diameter, which can be covered by petri dish. The fly's activity were recorded with a video camera and the number of squares crossed by each single fly, during a given time-window (30 s), was analyzed. The values represent the mean of five independent experiments.

Ex Vivo Assays

Homogenate Preparation

At the end of the treatment period (48 h), flies were anesthetized in ice. Heads were separated from the body using a sharp blade/cutter. Afterward, heads and bodies were homogenized in 0.9% NaCl solution, 1:5 (flies/volume; i.e. 50 heads or 50 bodies to 250 µL). The homogenates were centrifuged at 2500×*g* for 10 min at 4 °C, and the supernatant was used for biochemical assays. All biochemical determinations were performed in duplicates in 3–5 independent experiments.

Determination of TBARS

The lipid peroxidation end products were quantified as thiobarbituric acid reactive substances (TBARS) according to established previously [23] with some modifications. In brief, an aliquot of homogenate (100 µL) was incubated per 1 h at 37 °C in a water bath. Thereafter samples were incubated at 100 °C for 120 min in 200 µL of a medium containing equal volumes of trichloroacetic acid (10%, w/v) and thiobarbituric acid (0.6%, w/v) in 0.1 M HCl for color development. After boiling step, 20 µL 8.1% SDS was added. The reaction product was determined at 532 nm and the results were expressed as % of control after correction by the protein content.

Determination of Non-protein Thiol (NPSH)

The NPSH level was determined in the control and treated flies according to the method previously described [24]. For NPSH assay, 70 µL homogenates were precipitated with 70 µL TCA 10% (1:1 v:v) followed by centrifugation at 3000×*g* for 10 min at 4 °C. The reaction system was made up of 940 µL of 1 M dibasic potassium phosphate buffer, 50 µL of sample, and 10 µL of 5 mM DTNB. At the end of 10 min incubation at room temperature (25 °C), the absorbance was measured at 412 nm. The results were expressed as % of control after correction by the protein content.

Activity of Superoxide Dismutase (SOD)

The assay consists in the inhibition of superoxide driven oxidation of quercetin by SOD [25]. Briefly, the reaction medium contained 200 µL of buffer (160 µL 0.02 M phosphate buffer/0.08 mM EDTA (pH 7.4) mixed with 200 µL TEMED), 10 µL of 0.05 µM quercetin and 40 µL of sample (1:5 dilution). The reaction was analyzed in time 0 and 20 min at 406 nm. The results expressed as % of control after correction by the protein content.

Activity of Catalase

The catalase activity was measured spectrophotometrically according to the method of [26], by monitoring the disappearance of H_2O_2. Briefly, the reaction medium contained 960 µL of 0.05 M phosphate buffer (pH 7.0), 20 µL of 1 M H_2O_2, and 20 µL of sample (1:5 dilution). The reaction was analyzed for 2 min (15 s intervals) at 240 nm. Results were expressed as percentage of control after correction by the protein content.

Activity of Acetylcholinesterase (AChE)

AChE activity was determined according previously determined [26]. Briefly, the assay medium consisted of 70 µL of distilled water, 100 µL of system (0.5 M potassium phosphate buffer (pH 7.4) with 10 mM DTNB), 10 µL of sample and 20 µL of 8 mM acetylthiocholine as substrate. The degradation of acetylthiocholine iodide was analyzed for 2 min (30 s intervals) at 412 nm. The results were expressed as % of control after correction by the protein content.

MTT and Resazurin Reduction Assays

Dehydrogenases activity was measured by two different methods. Firstly, its activity was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reduction assay described previously [22]. Briefly, an aliquot of sample (50 µL) was incubated during 30 min (37 °C) with 20 µL MTT and 5 mM succinate. Thereafter, reaction was stopped by addition of 200 µL DMSO and incubated per 30 min (37 °C) to dissolve formazan salts. Then, the samples were centrifuged to 2000 rpm for 5 min, and the absorbance was monitored at 630 nm and 545 nm. To calculate the MTT reduction was used: $|545|\text{nm} - |630|\text{nm} = \text{Result} \times 100$. The results were expressed as % of control after correction by the protein content.

The second method used was the resazurin reduction assay which was performed as previously described [27]. In short, 20 flies were homogenized in 1 mL 20 mM Tris buffer (pH 7.0) and centrifuged at 2000 rpm for 10 min at 4 °C. After that, the supernatant was incubated in ELISA plates with 20 mM buffer Tris (pH 7.0) and resazurin for two hours. Fluorescence was recorded using EnsPireR multimode plate reader (Perkin Elmer, USA) at $_{ex}579$ nm–$_{em}584$ nm.

Protein Determination

The protein content was determined as described previously using bovine serum albumin (BSA) as standard [28].

Statistical Analysis

Behavior parameters were analyzed using Kruskal–Wallis (kw) followed by Dunn's multiple comparisons test when appropriate, once Kolmogorov–Smirnov's test indicated the absence of homogeneity of variance. Survival data were evaluated using Kaplan–Meier analysis and Gehan–Breslow–Wilcoxon test. Other data were analyzed by one-way ANOVA followed by Tukey's multiple range tests when appropriate. Differences between groups were considered significant when $P < 0.05$. Data of non-parametric analysis are represented as box and whisker plots (min to max); and data of parametric analysis as means and S.E.M. Graphics were created using Graph Pad Prisma 6.0.

Results

Phytochemical Composition

HPLC fingerprinting of the aqueous extract of IP revealed the presence of a series of major and minor components. We have confirmed gallic acid ($t_R = 9.83$ min, peak 1), catechin ($t_R = 15.07$ min, peak 2), chlorogenic acid ($t_R = 24.01$ min, peak 3), caffeic acid ($t_R = 26.54$ min, peak 4), caffeine ($t_R = 28.17$ min, peak 5), epigallocatechin ($t_R = 34.20$ min, peak 6), rutin ($t_R = 38.91$ min, peak 7), quercetin ($t_R = 49.76$ min, peak 8) and kaempferol ($t_R = 60.11$ min, peak 9) as shown in Fig. 1 and Table 1.

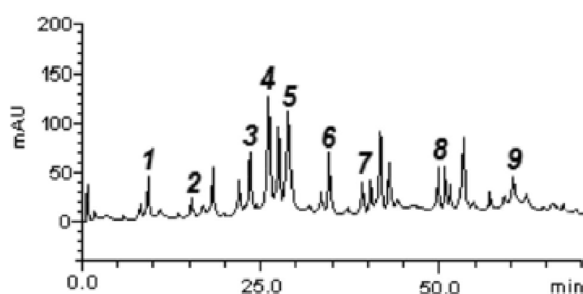

Fig. 1 Representative high performance liquid chromatography profile of IP extract. Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), caffeine (peak 5), epigallocatechin (peak 6), rutin (peak 7), quercetin (peak 8) and kaempferol (peak 9)

Table 1 Components of *Ilex paraguariensis*

Compounds	*I. paraguariensis* mg/g
Gallic acid	1.43 ± 0.02
Catechin	0.51 ± 0.05
Chlorogenic acid	2.26 ± 0.01
Caffeic acid	5.57 ± 0.01
Caffeine	5.08 ± 0.02
Epigalloca.techin	2.39 ± 0.03
Rutin	1.17 ± 0.01
Quercetin	1.42 ± 0.04
Kaempferol	1.09 ± 0.01

Results are expressed as mean ± standard deviations (SD) of three determinations

Effect of Treatments on Behavioral Profile in DM

Effect of IP Extract on MMA or MA Caused Mortality

Gehan–Breslow–Wilcoxon test from Kaplan–Meier survival curve revealed a significant difference among groups from MMA (Fig. 2a), MA (Fig. 2b), or IP (Fig. 2c) and controls. A significant effect of IP against MMA (Fig. 2d) or MA (Fig. 2e) toxicity was also found.

The survival rate in the groups exposed to MMA for 15 consecutive days was as follow: Control (34%), MMA 1 mM (5.5%), MMA 5 mM (3.62%) and MMA 10 mM (2.5%). Accordingly, MMA at all concentrations significantly increase fly mortality as compared to control. In turn, the median survival was as follow (in days): Control (11.5 days), MMA 1 mM (8 days), MMA 5 mM (6.5 days) and MMA 10 mM (7 days) (Fig. 2a).

The survival rate in the groups exposed to MA for 15 consecutive days was as follow: Control (33.5%), MA 1 mM (0%), MA 5 mM (0%) and MA 10 mM (0%). Accordingly, MA at all concentrations significantly increase fly mortality as compared to control. In turn, the median survival was as follow (in days): Control (11.4 days), MA 1 mM (6 days), MA 5 mM (6 days) and MA 10 mM (6 days) (Fig. 2b).

The survival rate in the groups exposed to IP for 15 consecutive days was as follow: Control (34.7%), IP 63 µg/mL (48.75%), IP 125 µg/mL (52.4%), IP 250 µg/mL (54.3%) and IP 500 µg/mL (55.5%). Accordingly, IP at 250 and 500 µg/mL significantly decrease spontaneous fly mortality. In turn, the median survival was as follow (in days): Control (11.6 days), IP 63 µg/mL (15 days), IP 125 µg/mL (15 days), IP 250 µg/mL (> 15 days) and IP 500 µg/mL (> 15 days) (Fig. 2c).

IP (250 µg/mL) protected against MMA (5 mM) induced fly mortality (Fig. 2d). The survival rate in the groups exposed to MMA and/or IP for 15 consecutive days was as

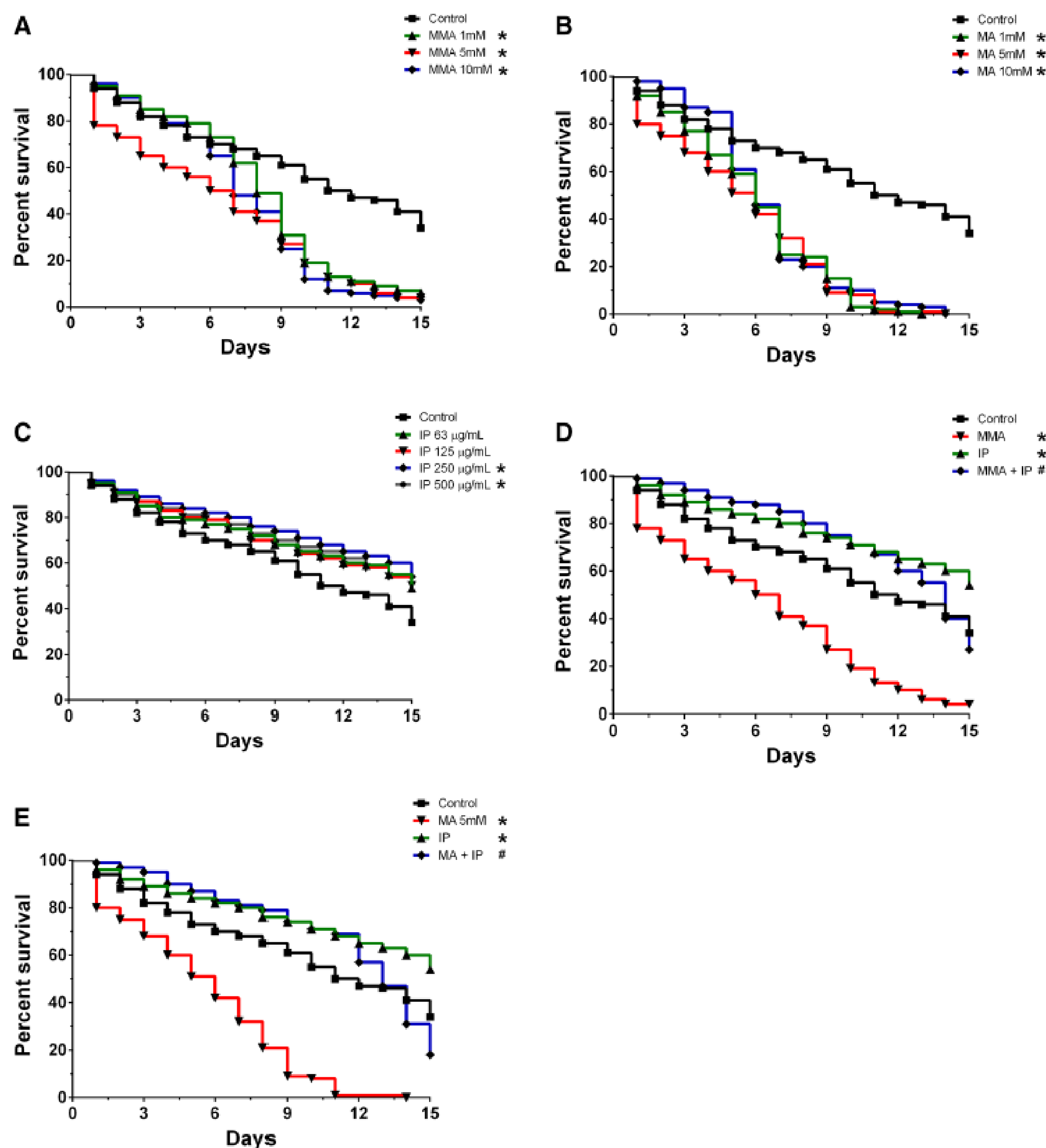

Fig. 2 **a** Effect of MMA (1–10 mM) on the survival rate of treated flies. **b** Effect of MA (1–10 mM) on the survival rate of treated flies. **c** Effect of IP (63–500 µg/mL) on the survival rate of treated flies. **d** Effect of IP (250 µg/mL) and/or MMA (5 mM) on the survival rate of treated flies. **e** Effect of IP (250 µg/mL) and/or MA (5 mM) on the survival rate of treated flies. Data were collected every 24 h for each group during 15 days and analyzed using a Kaplan–Meier survival plot. The total number of flies (200 per group) represents the sum of four independent experiments. The numbers of dead flies are represented as % of control. Statistical differences among groups were calculated by log-rank (Gehan–Breslow–Wilcoxon) test (*) represent significant difference of the respective curve as compared with the curve of control group. (#) represents significant difference between curves of MA vs. MA + IP or significant difference between curves of MMA vs. MMA + IP. Values are expressed as percentage

follow: Control (33.9%), MMA (2.65%), IP (50.06%) and MMA + IP (27%). In turn, the median survival was as follow (in days): Control (11.5 days), MMA (6.5 days), IP (> 15 days) and MMA + IP (14 days).

IP (250 µg/mL) also protected against MA (5 mM) induced fly mortality (Fig. 2e). The survival rate in the groups exposed to MA and/or IP for 15 consecutive days was as follow: Control (33.9%), MA (0%), IP (50.06%) and MA + IP (17.67%). In turn, the median survival was as follow (in days): Control (11.7 days), MA (6 days), IP (> 15 days) and MA + IP (13 days).

Effect of IP Extract on MMA or MA Induced Alterations in Negative Geotaxis and Open-Field Tests

The climbing behavior of flies in MMA and MA treated groups was significantly lower as compared to control group both at 1 mM (Fig. 3a; a condition in which no lethal toxicity was found) and at 5 mM (Fig. 3c). The effect of either MMA or MA was completely abolished by IP treatment. Additionally, no significant difference was observed in IP and control group.

In open-field test, Dunn's multiple comparisons test revealed that the flies treated with MMA and MA presented a significant increase in the number of crossings when compared to the control group both at 1 mM (Fig. 3b; a condition in which no lethal toxicity was found) and at 5 mM (Fig. 3d). Likewise on negative geotaxis test, the effect of MMA and MA was completely abolished by IP treatment. Statistically no significant difference in IP and control group was noted.

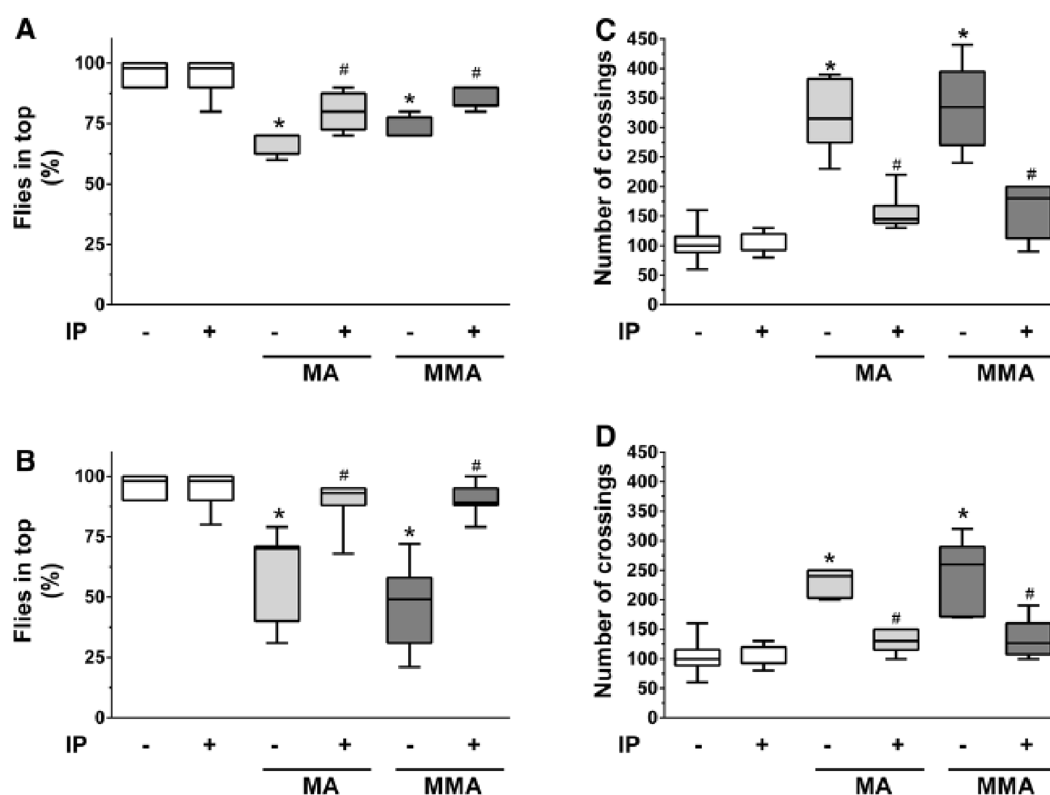

Fig. 3 **a** Effect of IP (250 µM/mL), MMA (1 mM) and MA (1 mM); and **b** Effect of IP (250 µM/mL), MMA (5 mM) and MA (5 mM) on geotaxis response (climbing). **c** Effect of IP (250 µM/mL), MMA (1 mM) and MA (1 mM); and **d** Effect of IP (250 µM/mL), MMA (5 mM) and MA (5 mM) on locomotor activity evaluated by open-field test. Data were collected after 48 h of exposure. The total number of flies (75 per group in negative geotaxis and 15 per group in open-field) represents the sum of five independent experiments. Values are expressed as median and range (interquartile interval). (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA + IP or MMA and MMA + IP (Kruskal–Wallis test followed Dunn' multiple comparisons test, P < 0.05)

Effect of Treatments on Ex Vivo Biochemical Analyses in DM

Effect of IP Extract on TBARS and NPSH Levels in MA or MMA Treated Flies

Tukey's multiple comparisons test revealed that treatment with MMA and MA caused a significant increase in TBARS levels and NPSH content as compared with control group. In fact, the increase was observed both in the head (Figs. 4a, 5a) and body regions (Figs. 4b, 5b), respectively. Of particular importance, the effect of MMA and MA on TBARS levels was normalized by IP treatment both in head and in body (Fig. 4a, b) regions, respectively. Similarly, the NPSH levels were also normalized in head and body (Fig. 5a, b) regions, respectively. Finally, there was no significant difference in

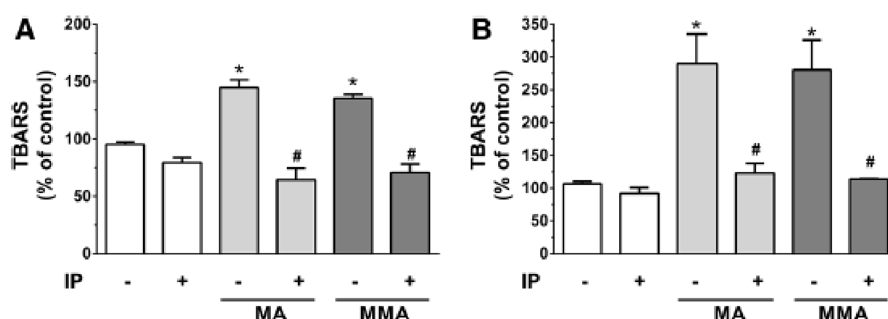

Fig. 4 Effect of IP on TBARS in **a** head and **b** body of flies exposed to MMA or MA. (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA + IP or MMA and MMA + IP ($P < 0.05$). (One-way ANOVA followed by Tukey's multiple range tests). Values are expressed as mean ± S.E.M. (n = 4)

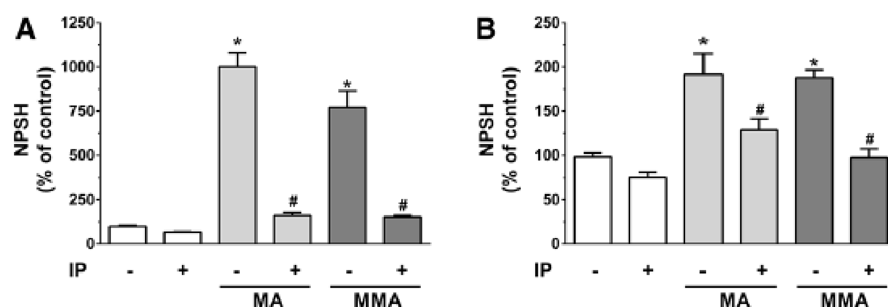

Fig. 5 Effect of IP on NPSH content in **a** head and **b** body of flies exposed to MMA or MA. (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA + IP or MMA and MMA + IP ($P < 0.05$). (One-way ANOVA followed by Tukey's multiple range tests). Values are expressed as mean ± S.E.M. (n = 5)

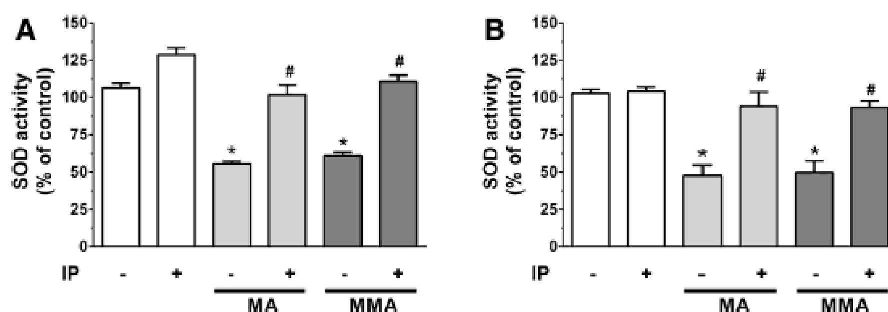

Fig. 6 Effect of IP on SOD activity in **a** head and **b** body of flies exposed to MMA or MA. (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA + IP or MMA and MMA + IP ($P < 0.05$). (One-way ANOVA followed by Tukey's multiple range tests). Values are expressed as mean ± S.E.M. (n = 4)

TBARS (Fig. 4) and NPSH (Fig. 5) levels, in IP (alone) and control group.

Effect of IP Extract on SOD, Catalase and AChE Activities in MA or MMA Treated Flies

Treatment with MMA and MA caused a significant decrease in SOD, catalase and AChE activities, both in head (Figs. 6a, 7a, 8a) and body (Figs. 6b, 7b, 8b) regions, respectively. Treatment with IP normalized SOD (Fig. 6a, b), catalase (Fig. 7a, b), and AChE activities (Fig. 8a, b) in both MMA and MA treated groups. Statistically, no significant alteration was noted in SOD (Fig. 6), catalase (Fig. 7) or AChE (Fig. 8) activities in IP (alone) and control group.

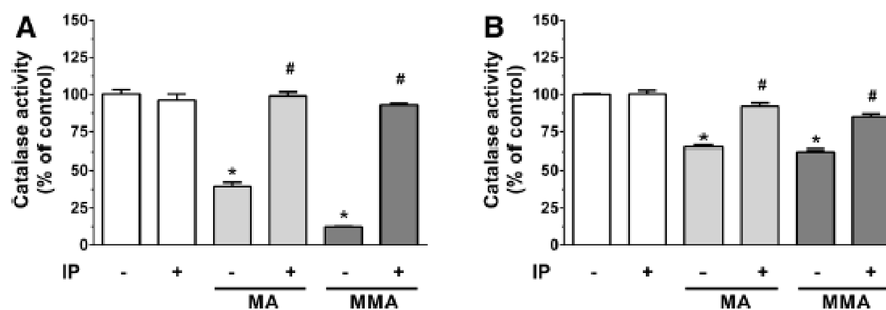

Fig. 7 Effect of IP on catalase activity in **a** head and **b** body of flies exposed to MMA or MA. (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA + IP or MMA and MMA + IP ($P < 0.05$). (One-way ANOVA followed by Tukey's multiple range tests). Values are expressed as mean ± S.E.M. (n = 4)

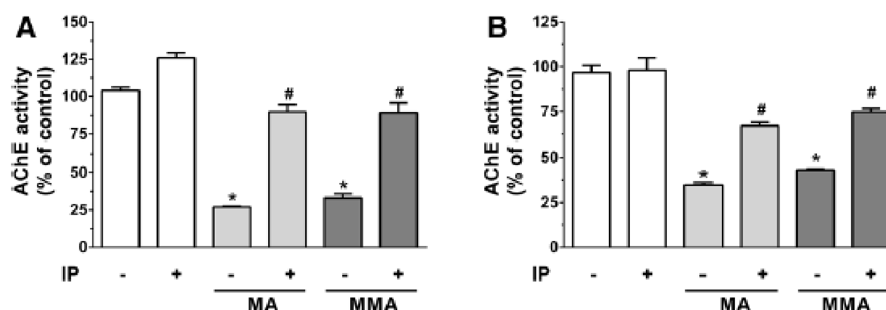

Fig. 8 Effect of IP on AChE activity in **a** head and **b** body of flies exposed to MMA or MA. (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA + IP or MMA and MMA + IP ($P < 0.05$). (One-way ANOVA followed by Tukey's multiple range tests). Values are expressed as mean ± S.E.M. (n = 5)

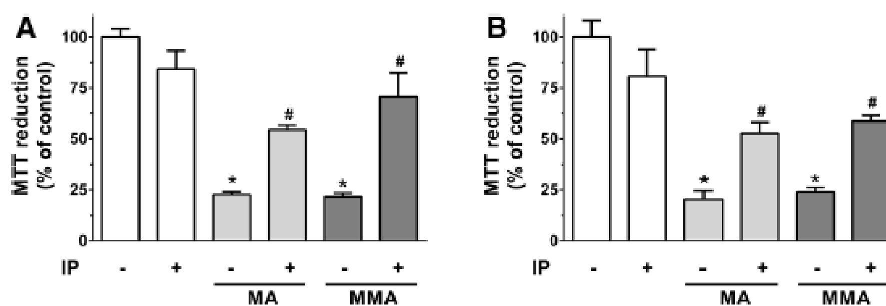

Fig. 9 Effect of IP on MTT reduction in **a** head and **b** body of flies exposed to MMA or MA. (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA + IP or MMA and MMA + IP ($P < 0.05$). (One-way ANOVA followed by Tukey's multiple range tests). Values are expressed as mean ± S.E.M. (n = 5)

Effect of IP Extract on MTT and Resazurin Reduction Assays in MA or MMA Treated Flies

The Fig. 9 represents the MTT reduction assay. Exposure to MMA and MA caused a significant decrease in MTT reduction, both in head (Fig. 9a) and body (Fig. 9b) respectively, which was partially normalized by IP treatment. No significant change in MTT reduction assay was noted in IP (alone) and control group (Fig. 9). The Fig. 10 represents the resazurin reduction assay. Exposure to MMA caused a significant decrease in resazurin reduction in head (Fig. 10a), whereas MA exposure resulted in a significant decrease in resazurin reduction in body (Fig. 10b). Treatment with IP normalized resazurin reduction in both MMA (Fig. 10a) and MA (Fig. 10b) treated groups. No significant change in resazurin reduction assay was noted in IP (alone) and control group (Fig. 10).

Discussion

Contributing to the scientific literature, this is the first report to not only describe the adverse effects of MMA and MA but also the protective efficacy of the aqueous extract of IP in DM model. One of the highlights in present study was that treatment with MMA and MA caused a time dependent increase in the cumulative number of dead flies. The MMA or MA induced mortality could be attributed to the cytotoxic effect of both drugs which has been previously reported. Involvement of oxidative stress, mitochondrial dysfunction, neuroinflammation and apoptotic processes are some of the reported mechanisms of their (MMA and MA) induced toxicities in mammals [29, 30].

Locomotor activity is a complex behavior that may be influenced by different neural systems in flies [31]. Of particular importance, it may be altered by toxicants [11, 12, 22, 26]. Here, in order to accurately evaluate behavioral changes, we choose either a condition in which the mortality was not significantly enhanced by MMA or MA treatment (i.e. at MMA or MA 1 mM; 48 h) and a condition in which the mortality was significantly enhanced by MMA or MA treatment (i.e. at MMA or MA 5 mM; 48 h). Accordingly, we found that either MMA or MA—at both concentrations—significantly impaired the performance of flies in the negative geotaxis assay (Fig. 3a, b). Furthermore, MMA and MA (either at 1 and 5 mM) significantly increased the number of crossings in the open-field test (Fig. 3c, d). Although, we cannot directly elaborate the mechanism (s) by which MMA and MA lead to changes in locomotor performance, this is the first report where we described such alterations in an invertebrate model (DM). Notably, nicotine-exposed flies presented reduced climbing activity that was associated to locomotor hyperactivity [32–35]. Of note, in the negative geotaxis, the nicotine effect was found to be mediated by dopaminergic system and cyclic adenosine monophosphate (cAMP)/cAMP-response element binding (CREB) pathway [32, 34]. In turn, dopaminergic neurons and a protein in the decapping complex (decapping protein 2) were found to play crucial roles in mediating nicotine-induced locomotor hyperactivity [33, 35]. So, considering that different pathways seems to be involved in the control of locomotor activity in flies, we suggest that MMA or MA could act by interfering with some of that, which deserves further investigation.

Dietary supplementation with aqueous extract of IP significantly reduced the MMA or MA mediated toxicity. IP not only decreased the mortality rate but also ameliorated the behavioral alterations associated with both drugs. Our results are strongly supported by an earlier report, where IP significantly extended lifespan and increased ability to resist to environmental stresses in flies [36], and also protected against paraquat induced mortality in *Caenorhabditis elegans* [37]. Importantly, caffeic acid, galic acid and epigallocatechin (some of the major content of IP) increased the lifespan and restored the impaired movement activity induced by paraquat in DM [38]. IP extract was also found to improve the locomotor deficits induced by the treatment with reserpine and MPTP in rodents, which was, at least in part, attributed to caffeine present in the extract [39]. Previous report also suggested that some polyphenols in IP extract are able to enhance GABAergic activity [40]. Therefore, the involvement of GABAergic pathway in IP effect cannot be denied.

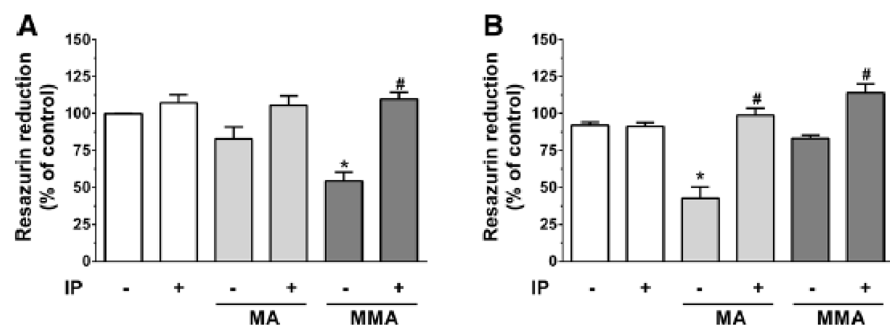

Fig. 10 Effect of IP on resazurin reduction in **a** head and **b** body of flies exposed to MMA or MA. (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA + IP or MMA and MMA + IP ($P < 0.05$). (One-way ANOVA followed by Tukey's multiple range tests). Values are expressed as mean ± S.E.M. ($n = 5$)

In an attempt to better understand the putative biochemical mechanism(s) of MMA and MA induced toxicity, we performed a series of assays to address the involvement of mitochondria and oxidative stress in both head and body regions of the treated flies. It is known that exposure to both MMA and MA can significantly influence mitochondrial dysfunction, increase TBARS, reactive species, total protein carbonylation and decrease in sulfhydryl content in mammals [2, 4, 5, 41, 42]. However, to the best of our knowledge, there is no data available about the effects of MMA or MA on flies. Thus, we observed that exposure to MMA and MA caused significant increase in TBARS levels, decreased SOD and catalase activities and also decreased the ability to reduce MTT and resazurin, either in head and body of treated flies. In strong contrast to above, treatment with MMA and MA resulted in increased NPSH levels in head and body regions, which may represent a primary compensatory response to oxidative insults.

Moreover, acute exposure to MMA and MA caused a significant decrease in AChE activity. Although, we cannot describe the exact mechanism by which MMA or MA decreased the enzymatic activity or their interference in cholinergic system, there are some reports which stated that MA can cause destruction of the basal forebrain cholinergic neurons [43]. In rats with renal failure treated to gentamicin, MMA was also found to increase susceptibility of activation of brain AChE, suggesting the involvement of cholinergic system on MMA toxicity [44].

Particularly important, dietary supplementation with IP extract significantly normalized all parameters (TBARS levels, SOD and catalase activity, NPSH levels and MTT or resazurin involved assay), thus suggesting the protective effect of IP against MMA or MA-induced mitochondrial impairment and/or oxidative stress in the treated flies. Notably, both caffeic acid and caffeine (two of major metabolites present in IP extract) were previously found to present antioxidant activity as well as to protect against mitochondrial dysfunction [45].

In order to explore some of the major constituents of IP, which may have a potential role against the MMA and MA induced damages, we performed HPLC analysis. As shown in (HPLC Fig. 1; Table 1), the IP extract contains gallic acid, catechin, chlorogenic acid, caffeic acid, caffeine, epigallocatechin, rutin, quercetin and kaempferol with concentrations ranging from 5.57 to 0.51 mg/g (for different constituents). In the present model we observed that dietary supplementation with IP significantly restored behavioral and biochemical parameters. Of particular importance, HPLC spectra of plant extract, as well as the UV–vis spectra, revealed that neither MA nor MMA were able to interact with the plant constituents, supporting the notion that plant antioxidants and the organic acids (MA or MMA) are not making complexes in the media (Data not Shown). So, we can assume that phenolic composition of IP extract may offer significant protection against MMA and MA induced damages.

In summary, IP protected against MMA and MA-induced toxicity in DM via reduction in mortality, inhibition of oxidative stress indices and maintenance of mitochondrial function. Moreover, dietary IP supplementation was associated with neuroprotection characterized by improvement in the AChE activity and locomotor function in flies. These observations highlights that IP extract may be a promising candidate against acute MMA or MA induce toxicity.

Conclusion

In fact, the use of IP can be of pharmacological importance because of the ease of availability and also the presence of different compounds that may have synergistic effects when combined. Additionally, our data reinforce the importance of DM model to toxicological studies and, based on presented data, to explore new therapeutic strategies to counteract MMA or MA toxicity under conditions where they may be present and/or causing deleterious effects, such as methylmalonic acidemia. However, additional studies are needed to understand the exact metabolic and neurological pathways involved in the protective role of IP.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there is no conflict of interest.

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4.2 Manuscrito: Ácido cafeico e a cafeína atenuam a toxicidade associada à exposição aos ácidos malônico ou metilmalônico em *Drosophila melanogaster*

Manuscrito a ser submetido para publicação no Periódico Industrial Crops and Products

Caffeic acid and caffeine attenuates the toxicity associated to malonic- or methylmalonic acids exposure in *Drosophila melanogaster*

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Abstract

We found recently that supplementation with *Ilex paraguariensis* aqueous extract offered protection against toxicity associated to methylmalonic (MMA) or malonic (MA) acids exposure in an invertebrate model (*Drosophila melanogaster*). Caffeic acid (CA) and/or caffeine (CAF), were the two of the major phytochemicals found in our *Ilex paraguariensis* crude extract. So, we used here both CA and/or CAF to test the hypothesis that supplementation with the isolated compounds (alone or combined) could also exert protective effect against MMA or MA induced toxicity *Drosophilas.* Therefore, flies were exposed to MA (5 mM) or MMA (5 mM) and concomitantly treated with CA (1.39 µg/mL) and/or CAF (1.27 µg/mL) during 4 days for survival, behavioral and/or biochemical assays. CAF treatment completely abolished mortality associated to either MMA or MA exposure, whereas CA treatment only protected against MA-induced mortality. CA and/or CAF completely abolished behavioral changes, as well as completely protect against changes in non-protein thiol (NPSH) content, thiobarbituric acid reactive substances (TBARS) levels, catalase (CAT) activity and MTT reduction ability, associated to MA or MMA exposure both in head and in body regions. However, CA and/or CAF failedto protect from changes in acetylcholinesterase (AChE) activity associatedto MA or MMA. Of note, CA and/or CAF significantly decreased AChE activity *per se*. Furthermore, CA was not able to protect against MA or MMA-induced changes in superoxide dismutase (SOD) activity, whereas CAF only restored SOD activity in head region of flies exposed to MA. CA and/or CAF also completely abolished the inhibitory effect of MMA on glutathione-S-transferase (GST) activity in body region of flies. Finally, CA and/or CAF were effective to protect from decrease in glucose and triglyceride levels associated to both MA and MMA exposure in hemolymph; however, CA and/or CAF have almost no effect on these parameters inthe carcasses. Together, our data confirm the hypothesis that supplementation with CA and/or CAF offers protection against detrimental changes associated to MMA or MA exposure in flies, being responsible, at least in part, by the protective effect of *I. paraguariensis* crude extract reported previously.

Keywords: Oxidative stress; behavioral changes; neurotoxicity; anticholinesterase.

Introduction

Methylmalonicacidemia is an inherited disorder resulting from the deficiency in the activity of the mitochondrial enzyme methylmalonyl-CoA mutase (MCM, EC 5.4.99.2), in which the affected subjects are unable to metabolize certain molecules properly. This condition is associated to accumulation of methylmalonic acid (MMA)[1-3]. However, part of the pathophysiology of this disease can be associated to other organic acids, including malonic acid (MA) [1, 4, 5]. From a mechanistic point of view, besides not completely understood, there are evidences that the exposures to MMA or MA are associated to oxidative stress and mitochondrial dysfunction [1-3, 5-10]. Importantly, brain accumulations of MMA or MA are also associated to neurodegeneration [1, 5, 8].

Considering the lack of effective treatment to counteract toxicity associated to MMA or MA, therapeutic strategies aimed to prevent or delay oxidative stress and/or mitochondrial dysfunction represent a reasonable choice [10-12]. Accordingly, there is a growing interest in the use of phytochemicals that might prevent damage associated with such conditions[13-17]. In line with this, we recently found that *Ilex paraguariensis* aqueous extract (crude extract) offers protection against MMA-or MA-induced toxicity in Drosophila model. Of note, the major phytoconstituents found in our *I. paraguariensis* extract were caffeic acid (CA) and caffeine (CAF)[13].

Caffeic acid (CA) (3,4-dihydroxycinnamic acid) is phenolic compound widely found in fruits, vegetables and plants [18]. Notably, it was previously reported that CA exhibit a variety of pharmacological activities including antioxidant and anti-inflammatory [19-22]. Particularly important, CA is a promising compound for treating neurodegenerative disorders and other conditions [19, 23-25].

Similarly, caffeine (CAF) (1,3,7-trimethylxanthine) is a alkaloid found in a variety of plants [26]. It was previously reported that CAF also exhibit a variety of pharmacological properties including antioxidant, anti-inflammatory and anti-apoptotic [27]. Importantly, previous studies have also reported that CAF exerts neuroprotective effects against various neurodegenerative disorders, is a potent reactive oxygen species (ROS) scavenger, and improves cognition in the rat brain by altering the endogenous antioxidant system[28, 29].

Considering the usefulness of *Drosophila melanogaster* model to test strategies to counteract the damage associated to exposure to different toxic agents[13, 15, 17, 30, 31], we performed this study to test the hypothesis that treatment with CA and/or CAF would result in protective effect against mortality, behavioral and/or biochemical changes associated to MA or MMA exposure in flies. Therefore, we evaluated the mortality and locomotor deficit following short-term dietary regimen. Furthermore, using the body and head region of flies, we evaluated some biochemical markers of toxicity such as acetylcholinesterase (AchE), catalase (CAT), superoxide dismutase (SOD) and glutathione-S-transferase (GST) activities, MTT reduction capacity, as well as levels of non-protein thiol (NPSH) and thiobarbituric acid reactive substances (TBARS), subsequent to MA or MMA exposure. In addition, using hemolymph and carcasses we determined the effect of different treatments on glucose and triglyceride levels, tentatively to understand the putative protective mechanism(s) of CA and/or CAF in this experimental protocol.

Material and methods

Chemicals

Malonic Acid (MA), Methylmalonic acid (MMA), Thiobarbituric acid (TBA), acetylthiocholine iodide, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB),caffeic acid (CA) and caffeine(CAF) were acquired from Sigma Chemical Co. (St. Louis, MO, USA). Methanol and acetic acid were purchased from Merck (Darmstadt, Germany). All the other chemicals were commercial products of the highest purity grade available.

Drosophila melanogaster Stock

Drosophila melanogaster wild-type (Harwich strain) was obtained from the National species Stock Center (Bowling Green, OH, USA). The flies were maintained and reared on cornmeal medium (1% w/v brewer’s yeast, 2% w/v sucrose, 1% w/v powdered milk, 1% w/v agar, and 0.08% v/w methylparaben) at constant temperature and humidity (22±1 °C; 60% relative humidity, respectively) under 12 h dark/light cycle conditions until treatment. All the experiments were carried out with the same fly strain.

MMA or MA Exposure and Treatment with CA and CAF

D. melanogaster (both sexes) aged 1 to 3 days old, were divided into twelve groups of 50 flies each: (1) control group (standard diet); (2) CA (1.39 µg/mL); (3) CAF (1.27 µg/mL); (4) CA (1.39 µg/mL) + CAF (1.27 µg/mL); (5) MA (5 mM); (6) MA (5 mM) + CA (1.39 µg/mL); (7) MA (5 mM) + CAF (1.27 µg/mL); (8) MA (5 mM) + CA (1.39 µg/mL) + CAF (1.27 µg/mL); (9) MMA (5 mM); (10) MMA (5 mM) + CA (1.39 µg/mL); (11) MMA (5 mM) + CAF (1.27 µg/mL); (12) MMA (5 mM) + CA (1.39 µg/mL) + CAF (1.27 µg/mL).The flies were exposed concomitantly to a diet containing MA or MMA and CA and/or CAF for four days (96 h). The CA (1.39 µg/mL) and (3) CAF (1.27 µg/mL) were placed into the fly’s food at a final indicated concentrations because were equivalent to those found in the crude extract of *I. paraguariensis* used previously [13]. The diet treatment consisted of 10 mL (1.4 g of sucrose, 0.7 g of milk powder, 1.4 g of agar in 70 mL of distilled water and methylparaben as antifungal) and was

replaced every 48 h. This protocol was repeated at least three times in triplicate and was performed at room temperature.

In Vivo Assays

Survival rate analysis

The survival rate of the flies exposed to treatments were observed counting the number of alive flies compared to the number of dead flies after four days of exposure. Approximately 150 flies/groups have been included in the survival data and the total number of flies represents the sum of three independent experiments (50 flies/each replicated treatments). Results were analyzed and plotted as % of survival.

Negative Geotaxis

Locomotor ability of flies was performed with a negative geotaxis assay as described previously [32], with some adaptations[15]. In short, flies were sorted under a brief ice anesthesia and placed in a vertical glass column (length: 10 cm, diameter: 1.5 cm/10 flies each). After the recovery from cold exposure (approximately 10 min) the flies were gently tapped to the bottom of the column. The flies that reached the top of the column (6.5 cm) and the flies that remained at the bottom were counted separately during 6 s. The scores represent the mean of the numbers of flies at the top (ntop) as percentage of the total number of flies (ntot). The test was repeated five times for each fly and 5 flies had been separated from each group to be evaluated. Four independent experiments were performed (a total of 20 flies per group were used) and the plotted value represents the mean (as % of flies) in top.

Open Field Test

To evaluate the behavioral and exploratory activity, 5 flies were used for each group, every fly was assessed individually after submitting it into a Petri dish divided by squares (1 cm x 1 cm) as described previously [13, 15]. The exploratory activity of the fly over a 30-second-period was evaluated according to the number of squares crossed / explored by each fly (a total of 20 flies per group were used).

Ex Vivo Assays

Homogenate Preparation

At the end of the treatment period (96 h), flies were anesthetized in ice. Heads were separated from the body using a sharp blade/cutter. Afterward, heads and bodies were homogenized in 0.9% NaCl solution, 1:5 (flies/volume; i.e. 50 heads or 50 bodies to 250 µL). The homogenates were centrifuged at 2500×g for 10 min at 4 °C, and the supernatant was used for biochemical assays. All biochemical determinations were performed in duplicates in 3 independent experiments.

Activity of acetylcholinesterase (AChE)

AChE activity was measured according to the method described previously [33]. The reaction was prepared with 0.5M potassium phosphate buffer (pH 7.4) and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) (5 mM) and this was added to the supernatant sample (20 µL) and 50 µL acetylthiocholine (AcSCh) (7.25 mM). The reaction was then monitored for 2 minutes at 412 nm. The enzymatic activity was expressed in % of control after correction by the protein content.

Activity of Glutathione-S-transferase (GST)

GST activity was assayed according to previously published procedure, using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate[34]. The assay reaction mixture was 20 µL of sample with 30mM TFK buffer (pH 7.4), 1mM GSH and 1mM CDNB. The reaction was monitored for 3 min (15s intervals) at 340 nm at room temperature (25 °C). GST activity was standardized per total protein level and expressed as % of control after correction by the total protein content.

Determination of Non-protein Thiol (NPSH)

NPSH were estimated based on spectrophotometry according to the method described previously [35]. For NPSH measures, 70 µL homogenates were precipitated with 70 µL TCA 10% (1:1 v: v) followed by centrifugation at 3000×g for 10 min at 4 °C. The reaction system was 50 µL of sample with 1 M dibasic potassium phosphate buffer, and 50 µM DTNB. At the end of 10 min incubation at room temperature (25 °C), the absorbance was measured at 412

nm. The results were expressed as % of control after correction by the protein content.

Determination of TBARS

Lipid peroxidation (LPO) was measured by the substances reactive to thiobarbituric acid (TBARS) assay, which was performed according to the method described previously[36]. An aliquot of homogenate (100 μL) was incubated during 1 h at 37 °C in a water bath. Thereafter samples were incubated at 100 °C for 120 min in 200 μL of a medium containing equal volumes of trichloroacetic acid (10%, w/v) and thiobarbituric acid (0.6%, w/v) in 0.1 M HCl for color development. After boiling step, 20 μL 8.1% SDS was added. The reaction product was determined at 532 nm and the results were expressed as % of control after correction by the protein content.

Activity of superoxide dismutase (SOD)

SOD activity was measured by evaluating the inhibition of quercetin auto-oxidation, according to the procedure previously described [37], with some modifications [13]. Briefly, the reaction medium contained 40 μL of sample with buffer 13 mM phosphate buffer (pH 7.4), 50 μM EDTA, 1 μM TEMED and 2 nM quercetin. The reaction was analyzed at time 0 and after 20 min at 406 nm at room temperature (25 °C). The results were expressed as % of control after correction by the protein content.

Activity of catalase (CAT)

CAT activity was measured spectrophotometrically according previously describes[38], by monitoring the disappearance of H_2O_2. Briefly, the reaction medium contained 960 μL of 0.05 M phosphate buffer (pH 7.0), 20 μL of 1M H_2O_2, and 20 μL of sample. The reaction was analyzed for 2 min (15 s intervals) at 240 nm. Results were expressed as % of control after correction by the protein content.

MTT reduction assay

Dehydrogenases activity was evaluated by using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reduction assay described

previously[15]. The assay reaction mixture was 50µL of sample with 65mM TFK (pH 7.4) and 0.1mg/mL MTT. This mixture was incubated at 37°C for 30 min. Thereafter, 200µL of DMSO was added and the mixture was incubated again for 30 min. Finally, 200µL of SDS 8.1% was added and the resulting supernatant was centrifuged 3000g for 20 min. Then, the samples were centrifuged to 2000 rpm for 5 min, and the absorbance was monitored at 630 nm and 545 nm. To calculate the MTT reduction was used: $||545||nm - ||630||nm = Result x 100$. The results were expressed as % of control after correction by the protein content.

Triglycerides and glucose determination

Hemolymph extraction was performed accordingly previously reported[39], with some modifications. Accordingly, treated flies (20 per group of each independent experiment) were subjected to three cycles of freezing and thawing, followed by centrifugation (3000g, 5 min at 4°C) to hemolymph extraction.

The rest of the bodies and heads (called here as carcasses) were homogenized in 1 ml of 50 mM TFK, pH 7.0 and centrifuged (3000g, 5 min at 4°C). Resulting supernatant and hemolymph were used to glucose and triglycerides determinations by using specific kits (Labtest® kit).

Protein Determination

The protein concentrations were determined based on the method described previously [40]. The sample (10 µL of body or head sample, dilution 1:10) and 190 µL of Bradford reagent were added. After 10 minutes of incubation at room temperature, the absorbance was measured at a wavelength of 595 nm in a spectrophotometer. The protein values were calculated with a standard curve by using bovine serum albumin as standard.

Statistical analysis

Data from the behavioral performance assays were evaluated by non-parametric methods because a Kolmogorov-Smirnov's test indicated the absence of Homogeneity of Variance. Accordingly, behavior parameters were analyzed using Kruskal–Wallis (kw) followed by Dunn's multiple comparisons

test when appropriate. All the remaining assays were analyzed using One-way ANOVA followed by Tukey's multiple range tests when appropriate. Differences between groups were considered significant when P<0.05. Data of non-parametric analysis are represented as box and whisker plots (min to max); and data of parametric analysis as means and S.E.M. Graphics were created using Graph Pad Prisma 6.0.

Results

CA and/or CAF attenuates fly mortality associated to MA or MMA exposure

Exposure of adult flies for an experimental period of four days to MA (5 mM) or MMA (5 mM) decreased the survival rates compared to those in the control group. Accordingly, the percent of mortality of the group exposed to MA was around 34% and of the group exposed to MMA was around 33% compared to control group. Importantly, CA (1.39 µg/mL) co-exposure significantly reduced fly mortality associated to MA exposure, being ineffective against MMA-induced mortality. In turn, CAF (1.27 µg/mL) co-exposure significantly reduced fly mortality associated to both MA and MMA exposure. Moreover, the combination CA + CAF does not offer additional protective effect. Statistical analysis also revealed that there was no significant effect of CA and/or CAF *per se* (Figure 1).

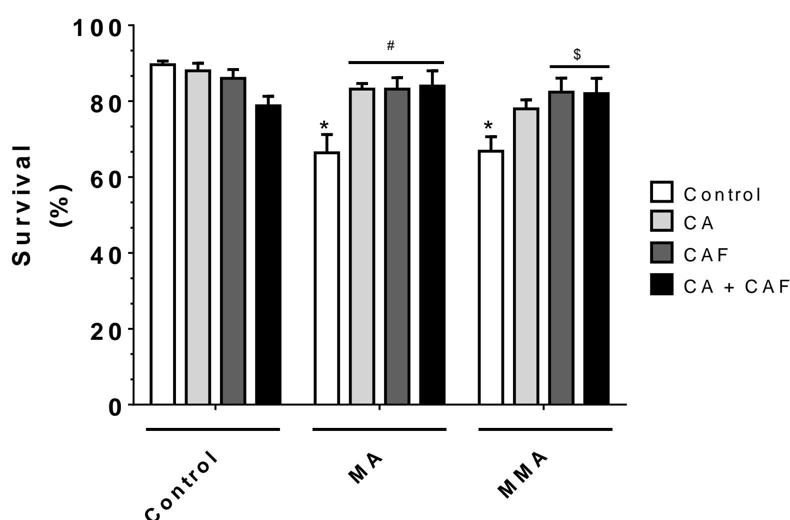

Figure 1: Effect of CA (1.39 µg/mL) and/or CAF (1.27 µg/mL) on MMA (5 mM) or MA (5 mM) induced mortality in flies. Data were collected after 96 hours of exposure for each group. The total number of flies (150 per group) represents the sum of three independent experiments. The numbers of alive flies are represented as % of survival and the values are expressed as mean ± S.E.M. (*) represent significant difference as compared with control group;(#) represents significant difference between MA and MA+CA or MA+CAF or MA+CA+CAF; ($) represents significant difference between MMA and MMA+CA or MMA+CAF or MMA+CA+CAF by One-way ANOVA followed by Tukey's multiple range test(P < 0.05 was considered significant).

CA and/or CAF attenuates locomotor impairment associated to MA or MMA exposure

Exposure of adult flies to MA or MMA significantly decreased the climbing behavior (ability of flies that reach the top of the vial) **compared to those in the control group. Importantly, both** CA and CAF co-exposure significantly prevented the negative effects associated to both MA and MMA exposure (Figure 2).

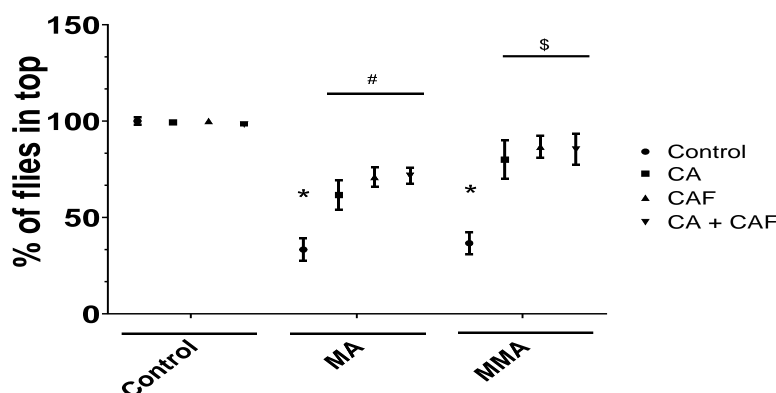

Figure 2: Effect of CA (1.39 µg/mL) and/or CAF (1.27 µg/mL) on MMA (5 mM) or MA (5 mM) on geotaxis response (climbing) in flies. Data were collected after 96h of exposure. The total number of flies (20 per group) represents the sum of four independent experiments. Values are expressed as median and range (interquartile interval). (*) represent significant difference as compared with control group;(#) represents significant difference between MA and MA+CA or

MA+CAF or MA+CA+CAF; ($) represents significant difference between MMA and MMA+CA or MMA+CAF or MMA+CA+CAF by Kruskal–Wallis test followed Dunn' multiple comparisons test(P < 0.05 was considered significant).

In open-field test, there was an increase in the exploratory activity through the crossing number of approximately 2.2 times in MA and 2.8 times in MMA treated flies. Likewise on negative geotaxis test, the effect of both MA and MMA was completely abolished by **both** CA and CAF co-exposure (Figure 3).

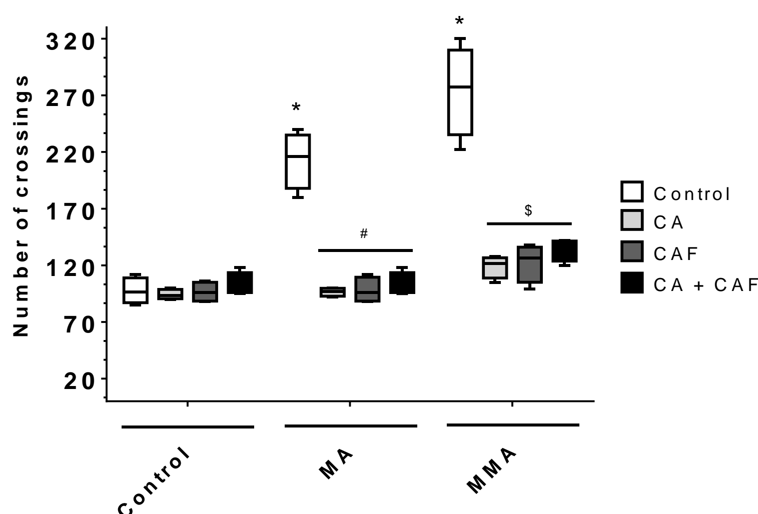

Figure 3: Effect of CA (1.39 µg/mL) and/or CAF (1.27 µg/mL) on MMA (5 mM) or MA (5 mM) on locomotor activity (open-field assay) of treated flies. Data were collected after 96h of exposure. The total number of flies (20 per group) represents the sum of four independent experiments. Values are expressed as median and range (interquartile interval). (*) represent significant difference as compared with control group;(#) represents significant difference between MA and MA+CA or MA+CAF or MA+CA+CAF; ($) represents significant difference between MMA and MMA+CA or MMA+CAF or MMA+CA+CAF by Kruskal–Wallis test followed Dunn' multiple comparisons test(P < 0.05 was considered significant).

Statistical analysis revealed that the combination CA + CAF does not offer additional protective effect against MA or MMA-induced locomotor changes. Additionally, statistical analysis also revealed that there was no

significant effect of CA and/or CAF *per se* on locomotor behavioral of flies (Figures 2 and 3).

CA and/or CAF attenuates biochemical changes associated to MA or MMA exposure

Treatment with MMA and MA caused a significant decrease in AChE activity, both in head (Figures 4A) and body (Figures 4B) regions. Treatment with CA and/or CAF was completely ineffective in normalized changes in AChE activity associated to MA or MMA exposure. Notwithstanding, statistical analysis revealed that there was a significant inhibitory effect of CA and/or CAF *per se* on AChE activity in body region (Figure 4B) and of CA in head region (Figure 4A).

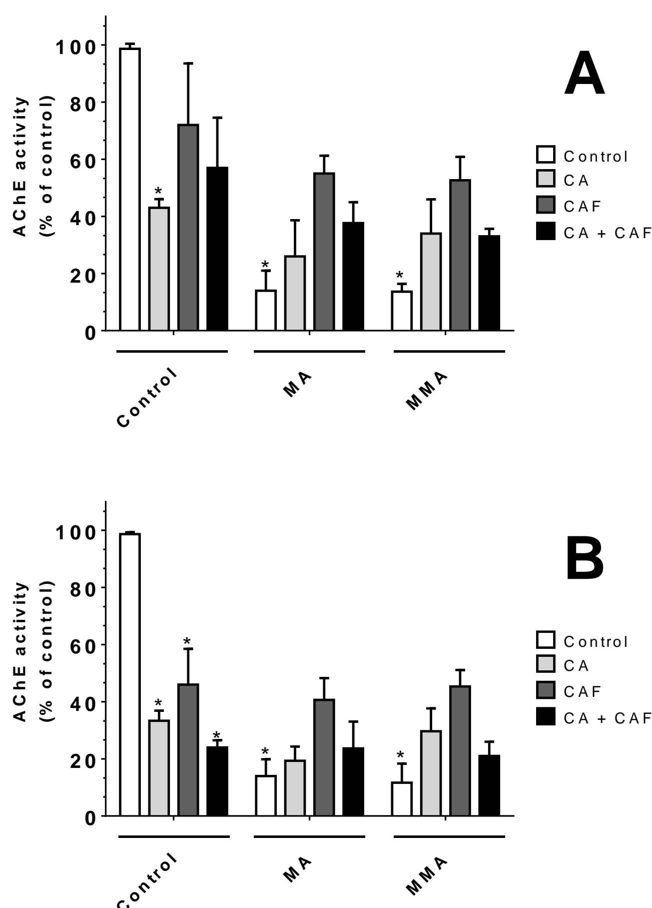

Figure 4: Effect of CA (1.39 µg/mL) and/or CAF (1.27 µg/mL) on AChE activity in (A) Head and (B) Body of flies exposed to MA (5 mM) or MMA (5 mM). Values are expressed as % of control and are expressed as mean ± S.E.M. (n =

3). (*) represent significant difference as compared with control group by One-way ANOVA followed by Tukey's multiple range test (P < 0.05 was considered significant).

Treatment with MMA caused a significant decrease in GST activity in body (Figures 5B) region. Treatment with CA and/or CAF completely normalized changes in GST activity associated to MMA exposure. Statistical analysis revealed that the combination CA + CAF does not offer additional protective effect against MA or MMA-induced changes in GST activity. Statistical analysis also revealed that there was no significant effect of CA and/or CAF *per se* on GST activity (Figure 5).

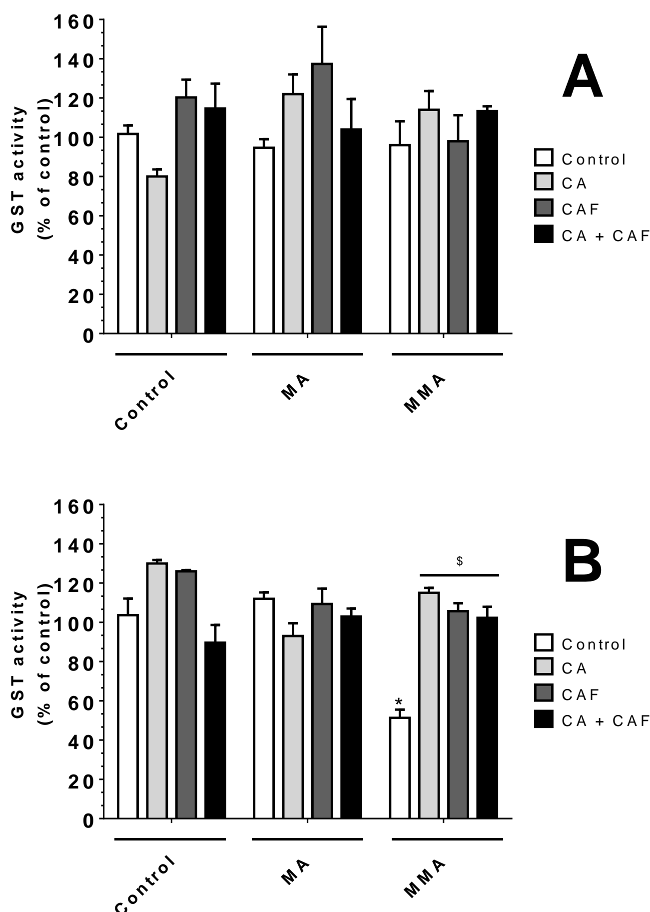

Figure 5: Effect of CA (1.39 µg/mL) and/or CAF (1.27 µg/mL) on GST activity in (A) Head and (B) Body of flies exposed to MA (5 mM) or MMA (5 mM). Values are expressed as % of control and are expressed as mean ± S.E.M. (n = 3). (*)

represent significant difference as compared with control group; ($) represents significant difference between MMA and MMA+CA or MMA+CAF or MMA+CA+CAF by One-way ANOVA followed by Tukey's multiple range test(P < 0.05 was considered significant).

Tukey's multiple comparisons test revealed that treatment with MA and MMA caused a significant increase in NPSH content as compared with control group. In fact, the increase was observed both in the head (Figure 6A) and body regions (Figure 6B). Of particular importance, the effect of MA and MMA on NPSH (Figure 6) levels was completely abolished by **both** CA and CAF co-exposure both in head and in body. Statistical analysis revealed that the combination CA + CAF does not interfere with the MA or MMA-induced changes in NPSH content. Statistical analysis also revealed that there was no significant effect of CA and/or CAF *per se* on this parameter (Figures 6).

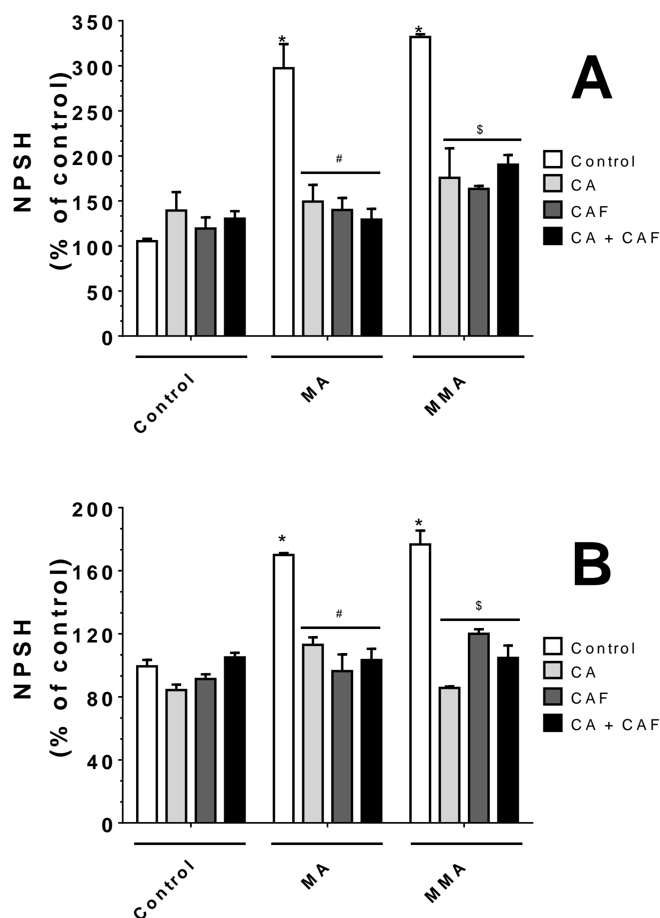

Figure 6: Effect of CA (1.39 µg/mL) and/or CAF (1.27 µg/mL) on NPSH levels in (A) Head and (B) Body of flies exposed to MA (5 mM) or MMA (5 mM). Values are expressed as % of control and are expressed as mean ± S.E.M. (n = 3). (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA+CA or MA+CAF or MA+CA+CAF; ($) represents significant difference between MMA and MMA+CA or MMA+CAF or MMA+CA+CAF by One-way ANOVA followed by Tukey's multiple range test($P < 0.05$ was considered significant).

Similarly, Tukey's multiple comparisons test revealed that treatment with MA and MMA caused a significant increase in TBARS levels as compared with control group. In fact, the increase was observed both in the head (Figure 7A) and body regions (Figure 7B), respectively. Of particular importance, the effect of MA and MMA on TBARS levels was completely abolished by either CA or CAF co-exposure both in head and in body. Statistical analysis revealed that the combination CA + CAF does not offer additional protective effect against MA or MMA-induced changes in TBARS levels. Statistical analysis also revealed that there was no significant effect of CA and/or CAF *per se* on this parameter (Figure 7).

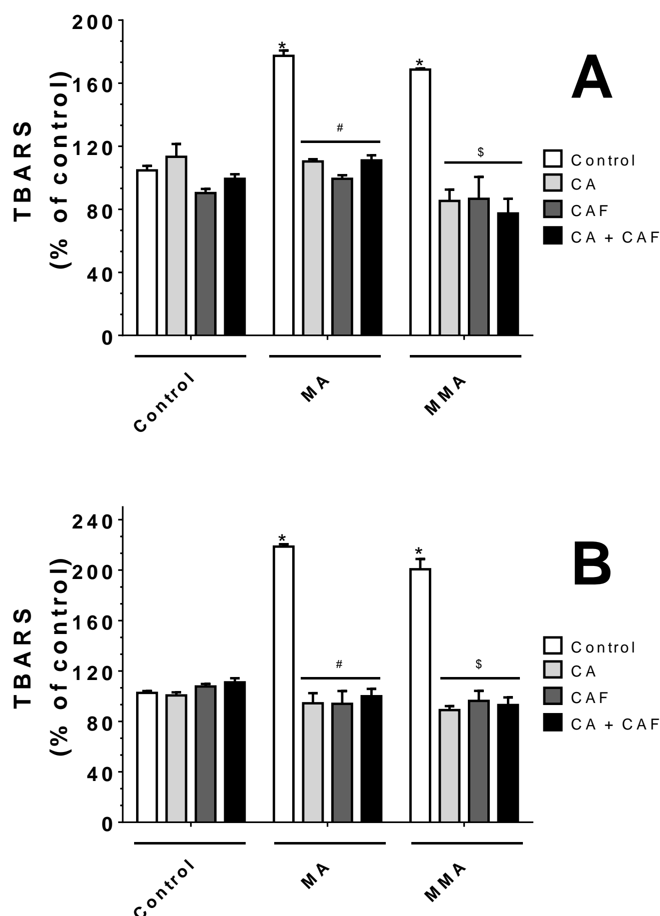

Figure 7: Effect of CA (1.39 µg/mL) and/or CAF (1.27 µg/mL) on TBARS levels in (A) Head and (B) Body of flies exposed to MA (5 mM) or MMA (5 mM). Values are expressed as % of control and are expressed as mean ± S.E.M. (n = 3). (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA+CA or MA+CAF or MA+CA+CAF; ($) represents significant difference between MMA and MMA+CA or MMA+CAF or MMA+CA+CAF by One-way ANOVA followed by Tukey's multiple range test(P < 0.05 was considered significant).

Treatment with MMA and MA caused a significant decrease in SOD activity, both in head (Figures 8A) and body (Figures 8B) regions. Treatment with CA and/or CAF was almost ineffective in normalized changes in SOD activity associated to MA or MMA exposure. Indeed, Tukey's multiple comparisons test revealed that the effect of MA was completely abolished only by CAF co-exposure merely in head (Figure 8A). Statistical analysis also

revealed that there was no significant effect of CA and/or CAF *per se* on SOD activity (Figure 8).

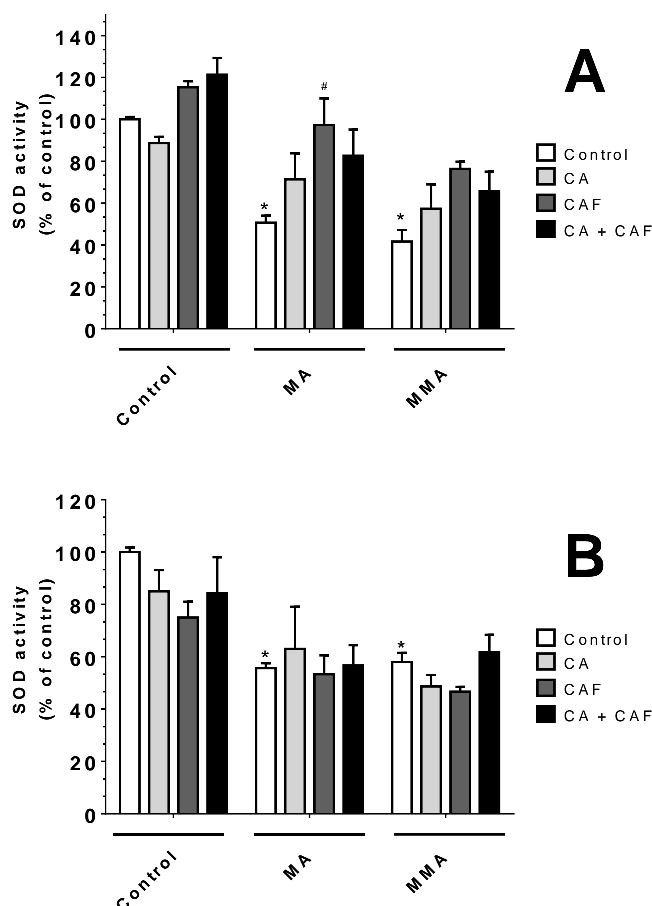

Figure 8: Effect of CA (1.39 μg/mL) and/or CAF (1.27 μg/mL) on SOD activity in (A) Head and (B) Body of flies exposed to MA (5 mM) or MMA (5 mM). Values are expressed as % of control and are expressed as mean ± S.E.M. (n = 3). (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA+CA or MA+CAF or MA+CA+CAF by One-way ANOVA followed by Tukey's multiple range test ($P < 0.05$ was considered significant).

Tukey's multiple comparisons test revealed that treatment with MA and MMA caused a significant decrease in CAT activity as compared with control group. In fact, the decrease was observed both in the head (Figure 9A) and body regions (Figure 9B). Of particular importance, the effect of MA and MMA on CAT activity was completely abolished by **both** CA and CAF co-exposure

both in head and in body. Statistical analysis revealed that the combination CA + CAF does not offer additional protective effect against MA or MMA-induced changes in CAT activity. In turn, statistical analysis also revealed that there was a significant increase in CAT activity in the head region of flies exposed to CAF (Figure 9A).

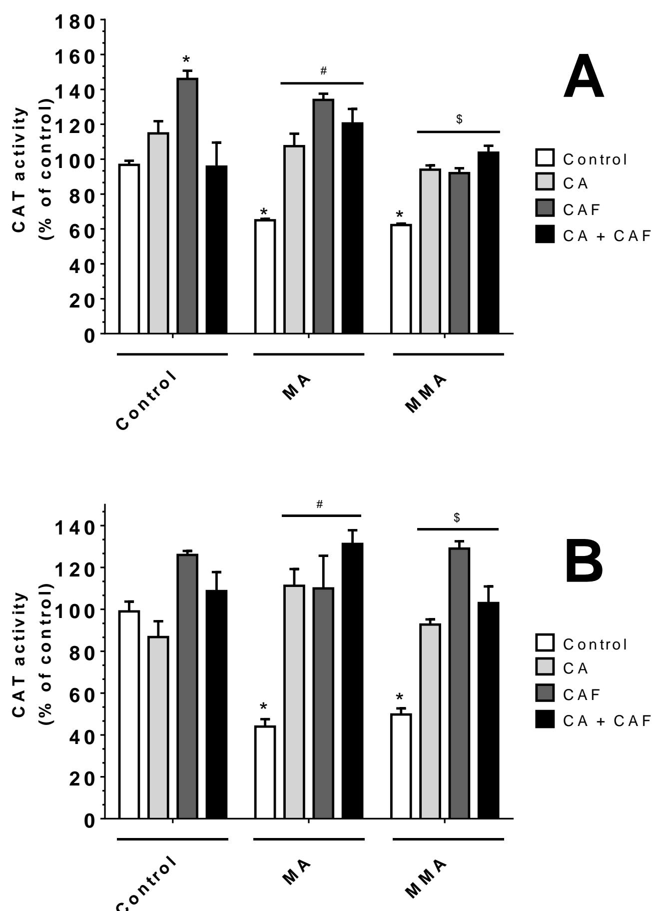

Figure 9: Effect of CA (1.39 µg/mL) and/or CAF (1.27 µg/mL) on CAT activity in (A) Head and (B) Body of flies exposed to MA (5 mM) or MMA (5 mM). Values are expressed as % of control and are expressed as mean ± S.E.M. (n = 3). (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA+CA or MA+CAF or MA+CA+CAF; ($) represents significant difference between MMA and MMA+CA or MMA+CAF or MMA+CA+CAF by One-way ANOVA followed by Tukey's multiple range test($P < 0.05$ was considered significant).

Tukey's multiple comparisons test revealed that treatment with MA and MMA caused a significant decrease in MTT reduction ability as compared with

control group. In fact, the decrease was observed both in the head (Figure 10A) and body regions (Figure 10B). Of particular importance, the effect of MA and MMA on MTT reduction ability was completely abolished by **both** CA and CAF co-exposure both in head and in body. Statistical analysis revealed that the combination CA + CAF does not offer additional protective effect against MA or MMA-induced changes in MTT reduction. Statistical analysis also revealed that there was no significant effect of CA and/or CAF *per se* on this parameter (Figure 10).

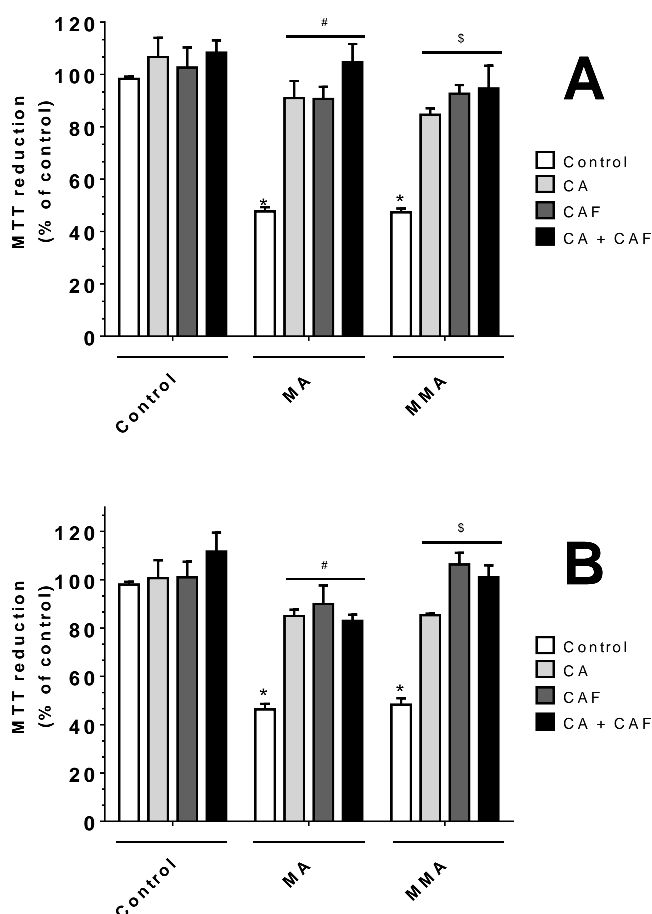

Figure 10: Effect of CA (1.39 µg/mL) and/or CAF (1.27 µg/mL) on MTT reduction capacity in (A) Head and (B) Body of flies exposed to MA (5 mM) or MMA (5 mM). Values are expressed as % of control and are expressed as mean ± S.E.M. (n = 3). (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA+CA or MA+CAF or MA+CA+CAF; ($) represents significant difference between MMA

and MMA+CA or MMA+CAF or MMA+CA+CAF by One-way ANOVA followed by Tukey's multiple range test($P < 0.05$ was considered significant).

Tukey's multiple comparisons test revealed that treatment with MA and MMA resulted in a significant decrease in glucose (Figures 11A and B) and in triglyceride (Figures 11C and D) levels as compared with control group, both in the hemolymph (Figures 11A and C) and in carcasses (Figures 11B and D). Of particular importance, the effect of MA and MMA on glucose levels were completely abolished by **both** CA and CAF co-exposure in hemolymph (Figures 11A). Similarly, the effect of MA and MMA on triglycerides levels was completely abolished by CA co-exposure in hemolymph, being CAF without effect on this parameter (Figures 11C).

However, CA and/or CAF practically does not protect from MA or MMA changes in glucose or triglycerides levels in the carcasses (Figures 11B and D, respectively). Indeed, only by the combination of CA and CAF co-exposure offers protection against MA-induced decrease in triglycerides levels in carcasses (Figures 11C). Statistical analysis also revealed that there was no significant effect of CA and/or CAF *per se* on these parameters (Figures11).

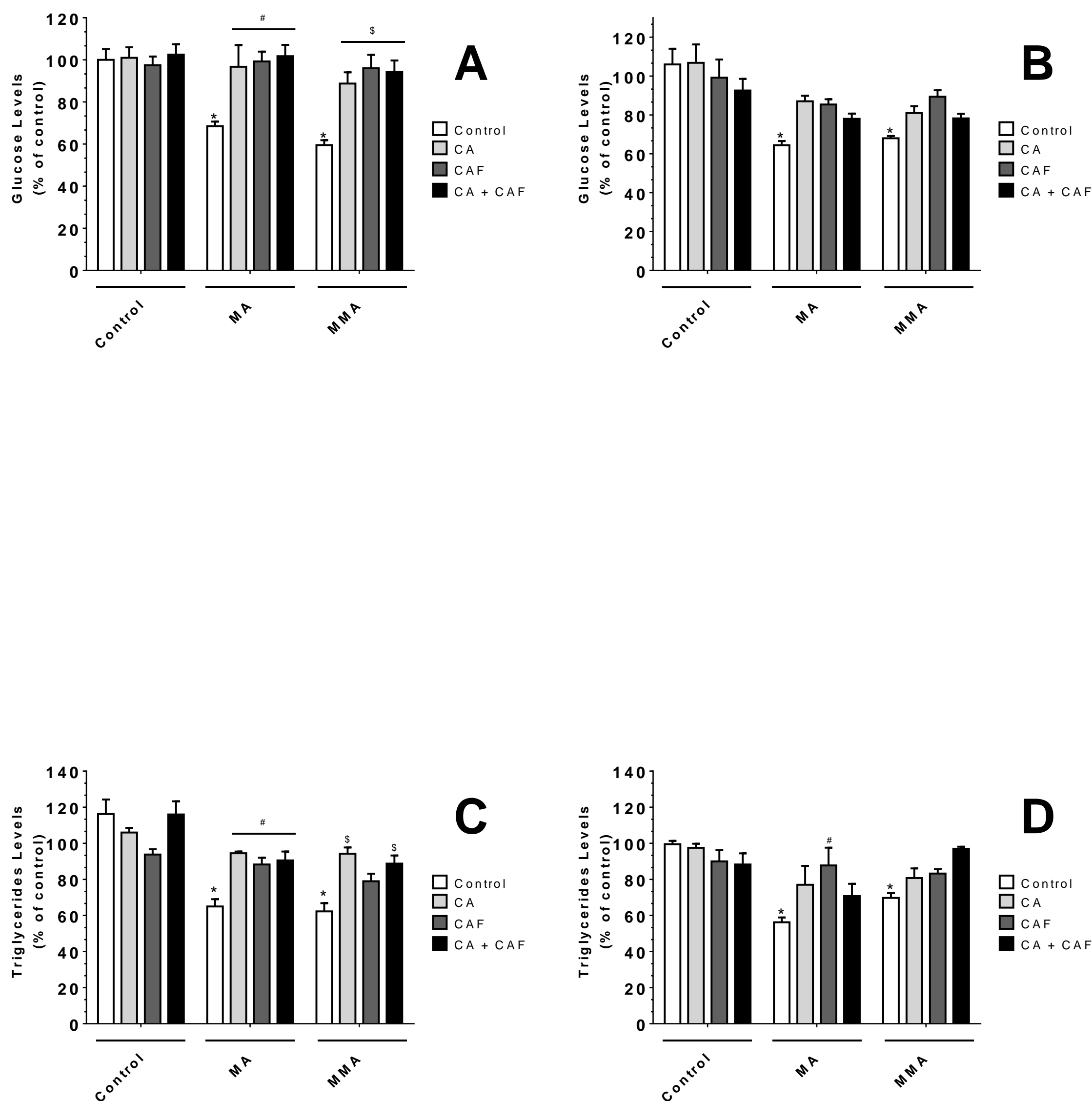

Figure 11: Effect of CA (1.39 µg/mL) and/or CAF (1.27 µg/mL) on total glucose in (A) hemolymph and in (B) carcasses; and on total triglycerides in (C) hemolymph and in (D) carcasses of flies exposed to MA (5 mM) or MMA (5 mM). Values are expressed as % of control and are expressed as mean ± S.E.M. (n = 3). (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA+CA or MA+CAF or MA+CA+CAF; ($) represents significant difference between MMA and MMA+CA or MMA+CAF or MMA+CA+CAF by One-way ANOVA followed by Tukey's multiple range test($P < 0.05$ was considered significant).

Discussion

We recently found that *D. melanogaster* is a usefulness model for study toxicological changes associated to MA or MMA exposure [13]. Accordingly, data presented here confirms that flies exposure to MA or MMA resulted in increased mortality and changes in motor behaviors. Moreover, we confirmed alterations in several toxicity assays which included MA- or MMA-induced changes in NPSH and TBARS levels; changes in AChE, SOD and CAT activities; a decreased ability to reduce MTT, and also changes in glucose and triglycerides pools. Particularly important, we found that both CA and/or CAF (the two major metabolites found in our *I. paraguariensis* extract [13]) attenuated the damage caused by either MA or MMA regarding survival, behavioral parameters and also restored the antioxidant defenses, mitochondrial function and metabolic fuels. Importantly, we found that CA and CAF does not act in synergistic/additive way and that both CA and CAF *per se* caused significant inhibition in AChE activity in body region, whereas CA also leads to inhibition in AChE activity in head region.

Indeed, in our experimental conditions, exposure to MA or MMA significantly reduced survival (Figure 1) and promoted changes in locomotor performance (Figures 2 and 3) of flies as well as promoted changes in biochemical parameters related to oxidative stress (Figures 6-9), mitochondrial dysfunction (Figure 10) and metabolic substrates (Figure 11). Accordingly, a growing body of evidence suggests that oxidative stress and mitochondrial dysfunction are the putative mechanism(s) associated to MA or MMA toxicity [7, 11, 13, 41-43]. However, although little is known about the precise mechanism(s) of action of MA or MMA in *D. melanogaster*, the involvement of oxidative damage and the mitochondrial dysfunction is supported by present data and also by our previous study [13].

In this work, we also found that under our regimen of exposure, MA and MMA leads to a significant decrease in the pool of glucose and triglycerides, both in hemolymph and in the carcasses of flies. Besides not investigated in a mechanistically point of view, these findings are supported by previously, in which it was found hypoglycemia associated to MMA accumulation [44]. In addition, it was previously reported that MA accumulation leads to

hypoglycemia, accumulation of ketone bodies and result in a decrease in triglycerides and free fatty acids in dogs[45], further supporting our findings.

Of particular importance, CA and/or CAF protected the flies from increased mortality (Figure 1), from changes in locomotor performance (Figures 2 and 3) and also in biochemical parameters (Figures 4-11), demonstrating to be a promising alternative to treat conditions associated to MMA or MA accumulation, such as in methyl malonica acidemia. These findings are in accordance to previous reports in which CA was found to protects against mortality, locomotor deficits and oxidative stress associated to different experimental protocols [46, 47]. Similarly, *I. paraguariensis* extract was also found to improve the locomotor deficits induced by the treatment with reserpine and MPTP in rodents, which was, at least in part, attributed to CAF present in the extract [48].We also suggest that the protective effect of CA and CAF could be due, at least in part, to its ability to protect against changes in the pool of metabolic substrates (glucose and triglycerides), thus further contributing to the maintenance the mitochondrial function closer to those of control flies.

So, despite of the scant in scientific literature regarding the cumulative number of dead flies as well about behavioral changes following CAF or CA treatment, we suggest that isolated compounds are safety and benefit to counteract MMA or MA associated toxicity under these experimental conditions. Accordingly, CA (a typical phenolic compound) and CAF (a typical alkaloid) are both known for their antioxidant potential [49-51] as well as for its ability to improve mitochondrial function [52, 53].The mechanisms that are proposed to contribute to the overall antioxidant activity of CAF include its ability to form adduct with different radicals[54]. Moreover, CAF biological activity could be also explained by other mechanisms than antioxidant. Of note, the most important mechanisms of action of CAF involves antagonism of adenosine receptors, phosphodiesterase inhibition, modulation of GABA receptor action, regulation of intracellular calcium levels and also inhibiting in a non-competitive manner the activity of AChE [55, 56]. Likewise, the activity of CA could be due to their unique structure that is capable present free radical scavenging properties by acting as hydrogen-donating molecules, in addition to other chemical characteristics such as metal chelator[57, 58].Of note, CA biological

activity could also be explained, at least in part, due its ability to inhibit AChE activity [59].

Accordingly, reversible AChE inhibition has been accepted as an effective treatment/management strategy against neurodegenerative disease (particularly Alzheimer's disease), and recent efforts have focused on plant phytochemicals as natural sources of effective AChE inhibitors with little or no side effects[59, 60].Importantly, our data (Figure 4) confirm the inhibitory efficacy of both CAF and/or CA to AChE. However, it is remarkable that MA or MMA exposure also resulted in AChE inhibition. So, we suggest that although all treatments results in an impairment in AChE activity, the inhibition obtained in the presence of CA and/or CAF are beneficial to flies probably because it is a reversible inhibition (as previously reported [59, 60]), whereas that caused by MA or MMA are detrimental to exposed organisms probably because it is an irreversible inhibition. Importantly, we suggest that both either CA and/or CAF are useful in ameliorating cholinergic impairments associated to MMA or MA exposure. Of note, it was previously found that MA or MMA exposure resulted in significantly impairment/dysfunction in cholinergic system[61-63], whereas CA and/or CAF were reported to significantly protect against cholinergic impairment [59, 60]. So, we suggest that CAF and/or CA may be considered in treatments of neurodegenerative diseases where AChE inhibition is employed because, in addition to their anticholinesterase action, they also present antioxidant activity.

In conclusion, the mechanism of both CA and CAF protection in MA or MMA-induced toxicity is attributed to their ability to counteract oxidative damage and mitochondrial dysfunction associated to both organic acids exposure. Together, the results revealed that CA and CAF protected against toxicity induced by MA or MMA by reducing flies mortality, protecting against abnormalities in flies behavior and also by inhibiting oxidative stress and maintaining mitochondrial activity. So, CA and/or CAF could be considered, at least in part, responsible for the protective activity of *I. paraguariensis* crude extract reported previously[13]and also could be considered a therapy strategy in conditions associated to MA or MMA accumulation.

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Conflict of interest

The authors declare that there is no conflict of interest.

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5. Discussão

Um dos principais objetivos deste trabalho foi avaliar o efeito protetor do extrato aquoso de *Ilex paraguariensis* contra a toxicidade induzida pelo ácido malônico e metilmalônico em *Drosophila melanogaster* .Neste viés, fomos os primeiros a mostrar os efeitos de toxicidade do MA e MMA em modelo alternativo, de DM, tanto sobre os parâmetros bioquímicos quanto os comportamentais. Os resultados apresentados no artigo já publicado, demonstram que o MA e MMA causam diminuição no tempo de vida (sobrevivência) (Figura 2, artigo), além de causar danos locomotores e comportamentais em moscas expostas aos mesmos (Figura 3, artigo). Esta toxicidade atribuída ao MA e MMA, deve-se ao envolvimento no estresse oxidativo, disfunções mitocondriais, além de danos e por fim apoptose (Gabbi P, 2017 e Valdeolivas S, 2013). Neste trabalho, suplementação dietética com extrato aquoso de IP reduziu significativamente a toxicidade por MA ou MMA, o extrato não apenas diminuiu a taxa de mortalidade, mas também melhorou as alterações comportamentais associadas aos ácidos. Na tentativa de entender melhor os possíveis mecanismos de toxicidade induzidas por MA e MMA, abordamos o estresse oxidativo e o seu envolvimento na mitocôndria. No entanto não há, dados na literatura, mostrando os efeitos por MA e MMA em DM, com isso observamos aumento significativo de TBARS (Figura 4 A e B, artigo) e NPSH (Figura 5 A e B, artigo) quando comparados ao grupo controle, ou seja, um efeito deletério e uma compensação ao insultos oxidativos. Além disso, observamos uma diminuição na atividade da SOD (Figura 6 A e B, artigo), CAT (Figura 7 A e B, artigo) AChE (Figura 8 A e B, artigo), e dos níveis de MTT (Figura 9 A e B, artigo) e Resazurina (Figura 10 A e B, artigo). Apesar de não podermos elaborar diretamente o(s) mecanismo(s) pelo qual MMA e MA levam a mudanças no desempenho locomotor e também aos insultos oxidativos, este é o primeiro relatório em que descrevem essas alterações em um modelo de invertebrado (DM). No entanto é importante demonstrar que a suplementação dietética com extrato aquoso de IP normalizou significativamente todos os parâmetros (TBARS, atividade da SOD e catalase, níveis de NPSH, MTT e Resazurina), sugerindo, assim, efeito protetor do extrato IP contra comprometimento mitocondrial e/ou estresse

oxidativo causado por MA ou MMA em DM. Acreditamos que os efeitos benéficos que o extrato de IP demonstra, está atrelado aos seus metabólitos (polifenóis) (Tabela 1, artigo) capazes de melhorar a atividade GABAérgica (testes comportamentais) e sua atividade antioxidante (ensaios bioquímicos), e por consequência em inibir tais efeitos deletérios (Milioli EM, 2007 e Branco C dos S,2013). A fim de elucidar quais os compostos que poderiam agir contra os danos causados pelos MA e MMA, desenvolvemos um segundo estudo, utilizando os dois principais metabólitos encontrados em nosso extrato bruto de IP, o Ácido Caféico e a Cafeína isolados ou combinados, para assim entender se um dos isolados ou a combinação destes poderia(m) proteger a DM contra os insultos oxidativos e comportamentais causados pelos ácidos. Neste segundo trabalho, o MA e MMA, em nossas condições experimentais, mantiveram seus efeitos deletérios para a *Drosophila melanogaster*, ou seja, os mesmos reduziram significativamente a sobrevivência (Figura 1, manuscrito), promoveram alterações no desempenho comportamental e locomotor (Figura 2 e 3, manuscrito), além de promoverem alterações nos parâmetros bioquímicos (Figuras 6-9, manuscrito), disfunções mitocondriais e metabólicas (Figuras 10 e 11, manuscrito). No entanto, pouco sabemos sobre os mecanismos de ação do MA e MMA sobre as DM, mas estes ensaios são suportados pelo trabalho anterior (Portela JL, 2019). Consequentemente, sugerimos que o estresse oxidativo e a disfunção mitocondrial são mecanismos de toxicidade associado ao MA e MMA (Kalonia H, 2010; Stepien KM, 2017; Portela JL,2019). De fato, o CA e/ou CAF, protegeram as moscas da mortalidade (Figura 1, manuscrito), das alterações comportamentais e locomotoras (Figura 2 e 3, manuscrito) e também de alguns parâmetros bioquímicos (Figuras 4-11, manuscrito) causados pelos ácidos. Esses achados estão de acordo com trabalhos anteriores nos quais a CA protege contra a mortalidade, déficit locomotores e estresse oxidativo associado a diferentes protocolos experimentais (Jimenez-Del-Rio M, 2010; Cruz LC,2016).

De fato, o CA (um composto fenólico típico) e o CAF (um alcaloide típico) são conhecidos por seu potencial antioxidante (Suh HJ,2017; Machado ML,2019), bem como por sua capacidade de melhorar a função mitocondrial (Elekofehinti OO, 2019; Samadi M, 2019). Além disso, a

atividade biológica do CA e CAF também pode ser explicada por outros mecanismos além do antioxidante. Os mecanismos de ação mais importantes da CAF envolvem antagonismo dos receptores de adenosina, inibição da fosfodiesterase, modulação da ação do receptor de GABA, regulação dos níveis intracelulares de cálcio e também inibindo de maneira não competitiva a atividade da AChE (Pohanka M, 2013; Monteiro JP, 2016). Importante ressaltar, que embora o tratamento com CA e CAF resultem numa inibição da atividade da AChE nas moscas, isto acaba sendo benéfico, pois é, provavelmente, uma inibição reversível (Oboh G, 2013; Akomolafe SF, 2017), enquanto a inibição causada pelo MA e MMA, acaba sendo prejudicial, provavelmente por ser uma inibição irreversível. Encontramos, também, que a exposição ao MA ou MMA resultou em comprometimento/disfunção no sistema colinérgico (Connop BP, 1997; Gabbi P, 2017), enquanto que CA e/ou CAF foram relatados em proteger significativamente contra o comprometimento colinérgico (Oboh G, 2013; Akomolafe SF, 2017). Baseado no exposto, sugerimos que CA e/ou CAF possam ser considerados em tratamentos de doenças neurodegenerativas em que a inibição da AChE é empregada, pois, além de sua ação anticolinesterase, eles também apresentam atividade antioxidante.

Portanto, apesar da escassez na literatura científica sobre o número acumulado de moscas que tiveram óbito, bem como sobre mudanças locomotoras e comportamentais após a exposição com MA e MMA, podem ser consideradas, pelo menos em parte, responsáveis pela atividade protetora do extrato bruto de *I. paraguariensis* relatado anteriormente (Portela, JL,2019) e também podem ser consideradas uma estratégia terapêutica em condições associadas ao acúmulo de MA ou MMA.

6. Conclusões

Baseado nos resultados apresentados no artigo: "*Ilex paraguariensis* Attenuates Changes in Mortality, Behavioral and Biochemical Parameters Associated to Methyl Malonate or Malonate Exposure in *Drosophila melanogaster*" pode-se concluir que:

- ✓ O extrato aquoso de *Ilex paraguariensis* (IP) possui na sua composição diversos compostos bioativos como ácido gálico, catequinas, ácido clorogênico, ácido caféico, cafeína, epigalocatequina, rutina, quercetina e kaempferol.

- ✓ O extrato de IP foi eficaz em diminuir a mortalidade e os danos locomotores e comportamentais das moscas expostas ao ácido malônico (MA) e ácido metilmalônico (MMA).

- ✓ A co-exposição com o extrato IP, reduziu os níveis de peroxidação lipídica (TBARS), normalizou os níveis de NPSH, MTT, Resazurina, protegeu a inibição da atividade da SOD, CAT e AChE causados pela exposição ao MA e MMA.

Baseado nos resultados apresentados no manuscrito: "Caffeic acid and caffeine attenuates the toxicity associated with malonic or methylmalonic acids exposure in *Drosophila melanogaster*" pode-se concluir que:

- ✓ O Ácido Cafeico (CA) e/ou Cafeína (CAF) foram eficazes em diminuir a mortalidade e os danos locomotores e comportamentais das moscas expostas ao ácido malônico (MA) e ácido metilmalônico (MMA).

- ✓ A co-exposição com CA e/ou CAF, reduziu os níveis de peroxidação lipídica (TBARS), normalizou os níveis de NPSH, MTT, Glicose e Triglicerídeos totais, preveniu a inibição na atividade da SOD, CAT e AChE, os quais estavam alterados pela exposição ao MA e MMA.

7. Perspectivas Futuras

- Testar a capacidade do extrato de IP, bem como do ácido cafeico e da cafeína, em prevenirem (protocolo de pré-exposição aos mesmos) ou reverterem (protocolo de pós-exposição aos mesmos) a toxicidade associada aos MA e MMA.

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