Campus São Gabriel

Nathane Rosa Rodrigues

Potencial protetor do Extrato de *Psidium guajava* frente à toxicidade induzida pelo organosfosforado Clorpirifós em *Drosophila melanogaster*.

São Gabriel

2015

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Dissertação apresentada ao programa de Pós-graduação Stricto sensu em Ciências Biológicas da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Mestre em Ciências Biológicas.

Orientador: Jeferson Luis Franco

São Gabriel

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RESUMO

Clorpirifós (CP) é um inseticida organofosforado amplamente utilizado no controle de pragas agrícolas e domésticas. O principal dano causado pelo CP é a neurotoxicidade induzida pela inibição da enzima acetilcolinesterase, o que ocasiona um aumento no neurotransmissor acetilcolina e promove uma hiperexitação no sistema nervoso central e junções musculares, levando a perturbações do funcionamento fisiológico. A exposição ocupacional é uma das principais formas de intoxicação humana por organofosforados e as terapias atuais para estes compostos não são totalmente eficientes. Nesse sentido a procura por compostos capazes de reverter esses danos tem se intensificado e alguns estudos têm focado seus esforços sobre os efeitos de proteção de plantas ou compostos naturais em várias condições neuropatológicas. *Psidium guajava* é uma planta amplamente utilizada na medicina popular e a sua atividade antioxidante foi descrita, no Brasil as folhas e os frutos são utilizados para a anorexia, cólera, diarréia, problemas digestivos, disenteria, insuficiência gástrica, inflamação das membranas mucosas, laringite, problemas de pele, dor de garganta, úlceras, entre outros. Neste estudo foi avaliado o potencial antioxidante e protetor do extrato hidroalcoólico de *P. guajava* (HEPG) contra a toxicidade induzida por CP na mosca da fruta *Drosophila melanogaster*. A atividade antioxidante de HEPG *in vitro* foi confirmada pelos ensaios de ABTS, DPPH, fenóis totais e FRAP. A exposição das moscas ao CP causou aumento da mortalidade, deficiências locomotoras e inibição da acetilcolinesterase. Moscas expostas ao CP apresentaram aumento de ROS e peroxidação lipídica, acompanhado por uma diminuição significativa na viabilidade mitocondrial. Como resposta ao aumento do estresse oxidativo, moscas expostas ao CP mostraram aumento da atividade da GST e nos níveis de GSH. A expressão de mRNA de NRF2 e MPK2 (que codifica $p38^{MAPK}$ em *D. melanogaster*) também foram significativamente super regulados. HEPG foi capaz de restaurar todos os danos e alterações bioquímicas/moleculares causados pelo CP. Os nossos resultados mostram pela primeira vez o potencial efeito protetor de *P. guajava* contra a toxicidade causada por clorpirifós, sugerindo a *Psidium guajava* como um tratamento alternativo adjunto para o envenenamento por compostos organofosforados.

Palavras-chave: Clorpirifós, *Psidium guajava, Drosophila melanogaster,* estresse oxidativo, NRF2, $p38^{MAPK}$.

ABSTRACT

Chlorpyrifos (CP) is an organophosphate insecticide widely used for control agricultural and household pests. The main damage caused by the CP is the neurotoxicity induced by inhibition of the enzyme acetylcholinesterase, which causes an increase in the neurotransmitter acetylcholine and promotes hiperexitação the central nervous system and muscle junctions, leading to disruption of physiologic function. Occupational exposure is a major form of human poisoning by organophosphates and current therapies for these compounds are not fully efficient. In this sense the search for compounds that can reverse this damage has intensified and some studies have focused their efforts on plants protection purposes or natural compounds in various neuropathological conditions. *Psidium guajava* is a plant widely used in popular medicine and its antioxidant activity was described in Brazil leaves and fruit are used for anorexia, cholera, diarrhea, digestive problems, dysentery, gastric insufficiency, inflammation of mucous membranes, laryngitis, skin problems, neck pain, ulcers, among others. In this study we evaluated the antioxidant and protective potential of the hydroalcoholic extract of *P. guajava* (HEPG) against CP induced toxicity in the fruit fly *Drosophila melanogaster*. HEPG *in vitro* antioxidant activity was confirmed by ABTS, DPPH, Total Phenolics and FRAP assays. The exposure of flies to CP caused increased mortality, locomotor deficits and inhibition of acetylcholinesterase. Flies exposed to CP presented elevated ROS and lipid peroxidation which was accompanied by a significant decrease in mitochondrial viability. As a response to increased oxidative stress, CP exposed flies showed increased in GST activity and GSH levels. The mRNA expression of NRF2 and MPK2 (which encodes *D. melanogaster* $p38^{MAPK}$) were also significantly up-regulated. HEPG was able to restore all the damage and biochemical/molecular alterations caused by CP. Our results show for the first time the potential of *P. guajava* protective effect against the toxicity caused by Chlorpyrifos, suggesting *Psidium guajava* as an adjunct alternative treatment for poisoning by organophosphorus compounds.

Key words: Chlorpyrifos, *Psidium guajava, Drosophila melanogaster,* oxidative stress, NRF2, $p38^{MAPK}$.

APRESENTAÇÃO

No item **INTRODUÇÃO**, consta uma breve revisão da literatura sobre os temas trabalhados nesta dissertação. A metodologia realizada e os resultados obtidos que fazem parte desta dissertação estão apresentados sob a forma de manuscrito, que se encontra no item **MANUSCRITO**. No mesmo constam as seções: Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas. O item **CONCLUSÕES**, encontrado no final desta dissertação, apresenta interpretações e comentários gerais sobre os resultados do manuscrito presentes neste trabalho. As **REFERÊNCIAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO** e **CONCLUSÕES** desta dissertação.

LISTA DE ABREVIATURAS E SIGLAS

ABTS- Ácido 2,2'-bis(3-etilbenzotiazolina-6-sulfonico/2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

ACh- Acetilcolina/Acetylcholine

AChE- Acetilcolinesterase/Acetylcholinesterase

ARE- Antioxidant response element

CAT- Catalase

CP- Clorpirifós/ Chlorpyrifos

DCF-DA- 2',7'-diacetato de diclorofluoresceína

DNA- Ácido desoxirribonucléico

DPPH- 2,2-Difenil-1-Picrilhidrazil/2,2-Diphenyl-1-Picrylhydrazyl

EHPG- Extrato hidroalcoólico de *Psidium guajava*

ERA- Elemento de resposta antioxidante

ERK- Quinase regulada por sinal extracelular/Extracellular signal regulated kinase

ERN- Espécies Reativas de Nitrogênio

ERO- Espécies Reativas de Oxigênio

FRAP- Poder Antioxidante pela Redução de Ferro/Ferric ion reducing antioxidant power

GPDH- Glicerol 3 fosfato desidrogenase/Glycerol 3 phosphate dehydrogenase

GSH- Glutationa reduzida/Glutathione reduced

GST- Glutationa S-transferase/ Glutathione S-transferase

HEPG- Hydroalcoholic Extract of *Psdium guajava*

HO^{-1}- Heme oxigenase I/Heme oxygenase I

JNK- Quinase c- Jun N-terminal/c-Jun N-terminal Kinase

Keap-1- Kelch-como ECH associada a proteína 1/Kelch-like ECH-associated protein 1

MTT- (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

NRF2- Fator nuclear eritróide 2/Nuclear factor (erythroid-derived 2)-like 2

OP- Organofosforados/organophosphates

P38 MAPK- Proteínas quinases P38 ativadas por mitógenos/P38 mitogen-activated protein kinases

Pg- *Psidium Guajava*

PKC- Proteína quinase C/Protein Kinase C

RNA- Ácido ribonucléico

ROS- Reative Oxygen Species

SOD- Superóxido dismutase/Superoxide dismutase

TBOOH- Hidroperóxido de terc-butila/ Tert-butyl hydroperoxide

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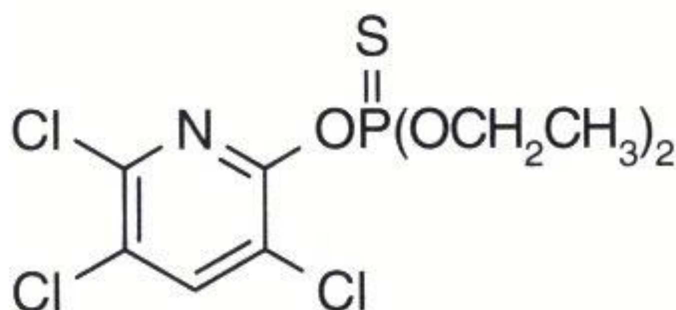
1. INTRODUÇÃO

1.1 Clorpirifós

A contaminação ambiental gerada pelo uso de agroquímicos tem se intensificado devido ao aumento nas atividades agrícolas. Além da poluição ambiental grave, o uso de pesticidas em lavouras representa sérios problemas à saúde humana (PERIS-SAMPEDRO et al., 2015).

O clorpirifós (O,O-diethyl O-3,5,6-trichloro-2-pyridylphosphorothioate) é classificado como inseticida de Classe II, pertencente ao grupo dos organofosforados. A família dos organofosforados representa um grande número de pesticidas e inseticidas, devido à sua baixa persistência e alta eficácia, eles são amplamente empregadas na agricultura, além disso, alguns exibem toxicidade aguda elevada em seres humanos (MARRAZA, 2014), constituindo um dos principais responsáveis por intoxicações ocupacionais no campo.

Figura 1. Estrutura química do Clorpirifós.



Fonte: Anvisa http://www4.anvisa.gov.br/base/visado...

O clorpirifós (CP) é um dos inseticidas mais utilizados em todo mundo (PERIS-SAMPEDRO, et al.,2015), no Brasil encontra-se entre os principais inseticidas comercializados, indicado para aplicações via irrigação por aspersão (quimigação), age sobre os insetos por contato, ingestão e inalação (PENA et al. 2003).

O principal dano causado pelo CP é a neurotoxicidade induzida pela inibição da enzima acetilcolinesterase, o que ocasiona um aumento no neurotransmissor acetilcolina e promove uma hiperexitação no sistema nervoso central e junções musculares, levando a perturbações do funcionamento fisiológico (YU et al, 2008). Além disso, a exposição a estes compostos

podem levar a anomalias imunológicas (TRASHER, HEUSER, BROUGHTON, 2002), danos aos tecidos (JETT & NAVOA, 2000) e induzir o estresse oxidativo através da produção de espécies reativas de oxigênio (ERO), depleção de defesas antioxidantes e comprometimento de enzimas antioxidantes (GOEL, DANI, DHAWAN, 2005).

O tratamento das intoxicações por CP baseia-se principalmente no uso de atropina, um antídoto sintomático e, com menor frequência, das oximas (pralidoxima) (PETER, SUDARSAN, MORAN, 2014). Substâncias antioxidantes que possam reverter a ação de ERO ou amenizar a intoxicação por organosfosforados são de suma importância. Neste sentido a procura por novos compostos naturais para aplicação biotecnológica e na saúde tem se intensificado (ANDRADE, CAMPOLINA, DIAS, 2001; WILLIAMS, SPENCER, RICE-EVANS, 2004; WAGNER et al., 2006).

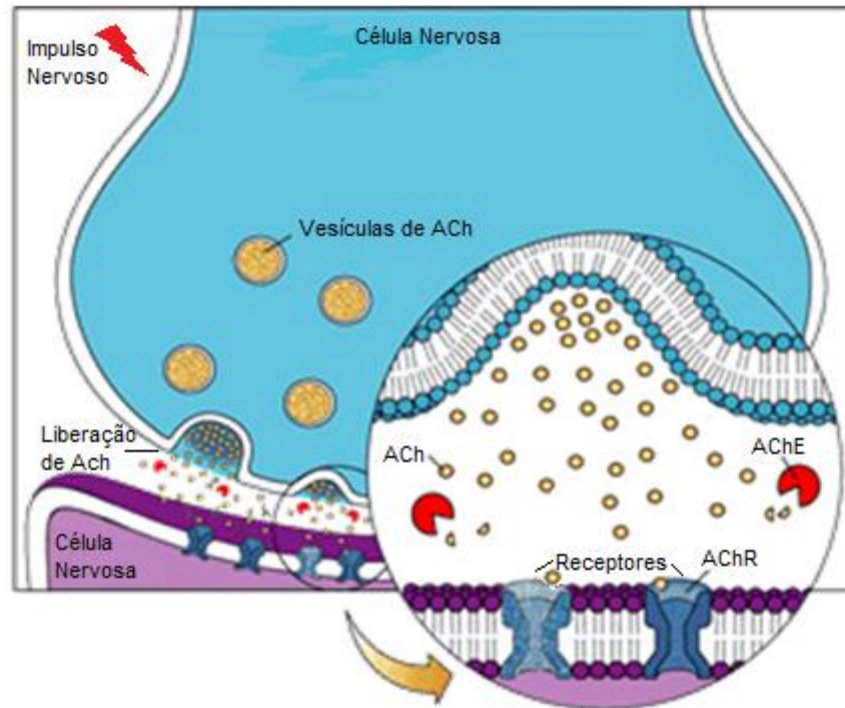
1.2 Acetilcolinesterase

A acetilcolinesterase (AChE) (EC 3.1.1.7), uma serina hidrolase, pertencente a família das colinesterases, está presente no sistema nervoso central e periférico e é responsável pela finalização da transmissão dos impulsos nervosos nas sinapses colinérgicas pela hidrólise do neurotransmissor acetilcolina (ACh) (RANG, DALE, RITTER, 2001).

A acetilcolina (ACh) é um neurotransmissor que desempenha um papel importante em todo o sistema nervoso. No sistema nervoso periférico, ACh é liberada a partir de células nervosas e se liga aos receptores, que em última análise provoca a contração do músculo. No sistema nervoso central, a ACh está envolvida em processos complexos, tais como cognição, aprendizagem e memória (SARTER & BRUNO, 1997). Antes que ocorra nova liberação de acetilcolina, a molécula previamente liberada deve ser hidrolisada pela AChE a fim de evitar o excesso de transmissão nervosa, que pode levar a problemas de funcionamento do corpo (PATRICK, 2001).

A inibição da AChE, resulta em níveis elevados de ACh, o que provoca uma variedade de efeitos adversos, incluindo aumento da salivação, lacrimejamento, incontinência urinária e fecal, distúrbio gastrointestinal e paralisia muscular. Estes efeitos adversos estão associados com medicamentos inibidores reversíveis de AChE, juntamente com inibidores irreversíveis, como pesticidas e agentes de guerra química. Os inibidores da acetilcolinesterase podem ser sintetizados ou encontrados em uma variedade de vegetais (HOUGHTON et al., 2006; MUKHERJEE et al., 2007).

Figura 2. Ilustração de uma junção entre células nervosas mostrando a degradação da Acetilcolina pela acetilcolinesterase- A acetilcolina é armazenada em vesículas dentro da célula. Quando a célula recebe o impulso nervoso o neurotransmissor ACh é secretado na fenda entre as células nervosas em direção aos receptores a fim de regenerar o impulso nervoso e dar continuidade a transmissão. Na fenda entre as células nervosas a AChE recolhe a acetilcolina, interrompendo o processo de interação da ACh com o receptor para evitar o excesso de transmissão nervosa. A inibição da enzima acetilcolinesterase promove o acúmulo de acetilcolina na fenda sináptica, causando um colapso do sistema nervoso.



Fonte: Modificado de http://4.bp.blogspot.com/_4OE6-Q2PDhs...

A inibição irreversível da AChE é o mecanismo principal de ação de muitos organofosforados, incluindo pesticidas e agentes nervosos altamente tóxicos. Estes compostos exercem a sua toxicidade aguda através fosforilação do sitio ativo de serina da AChE (HOLMSTEDT, 1959; TAYLOR et al., 1995).

1.3 Estresse Oxidativo e Sistema de Defesa Antioxidante

Os radicais livres são agentes oxidantes caracterizados como espécies atômicas ou moleculares que possuem um ou mais elétrons desemparelhados na sua orbital externa, tornando-as espécies altamente reativas que agem como eletrófilos (GILLHAN et al., 1997). Dentre os oxidantes mais importantes envolvidos em processos patológicos estão às espécies reativas de oxigênio (ERO) e as espécies reativas de nitrogênio (ERN) (GILLHAM, PAPACHRISTODOULOU, THOMAS, 1997; SIES, 1997).

Em condições normais, as ERO podem desempenhar importante papel fisiológico na regulação da resposta imunológica, participando do processo fagocítico de defesa contra infecções e atuando como fatores de transcrição na sinalização intracelular, induzindo a apoptose (HALLIWELL, 1994; BIESALSKI, 2002). Porém, quando produzidas em excesso podem ocasionar uma grande variedade de reações deletérias no organismo podendo assim lesionar diferentes estruturas celulares como exercer efeitos citotóxicos sobre os fosfolipídios de membrana resultando em peroxidação lipídica, oxidação protéica e alterações na atividade das enzimas antioxidantes (ALLEN, 1998; ANANTHAN et al., 2003; SEVEN et al., 2004; OGA, CAMARGO, BATISTUZZO, 2008). No entanto, esse aumento na sua produção e/ou diminuição na sua eliminação gera um desequilíbrio fisiológico, e caracteriza o estresse oxidativo que quando aliado a exposição a agentes tóxicos, como agroquímicos, leva a mutações, danos a biomembranas e biomoléculas (proteínas, ácidos nucléicos e lipídios) ocasionando, consequentemente, morte celular por apoptose (FINKEL & HOLBROOK, 2000; HALLIWELL & GUTTERIDGE, 2000; JUNQUEIRA & RAMOS, 2005).

O estresse oxidativo decorre de um desequilíbrio entre a geração de compostos oxidantes e a atuação dos sistemas de defesa antioxidante, os mecanismos de defesa antioxidantes têm o objetivo de limitar os níveis de ERO e ERN e controlar a ocorrência de danos (BARBOSA et al., 2010). Fisiologicamente, é muito baixa a concentração de ERO dentro das células, devido a presença de sistemas antioxidantes celulares que catalisam a remoção ou impedem a formação de ERO (OGA, CAMARGO, BATISTUZZO, 2008). No decorrer da evolução, os seres vivos desenvolveram mecanismos adaptativos capazes de lhes permitirem co-existir com a exposição a ERO. Além dos antioxidantes naturais oriundos da dieta, as células possuem a capacidade de detoxificação de ERO específicas, ajudando a manter os níveis fisiológicos destas espécies reativas.

Entre os mecanismos de defesa antioxidante celular, sistemas não enzimáticos como o tripeptídeo glutationa (GSH) e enzimas antioxidantes como a superóxido dismutase (SOD), glutationa peroxidade (GPx), glutationa S-transferase (GST) e catalase (CAT) podem ser destacados (HALLIWELL & GUTTERIDGE, 2007, OGA, CAMARGO, BATISTUZZO, 2008). Quando há limitação nestes sistemas antioxidantes, podem ocorrer diversas lesões cumulativas, levando a disfunção e morte celular (ATOUI et al., 2005).

A enzima CAT atua na remoção de H_2O_2 estando relacionada a diversas patologias ligadas ao estresse oxidativo como neoplasias e doenças neurodegenerativas. Esta enzima possui o mais alto poder de catálise conhecido dentre as enzimas, dismutando o H_2O_2 em oxigênio e água diminuindo assim sua permanência nas células (ROJKIND et al., 2002; KLICHKO, RADYUK, ORR, 2004). A enzima SOD possui por função catalisar a dismutação do radical superóxido; a GPx, catalisar a redução de H_2O_2 através da glutationa reduzida (GSH) para formação de água e glutationa oxidada (GSSH) (OGA, CAMARGO, BATISTUZZO, 2008). A GST causa a polarização de xenobióticos, tornando-os mais solúveis para sua eliminação pelos organismos, como, por exemplo, em insetos a maioria dos estudos relacionados à GST diz respeito ao envolvimento desta enzima no aumento da resistência destes animais aos inseticidas (TOWNSEND, TEW, TAPIERO, 2003) As GSTs promovem a conjugação de GSH com produtos endógenos causadores de danos oxidativos, como radicais hidroxila citotóxicos, peróxidos de lipídios de membrana e produtos de degradação oxidativa do DNA, visando sua detoxificação (YAMUNA, BHAVAN, GERALDINE, 2012)

O estresse oxidativo tem demonstrado possuir um papel importante nos mecanismos implicados à toxicidade do clorpirifós principalmente pela produção de ERO (CHIAPELLA, et al., 2013)

1.4 Via de Sinalização de NRF2 e P38 ^{MAPK}

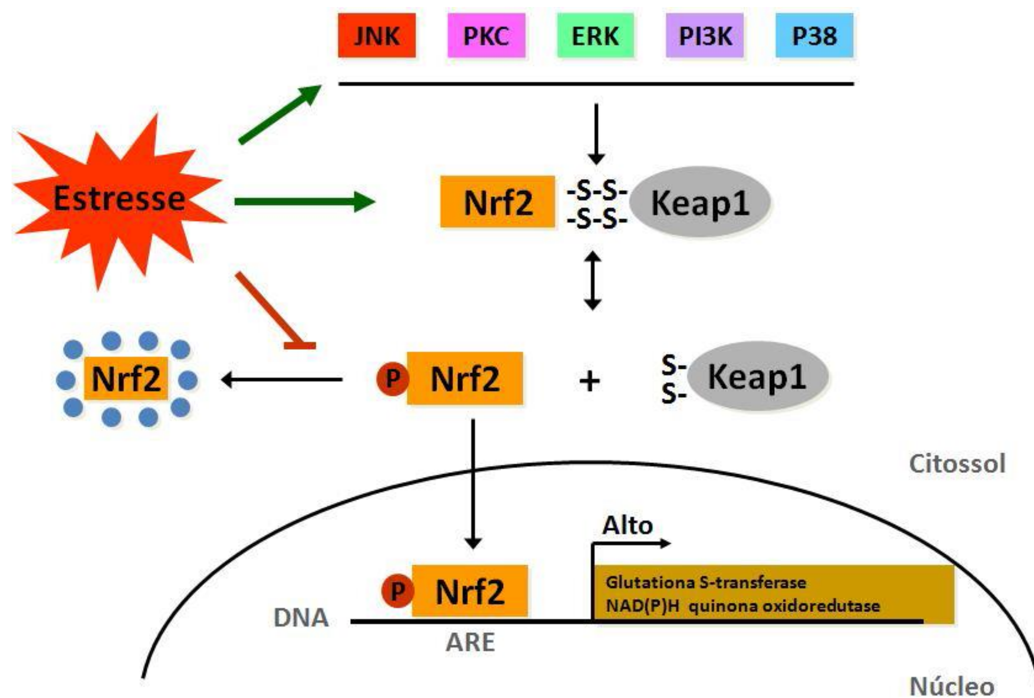
A regulação de sistemas antioxidantes e de resposta celular ao estresse oxidativo envolve diversas moléculas sinalizadoras, entre elas o NRF2 (fator nuclear eritróide 2), fator de transcrição que regula a expressão de uma variedade de enzimas mediante ligação ao ERA (elemento de resposta antioxidante) (CHEN et al., 2015). A partir da interação do NRF2 com o ERA são expressas enzimas antioxidantes e de detoxificação de fase II (GST, GPX, SOD, TRX, entre outras) envolvidas no metabolismo de xenobióticos eletrofílicos (OSBRURN & KENSLER, 2008).

Em condições normais o NRF2 está localizado no citoplasma formando um complexo inativo com a proteína Keap-1 que suprime a atividade transcricional do NRF2. A exposição a estressores como ERO promove a liberação do NRF2 do Keap-1 o que permite sua translocação para o núcleo onde ocorre a interação com o ERA, ativando a transcrição (COPPLE et al., 2008).

A interação de Keap-1 com ERO permite a oxidação de resíduos de cisteína críticos na manutenção da ligação de Keap-1 ao NRF2. A liberação deste fator pode ser facilitada pela fosforilação de resíduos específicos de serina ou treonina de forma que NRF2 dissocia-se do complexo. (LEVONEN et al, 2004; FOURQUET et al, 2010)

A via do NRF2 é considerada a mais importante na defesa celular contra o estresse oxidativo (ZANG, 2006; KOBAYASHI & YAMAMOTO, 2006; COPPLE et al, 2008). O estresse oxidativo pode agir diretamente no complexo NRF2-Keap-1 ou alternativamente ativar as quinases (quinase c- Jun N-terminal (JNK); proteína quinase C (PKC); quinase regulada por sinal extracelular (ERK); MAP quinase p38 (p38); fosfatidilinositol-3 quinase (PI3K)) causando a liberação de NRF2 do seu estado inibitório (Son, Camandola, Mattson, 2008).

Figura 3. Modelo de ativação de Nrf-2 mediada por estresse oxidativo. O estresse oxidativo pode agir diretamente no complexo Nrf-2-Keap-1, ou alternativamente ativar as quinases PI3K, p38, ERK, PKC e JNK, causando a liberação de NRF-2 do seu estado inibitório. O fator de transcrição NRF-2 ativado, transloca-se para o núcleo, onde este se liga ao elemento responsivo ao estresse (ERA) na região promotora de genes alvo, tais como genes antioxidantes ou de detoxificação de fase II.



Fonte: Adaptado de SON, CAMANDOLA, MATTSON, 2008.

Proteínas quinases ativadas por mitógenos (MAPKs) compreendem um grande número de quinases serina/treonina envolvidas na regulação da proliferação, diferenciação, adaptação ao estresse e apoptose. Em *Drosophila melanogaster*, a via da MAPK é conhecida por estar envolvida em numerosos processos durante o desenvolvimento normal e na regulação da resposta imunitária (STRONACH & PERRIMON, 1999). MAPKs são efetoras dowstream nas respostas antioxidantes e suas atividades são manifestadas na ativação de vários fatores de transcrição incluindo NRF2 (LIMÓN-PACHECO et al, 2002). ERK e $P38^{MAPK}$ também regulam positivamente a atividade de NRF2 para iniciar a transcrição de genes antioxidantes (ZIPPER & MULCAHY, 2000)

1.5 *Psidium Guajava*

Alguns estudos têm focado seus esforços sobre os efeitos de proteção de plantas ou compostos naturais em várias condições neuropatológicas, particularmente importante é o fato de que tem sido evidenciado que as plantas/compostos naturais são capazes de neutralizar a neurotoxicidade induzida por diversos agentes tóxicos sob condições *in vivo* (GUPTA & FLORA, 2006; XU et al., 2005)

O Brasil é o país com a maior biodiversidade de plantas do mundo, número superior a 55 mil espécies descritas, o que corresponde a 22% do total mundial. Esta rica biodiversidade é acompanhada por uma longa aceitação de uso de plantas medicinais e conhecimento tradicional associado (BRASIL, decreto nº 5.813, 2006).

Psidium guajava L., pertencente à família Myrtaceae, é conhecida popularmente como goiabeira. Espécie arbustiva a arbórea de pequeno porte, nativa da América tropical é empregada na medicina tradicional, as folhas de *P. guajava* são empregadas popularmente para tratar desordens gastrointestinais, prática herdada originariamente da medicina asteca no México (LOZOYA et al., 2002). Infusos preparados com folhas frescas ou desidratadas são indicados para diarréia, disenteria, flatulência e cólica abdominal. O efeito espasmolítico e antidiarréico está relacionado com o conteúdo em flavonóides, em particular de derivados da quercetina, que atuam como antagonistas do cálcio nas fibras musculares lisas (MORALES & LOZOYA, 1994). Adicionalmente, comprovaram-se as atividades antimicrobiana, antitussígena (JAIARJ et al., 1999), sedativa (LUTTERODT & MALEQUE, 1988), antioxidante (ANJANEYULU & CHOPRA, 2004; WOODMAN & CHAN, 2004) e antiproliferativa para células cancerígenas (MANOSROI, DHUMTANOM, MANOSROI, 2005).

Figura 4. ***Psisium guajava.*** A- Fruto da goiaba vermelha. B- Folhas de Goiabeira

A

B

Fonte: (A) http://www.brasilescola.com/upload/e/... (B) http://www.clickmudas.com.br/media/ca...

Um estudo fitoquímico de folhas de goiabeira demonstrou muitos compostos incluindo lipídios, hidratos de carbono, proteínas, vitaminas, óleos essenciais, taninos, saponinas, flavonóides, esteróis e triterpenos (OKUDA et al., 1987; ABDEL et al., 2004). Estes compostos polifenólicos são considerados como sendo as principais substâncias que fornecem à planta elevadas propriedades antioxidantes (MUIR, 1997; FAURE et al., 1990).

1.6 *Drosophila melanogaster*

Grande parte do entendimento dos mecanismos que levam ao desenvolvimento das doenças tem sido obtido graças aos modelos experimentais realizados com animais, sejam eles vertebrados ou invertebrados. O modelo experimental se faz valer pela capacidade deste em representar com fidelidade o fenômeno natural. Esse modelo animal, obrigatoriamente, deve permitir a avaliação de fenômenos biológicos naturais ou comportamentais induzidos, que possam ser comparados aos fenômenos em questão que ocorrem naturalmente. (FERREIRA, HOCHMAN, BARBOSA, 2005).

O grande número de pesquisas realizadas a partir da utilização de insetos contribui para a compreensão dos mecanismos biológicos existentes em quase todos os seres vivos (MORALES, 2008). O uso de insetos como plataformas de pesquisas de toxicidade ambiental tem sido encorajado uma vez que eles respondem à contaminantes de um modo semelhante em comparação com roedores e contribui para a compreensão dos mecanismos biológicos existentes em quase todos os organismos vivos (AHMAD, 1995).

Um invertebrado de grande utilização em estudos de toxicologia é a *Drosophila melanogaster*, durante a última década, este gênero emergiu como um dos melhores organismos para estudos de doenças humanas e pesquisas toxicológicas (SIDDIQUE et al., 2005). Dessa forma, tem sido utilizado não só porque as populações naturais são resistentes às toxinas lançadas pelos humanos no ambiente, mas também devido às vantagens advindas de seu ciclo biológico, como o rápido desenvolvimento e a fácil manipulação. Outra vantagem é a ausência de mitose celular nas moscas em fase adulta, desse modo, a mosca na fase adulta tem envelhecimento sincronizado das suas células, exceto as células nas gônadas e algumas no intestino (JIMENEZ-DEL-RIO, MARTINEZ, PARDO, 2009).

Figura 5. ***Drosophila melanogaster*****.** Modelo experimental.

Fonte: https://encrypted-tbn0.gstatic.com/im...

Sendo assim *a Drosophila melanogaster* é um modelo conveniente para responder questões de como o organismo se defende contra o excesso de um determinado poluente, uma vez que muitos aspectos da homeostase, principalmente dos metais, são conservados entre as moscas e os humanos (YEPISKOPOSYAN et al., 2006).

2. OBJETIVOS

2.1 Objetivo Geral

Avaliar o potencial antioxidante do extrato hidroalcoólico de *Psidium guajava pomífera L.* frente a toxicidade do organofosforado Clorpirifós, no modelo experimental *Drosophila melanogaster.*

2.2 Objetivos específicos

- ✓ Avaliar o potencial antioxidante do extrato hidroalcoólico de *Psidium guajava*.
- ✓ Avaliar o efeito da ingestão de Clorpirifós sobre a viabilidade de *Drosophila melanogaster*;
- ✓ Analisar a atividade de enzimas antioxidantes em *D. melanogaster* após a ingestão de CP em conjunto com o extrato de *P. guajava*;
- ✓ Determinar a indução e/ ou reversão de lipoperoxidação em *D. melanogaster* frente aos tratamentos, bem como a produção de espécies reativas de oxigênio;
- ✓ Avaliar a expressão de genes relacionados ao estresse e transdução de sinal.

3. RESULTADOS

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de manuscrito. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas, encontram-se no manuscrito, **o qual está disposto na forma em que foi submetido para publicação.**

Manuscrito submetido à revista Arabian Journal of Chemistry. Fator de Impacto 3.7

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Involvement of p38MAPK and NRF2 signaling pathways in the toxicity induced by chlorpyrifos in *Drosophila melanogaster*: Protective effects of *Psidium guajava pomífera L.* (Myrtaceae) hydroalcoholic extract

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Abstract

Chlorpyrifos (CP) is an organophosphate insecticide widely used in the control of agriculture and domestic pests. Occupational exposure is a major form of human poisoning by organophosphates and current therapies for these compounds are not completely efficient. *Psidium guajava* is a plant widely used in folk medicine and its antioxidant activity has been described. In this study we evaluated the antioxidant and protective potential of the hydroalcoholic extract of *P. guajava* (HEPG) against CP induced toxicity in the fruit fly *Drosophila melanogaster*. HEPG *in vitro* antioxidant activity was confirmed by ABTS, DPPH, Total Phenolics and FRAP assays. The exposure of flies to CP caused increased mortality, locomotor deficits and inhibition of acetylcholinesterase. Flies exposed to CP presented elevated ROS and lipid peroxidation which was accompanied by a significant decrease in mitochondrial viability. As a response to increased oxidative stress, CP exposed flies showed increased GST activity and GSH levels. The mRNA expression of NRF2 and MPK2 (which encodes *D. melanogaster* $p38^{MAPK}$) were also significantly up-regulated. HEPG was able to restore all the damage and biochemical/molecular alterations caused by CP. Our results show for the first time the *P. guajava* potential protective effect against the toxicity caused by chlorpyrifos.

Keywords: organophosphate compound, oxidative stress, natural compounds, protective effects.

1. *Introdution*

Organophosphate pesticides (OP) are neurotoxic agents widely used for agricultural, industrial, household and warfare purposes. They are active constituents of several household insecticides being still widely used in developing countries despite its controlled use (Soltaninejad and Shadnia, 2014). Chlorpyrifos (CP) is an agrochemical belonging to the class of OP widely used in agriculture as an insecticide due to its lower persistency and higher biodegradability as well as its broad spectrum of activity against arthropods (Breslinet al., 1996). Occupational exposure is a major form of human contamination by organophosphates (Hernández et al., 2008).

Chlorpyrifos exerts its toxicity by inhibiting the enzyme acetylcholinesterase (AChE), which is involved in the control of cholinergic neurotransmission (Li and Han, 2004; Yu et al., 2008). Inhibition of AChE leads to accumulation of the neurotransmitter acetylcholine in the synaptic cleft and promotes hyperexcitation at central nervous system and neuromuscular junctions, causing disturbance of normal physiological functioning (Chakraborty et al., 2009). Another mechanism of toxicity attributed to the CP is the generation of reactive oxygen species (ROS) and depletion of antioxidant defense systems, characterizing an oxidative stress condition (Jett and Navoa, 2000; Goel et al., 2005). Under normal physiological conditions (Bachschmid et al., 2013), ROS are important for normal cell function, but in high amounts they can lead to cellular and tissue damage (Gupta et. al., 2010). The mechanisms of cell response to oxidative stress induced by CP and other OP compounds are not fully understood and the understanding of such mechanisms is key factor in the development of effective therapeutic strategies.

The organisms have systems responsible for protecting against damage caused by reactive species. The signaling pathway of NRF2 transcription factor (nuclear factor erythroid 2-like 2) is considered the utmost defense system against oxidative stress and toxicants (Zang et al., 2015). NRF2 is a transcription factor that controls both basal and inducible expression of a variety of antioxidant and detoxification enzymes, including glutathione S-transferase, superoxide dismutase, catalase, thioredoxin reductase, glutathione peroxidase and others (Chen et al.. 2015).

Treatment of OP poisoning is based primarily on the use of benzodiazepines atropine and oximes (Peter et al., 2014). However, these therapies are not completely effective and several aspects other than cholinesterase inhibition (e.g. oxidative stress) caused by OP poisoning may not be significantly influenced by current therapeutic strategies. Therefore,

there is a need for the search of alternative treatments for the mitigation of OP toxicity. In this sense the search for natural compounds with antioxidant and protective activity for biotechnological and health applications has been intensified (Williams et al., 2004; Wagner et al., 2006).

In a worldwide comparison, Brazil has the highest plant biodiversity, with an estimated over 20% of the total number of botanical species on the planet. ***Psidium guajava pomífera L. (Myrtaceae)***, popularly known as guava, is found throughout South America, at tropical and subtropical regions and adapts to different climatic conditions (Gutiérrez et al., 2008).

The medicinal properties of guava have been investigated by scientists since the 1940's (Gutiérrez et al., 2008). *P. Guajava* is widely used in folk medicine against several conditions such as diarrhea, cramps, colitis, dysentery anorexia, cholera, digestive problems, dysentery, gastric insufficiency, inflammation, laryngitis, skin problems, ulcers, and others (Cybele et al., 1995; Holetz et al., 2002; Gutiérrez et al., 2008). The antioxidant activity of guava has been described and attributed mainly to constituents found in the leaves and fruits as phenolic compounds such quercetin, ascorbic acid, gallic acid and caffeic acid (Jimenez et al., 2001).

In the present study we aimed to evaluate the antioxidant and protective potential of the hydroalcoholic extract of *Psidium guajava* (HEPG) against organophosphate chlorpyrifos induced toxicity in the fruit fly *Drosophila melanogaster*.

Drosophila melanogaster is a convenient animal model for answering questions such as how organisms defend themselves against certain pollutants, since many aspects of homeostasis are conserved between flies and human (Yepiskoposyan et al., 2006) and has emerged as one of the most suitable organisms to study human disease and toxicological condition (Siddique et al., 2005). It has been used not only because natural populations of flies are resistant to toxins released by humans into the environment, but also due to the advantages arising from its biological cycle, as the rapid development and easy handling. Another advantage relies on the fly genome, which is completely characterized and the absence of cellular mitosis in flies adulthood, resulting in synchronized aging of its cells (Jimenez-Del-Rio et al., 2009).

2. *Material and Methods*

2.1. *Chemicals*

Chlorpiryfos Pestanal® (45395), sucrose (S5016), Reduced glutathione (GSH; G4251-5G), tetramethylethylenediamine (TEMED; T9281), Quercetin (Q4951), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; M2128-1G), 5,5-dithiobis (2-nitrobenzoic acid) DTNB (D8130), acetylthiocholine iodide (A5751), 1-Chloro, 2,4-dinitrobenzene (CDNB; 237329), 2',7'-dichlorofluorescein diacetate (DCFH-DA; 35845), D-Manitol (m9647), K2KO4P (1110216), KH2PO4 (P0662), HEPES (Titration; H3375), Albumin from bovine serum (BSA; A6003), Resazurin sodium salt (R7017), Triton X-100 (T8532) were obtained from Sigma-Aldrich (São Paulo, SP, Brazil). SYBR Select Master Mix Applied (4472908) from Biosystems by Life Technologies, DNAse I Amplification Grade – Invitrogen (18068-15) by Life Technologies and iScript cDNA Synthesis kit (1708891) from Biorad. All other chemicals and reagents used here were of the highest analytical grade.

2.2. *Drosophila melanogaster Stock and Media.*

Drosophila melanogaster (Harwich strain) was obtained from the National Species Stock Center, Bowling Green, OH, USA. The flies were maintained in incubators at 25 ± 1°C, 12h dark-light photoperiod and 60–70% relative humidity. The basic corn meal diet was composed of cereal flour, corn flour, water, antifungal agent (Nipagin) and supplemented with dried yeast as previously described (Paula et al., 2012).

2.3. Plant material and Hydroalcoholic Extract of Psidium guajava (HEPG). The plant material of *Psidium guajava pomífera L.*, was collected in the Horto Botânico de Plantas Medicinais do Laboratório de Pesquisa de Produtos Naturais (LPPN) of Universidade Regional do Cariri (URCA), Ceará State, Brazil. The plant material was identified, and a voucher specimen was deposited in the Herbarium Dardano Andrade Lima of URCA, under #3930. The extract was prepared by immersing 482g leaves in ethanol and water (1:1) for 72 hat room temperature, which was filtered and concentrated using a vacuum rotary evaporator (model Q-344B- Quimis, Brazil) and warm water bath (model Q214M2- Quimis Brazil), obtaining a yield of crude extract of 8g.

2.4. In vitro antioxidant activity determination

2.4.1. DPPH˙ Radical Scavenging Assay.

The scavenging activity towards 2,2-diphenyl-1 picrylhydrazyl (DPPH·) radical was evaluated according to the method of Baltrušaitytė et al. (2007) with minor modifications. In brief, 100 μL of DPPH˙ (300 μM) diluted in ethanol was mixed with 20 μL of HEPG (200 μg/mL) in a 96 wells microtitre plate. The final volume of each well was adjusted to 300 μL with ethanol. Ascorbic acid was used as a positive control. The absorbance was determined at 517nm after 45 min incubation. The results were expressed as mg of ascorbic acid equivalents (AAEs) per 100 mg HEPG (Piljac-Žegaracet al., 2009).

2.4.2. $ABTS^{\cdot+}$Radical Scavenging Assay.

The antioxidant activity of HEPG in the reaction with $ABTS^{\cdot+}$radical was determined according to the method of Baltrušaitytė et al. (2007) with some modifications. ABTS·+ radical solution was generated by oxidation of solutions prepared of 1mL of 7mM 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt stock solution with 17.5 µL of 140mM potassium persulfate (K2S2O8). 200 µL of ABTS·+ solution was mixed with 10 µL of HEPG (200 µg/mL) in a microplate and the decrease in the absorbance was measured after 10 min. Ascorbic acid (1mM) was used as a positive control. The results were expressed as mg of ascorbic acid equivalents (AAEs) per 100 mg HEPG (Piljac-Žegarac et al., 2009).

2.4.3. Total Phenolics.

Phenolic compounds from HEPG samples were detected by the Folin-Ciocalteu method with minor modifications (Cruz et al., 2014). HEPG (200 µg/mL) was mixed with 35 µL 1N Folin-Ciocalteu’s reagent. After 3min, 70 µL 15% Na_2CO_3 solution was added to the mixture and adjusted to 284 µL with distilled water. The reaction was kept in the dark for 2h, after which the absorbance was read at 760 nm. Gallic acid was used as standard (10–400 µg/mL). The results were expressed as mg of gallic acid equivalents (GAEs) per 100 g HEPG.

2.4.4. Ferric Reducing Antioxidant Power (FRAP). The reducing capacity of HEPG was assayed with the original method of Benzie and Strain (1996), adjusted to analysis of extract samples. 9 µL of HEPG (200 µg/mL) was mixed with of 270 µL of freshly prepared FRAP reagent. The FRAP reagent was prepared by mixing 2.5mL of 0.3 M acetate buffer pH 3.6 with 250 µL of 10mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) solution and 250 µL of $FeCl_3 \cdot 6H_2O$. The mixture was shaken and left in a water bath for 30min and the absorbance readings were taken at 595 nm. Ammonium iron (II) sulfate hexahydrate was used to calculate

the standard curve (100–2000 μM). The reducing ability of extract was expressed as μM of Fe (II) equivalent/100 g HEPG (Cruz et al., 2014).

2.4.5. Mitochondrial activity (MTT reduction test) and ROS production (DCF-DA assay) in vitro.

For the determination of mitochondrial activity *in vitro*, the MTT test was employed and DCF-DA assay was used for monitoring ROS production. A mitochondria enriched homogenate was obtained by homogenizing 1000 flies in 8ml of mitochondrial Isolation Buffer (220 mM mannitol, sucrose 68mM, 10mM KCl, 10mM HEPES, 1% BSA) in a glass-glass tissue grinder (Kimble Chase, Mexico) and then centrifuged at 1000g for 10 min. The obtained supernatant was isolated and incubated for 1h with 500μM TBOOH and/or HEPG 3,3μg / ml. After the incubation period, the MTT and DCF-DA tests were carried out according to described elsewhere (Franco et al., 2007; Pérez-Severiano et al., 2004).

2.5. Experimental Procedure In vivo

Adult female flies (1-4 days) were left, overnight, in glass tubes containing filter paper soaked in 1% sucrose for acclimation to new diet. For the experiments, 30 flies per group were kept in glass tubes containing filter paper soaked with 250μl of each treatment solution. The experimental groups were: Control (received 1% sucrose solution only), CP 0.75ppm (diluted in 1% sucrose solution), HEPG at concentrations of 10, 20 and 50 mg/ml (diluted in 1% sucrose solution) and CP + HEPG (at concentrations previously mentioned). After the period of treatment (24h), mortality, behavioral tests, biochemical and molecular analysis were performed. Lethal chlorpyrifos concentrations were previously determined by our group (unpublished data). The LC_{50} 24h in adult female flies was 1.182 ppm, so for this study the sublethal concentration of 0.75ppm CP during 24h was chosen.

2.6. Mortality and locomotor activity

At the end of treatments, the number of dead flies was recorded and expressed as percentage of survived flies compared to the control (considered 100%). Locomotor activity was determined as negative geotaxis behavior assays (climbing ability) in both individual flies (Bland et al., 2009) and collective flies (Coulomn and Birman, 2004) with some modifications. For the individual test, a total number of 20 flies per group were anesthetized in ice and individually placed in vertical glass tubes (length 25 cm, diameter 1.5 cm) closed with cotton wool. After 30 min of recovery the flies were gently tapped to the bottom of the

tube and the time taken by each fly to climb 6 cm in the glass column was recorded. The test was repeated 3 times with 20 second intervals for each fly. To test the collective negative geotaxis, 10 flies per group were anesthetized and placed in a glass tube. After 30 minutes of recovery the flies were gently tapped to the bottom of the tube and the number of flies able to climb over a 6 cm mark in the tube was computed. The tests were repeated 3 times with 20 second intervals for each group of 10 flies. Eight groups of each treatment were counted. The results were expressed as percentage of control.

2.7. Sample preparation

After treatments, twenty flies per group were homogenized in 1000µl of mitochondrial isolation buffer (220 mM mannitol, sucrose 68mm, KCl 10mM, 10mM HEPES, 1% BSA) following centrifugation at 1000g for 10 min (4°C). The mitochondrial-enriched supernatant was used for determination of mitochondrial viability (Rezasurin and MTT tests), ROS formation (DCF-DA assay), lipid peroxidation (TBARS) and thiols content. For measurements of enzymes activity, twenty flies per group were homogenized in 20 mM HEPES buffer (pH 7.0). The homogenate was passed through a thin mesh fabric to remove debri and centrifuged at 1000g for 10 min (4 °C). An aliquot of the first supernatant (S1) was used for measurements of cholinesterase activity; the remaining S1 was centrifuged at 20000g for 30 min (4 °C) (Eppendorf 5427R, rotor FA-45-30-11). The supernatant was isolated and used for measuring the activity of antioxidant enzymes (SOD, CAT and GST) based on protocols previously described. The protein concentration at all samples was determined by the method of Bradford (1976).

2.8. Enzymatic assays

Acetylcholinesterase (AChE) activity was assayed following protocols previously described (Ellman et al., 1961) Glutathione transferase activity (GST) was assayed following the procedure of Habig and Jakoby (1981) using 1-chloro 2,4-dinitrobenzene (CDNB) as substrate. The assay is based on the formation of the conjugated complex of CDNB and GSH at 340 nm. The reaction was conducted in a mix consisting of 0.1 M phosphate buffer pH 7.0, 1 mM EDTA, 1 mM GSH, and 2.5 mM CDNB. Catalase activity was assayed following the clearance of H_2O_2 at 240 nm in a reaction media containing 0.05 M phosphate buffer pH 7.0, 0.5 mM EDTA, 10 mM H_2O_2, 0.012% TRITON X100 according to the procedure of Aebi (1984). Superoxide dismutase (SOD), activity was based on the decrease in cytochrome *c*

reduction (Kostyuk and Potapovich, 1989). All spectrophotometric assays were performed in a Agilent Cary 60 UV/VIS spectrophotometer with a 18 cell holder accessory coupled to a Peltier Water System temperature controller.

2.9. Thiol status

Glutathione (GSH) was measured as non-protein thiols based on Ellman (1959) with minor modifications (Franco et al., 2006). Protein thiols (PSH) were measured spectrophotometrically using Ellman's reagent. The pellet from the GSH assay was washed with 0.5 M perchloric acid and incubated for 30 min at room temperature in the presence of a solution containing 0.15 mM DTNB, 0.5 M Tris–HCl, pH 8.0, and 0.1% SDS. PSH was estimated using the molar extinction coefficient of 13600 M^{-1} cm^{-1}. A sample blank without Ellman's reagent was run simultaneously.

2.10. Mitochondrial activity assays

The viability of mitochondrial enriched-fractions obtained from treated flies was used as an index of toxicity induced by exposure to CP. Mitochondrial activity was measured by two tests: resazurin assay (fluorescence) and MTT reduction assay (colorimetric). Rezasurin assay is based on the ability of viable mitrochondria to convert resazurin into a fluorescent end product (resorufin). Nonviable samples rapidly lose metabolic capacity and thus do not generate a fluorescent signal (O'Brien et al., 2000). The fluorescence was monitored at regular intervals of 1h using a fluorescence plate reader (Perkin Elmer Enspire 2300) at 544 nm_{ex} / 590 nm_{em}. Similarly, Mitochondrial activity was assessed by incubation for 1h of the mitochondrial-enriched fraction with the metabolic probe MTT as previously described (Franco et al., 2007) with same modifications. When viable, mitochondria convert the MTT to a colorful formazan, which can be detected at 550 nm. The values were normalized by protein concentration.

2.11. Determination of Lipid Peroxidation and DCF-DA Oxidation

Lipid peroxidation end products were quantified as thiobarbituric acid reactive substance (TBARS) following the method of Ohkawa et al. (1979) with minor modifications. After centrifugation, the supernatant was incubated in 0.45 M acetic acid/HCl buffer pH 3.4, 0.28% thiobarbituric acid, 1.2% SDS, at 95°C for 60 min and absorbance then measured at 532 nm. The TBARS values were normalized by protein concentration. The results were

expressed as % of control. We also quantified 2',7'-dichlorofluorescein diacetate (DCFDA) oxidation as a general index of ROS production following Pérez-Severiano et al.(2004). The fluorescence emission of DCF resulting from DCF-DA oxidation was monitored at regular intervals at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The rate of DCF formation was calculated as a percentage of the DCF formation in relation to the sucrose-treated control group and values were normalized by protein concentration.

2.12. Quantitative Real-Time qRT-PCR and Gene Expression Analysis

Approximately 1 µg of total RNA from 20 flies was extracted using the Trizol Reagent (Invitrogen) accordingly to the manufacturer's suggested protocol. After quantification, total RNA was treated with DNase I (DNAse I Amplification Grade - Invitrogen, NY) and cDNA was synthesized with iScript cDNA Synthesis Kit and random primers again accordingly to the manufacturer's suggested protocol (BIORAD). Quantitative real-time polymerase chain reaction was performed in 11µl reaction volumes containing water treated with diethyl pyrocarbonate (DEPC), 200 ng of each primer (described in Table I), and 0,2 x SYBR Green I (molecular probes) using a 7500 real time PCR system (Applied Biosystems, NY). The qPCR protocol was the following: activation of the reaction at 50°C for 2 min, 95° for 2 min, followed by 40 cycles of 15 s at 95°C, 60 s at 60°C, and 30 s at 72°C. All samples were analyzed as technical and biological triplicates with a negative control. Threshold and baselines were automatically determined SYBR fluorescence was analyzed by 7500 software version 2.0.6 (Applied Biosystems, NY), and the CT (cycle threshold) value for each sample was calculated and reported using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).The GPDH gene was used as endogenous reference genes presenting no alteration in response to the treatment. For each well, analyzed in quadruplicates, a ΔCT value was obtained by subtracting the GPDH CT value from the CT value of the interest gene (sequences of tested genes are represented in Table 1). The ΔCT mean value obtained from the control group of each gene was used to calculate the ΔΔCT of the respective gene ($2^{-\Delta\Delta CT}$).

2.13. Statistical Analysis

Statistical analysis was performed using a one- or two-way ANOVA followed by Tukey's post hoc test. Differences were considered to be significant at the $p<0.05$ level. All experiments were repeated 3-8 times per group (n=3-8), depending on the experiment.

3. *Results*

3.1. *HEPG Antioxidant Activity in vitro*

Unpublished data from our research group (under consideration for publication elsewhere) revealed the presence of several phenolics and flavonoids compounds in the HEPG, including caffeic acid, ellagic acid, isoquercitrin and quercetin. The antioxidant activity of HEPG is directly related to its chemical composition, especially to the presence/concentrations of phenolic compounds. The total phenolic content was 32.2 mg of GAE/100 g HEPG. The ferric reducing antioxidant power (FRAP) of HEPG was tested, the values obtained were 480 µM of Fe (II)/100 g of extract. In order to complement the evaluation of antioxidant activity of HEPG, the $DPPH^{\cdot}$ and $ABTS^{\cdot+}$radical scavenging capacity were also tested. HEPG exhibited scavenging potential towards both radicals, the mean AAE value determined in the ABTS assay (174.7 µM AAE/100 mg) was higher than the mean AAE determined in the DPPH assay (78 µM AAE/100 mg). These results are shown in Table 2.

In order to evaluate the antioxidant and protective potential HEPG *in vitro*, we incubated a drosophila mitochondrial enriched fraction with TBOOH, an organic hydroperoxide, in the presence or absence of the plant extract. Then, ROS formation and mitochondrial activity were quantified. Incubation of flies mitochondria with TBOOH caused a significant increase in ROS as assessed by DCF-DA assay (Figure 1A). Simultaneous incubation with HEPG completely inhibited ROS induction by TBOOH, demonstrating a high antioxidant capacity of HEPG against the oxidizing action of TBOOH (Figure 1A). HEPG also showed significant ability to block the mitochondrial dysfunction caused by TBOOH when compared to control (Figure 1B), indicating a protective potential to HEPG against oxidative stress *in vitro*.

3.2. *In vivo experiments*

Oxidative stress has been frequently attributed as a major mechanism during organophosphate compounds poisoning (Lukaszewicz-Hussain, 2010). However, the mechanisms by with organisms respond to oxidative stress elicited by OP compounds are not fully understood. For that reason, we performed a series of *in vivo* experiments in order to check for mechanisms of adaptive response of fruit flies against the deleterious effects caused by chlorpyrifos. Considering the important antioxidant and protective effects caused by

HEPG, as described above and the fact that current therapies do not effectively block the pro-oxidative damage caused by OP intoxications, we also investigated whether HEPG would protect flies against the toxicity induced by CP in our experimental model.

For survival analysis, flies were exposed for 24 hours to concentrations of 10, 20 and 50 mg/ml of HEPG alone or co-administered with CP 0.75ppm. Exposure of flies to CP 0.75ppm caused a significant decrease in the percentage of survived flies ($p<0.001$) compared to the control. None of the tested HEPG concentrations caused significant changes in mortality compared to control, but when co-administered with CP 0.75ppm the concentration of 50 mg/ml HEPG completely reversed the mortality of flies induced by CP (Figure 2).

The locomotor activity of the flies was evaluated as a marker of CP induced toxicity. The collective negative geotaxis was evaluated by the number of flies able to attain the top of a marked tube. Under normal conditions, flies exhibit a tendency to climb a glass column. This natural behavior is called negative geotaxis. CP exposure caused a significant decrease in the percentage of flies on top ($p<0.05$) compared to the control. Simultaneous treatment with HEPG was able to reverse the locomotor deficit (Figure 3 A). For a clear view, data obtained from flies exposed to CP and/or HEPG 50 mg/ml is depicted in Figure 3B. The highest HEPG concentration tested was able to completely reverse ($p< 0.001$) locomotor deficits elicited by CP (Figure 3B). Negative geotaxis was also evaluated in single flies. CP exposure caused a significant increase in the time taken by each fly to cross a 6 cm mark in a glass column (climbing time). Similarly, HEPG was able to reverse the locomotor deficit induced by CP (Figure 3C). Based on the results obtained during mortality, the concentration of 50mg/ml HEPG was chosen for subsequent analysis.

The acetylcholinesterase activity (AChE), a hallmark for OP poisoning, was measured. The enzyme activity was significantly inhibited ($p<0.01$) in flies exposed to CP ($\approx 25\%$ decrease in AChE). As shown in Figure 4, the co-exposure to the concentration of 50mg/ml HEPG reversed this inhibition to the control level.

Exposure of flies to CP caused a significant loss of mitochondrial viability ($p<0.01$) compared to the control. The simultaneous exposure to HEPG re-established mitochondrial activity to control levels (Rezasurin assay- Figure 5 A; MTT assay-Figure 5B). The production of reactive oxygen species ($p< 0.05$) and lipid peroxidation end products ($p<0.001$) were significantly increased by CP. HEPG administration to flies significantly blocked both ROS and TBARS levels (Figure 6A and Figure 6B).

Non-protein thiols (NPSH) content, which consist mainly in GSH, was significantly increased in flies exposed to CP ($p<0.01$) compared with the control, the co-exposure with HEPG restored GSH levels (Figure 7A), while protein thiols (PSH) were not changed (Figure 7B).

The activity of three major antioxidant enzymes known to be modulated under oxidative stress conditions was measured. Glutathione S-transferase, a group of enzymes involved in detoxification of xenobiotics was significantly increased after exposure to CP ($p<0.001$). Treatment of flies with HEPG was able to reverse the GST activity to control levels (Figure 8A). The activity of superoxide dismutase ($p = 0.4468$) and catalase ($p = 0.6538$) had no significant changes in their activities (Figures 8B and C, respectively).

We evaluated the expression of genes involved in cellular stress response in flies exposed to CP for 24h in the presence/absence of the HEPG concentration of 50mg / ml. qRT-PCR analysis revealed a significant increase in gene expression of NF-E2-related factor2 (NRF2) in flies exposed to CP ($p<0.001$). This effect was blocked in the presence of HEPG 50mg / ml ($p<0.05$) when compared to the control group (Figure 9A). MPK2 (which encodes *D. melanogaster* $p38^{MAPK}$) also presented an increased expression in flies exposed to CP ($p<0.01$). HEPG was also able to reverse this increase to control levels (Figure 9B).

The mRNA expression of ERK-rolled, JNK, HO-1, NFKB and CAT showed no significant changes (Table 3).

4. *Discussion*

Oxidative stress has been extensively implicated as a major factor in the toxicity induced by organophosphate compounds (dos Santos et al., 2011; Nurulain et al., 2013; Čolovićet al., 2015). Studies have concluded that antioxidants can be used as an adjunct therapy during OP poisoning (Nurulain et al., 2013). However, the mechanisms by which organisms respond to oxidative stress induced by OP and the actual effectiveness of antioxidants against OP poisoning are still unclear. Improving the knowledge on the biochemical and molecular responses elicited by both OP and antioxidant compounds in complex organisms would improve the understanding of their mode of action, thus, opening novel therapeutic possibilities. Thereby, studies are necessary in order to elucidate such important questions.

Plant extracts have been used as a source of medicines for a wide variety of human illnesses and toxicological conditions. Herbal and natural products have recently received increased attention because of their biological and pharmacological activities (Amirghofran,2012). *Psidium guajava* is a plant of popular use in different countries and has been described for various medicinal properties (Gutiérrez et al., 2008).

In this study HEPG antioxidant potential was evaluated by their ability to scavenge free radicals such as ABTS and DPPH, expressed equivalently to the known antioxidant, ascorbic acid. The DPPH radical has been widely used to test the free radical scavenging ability of various natural products and has been accepted as a model compound for free radicals originating in lipids (Porto et al., 2000). The ABTS assay is an excellent method used for determining the antioxidant activity of a broad diversity of substances, such as hydrogen-donating antioxidants or scavengers of aqueous phase radicals and of chain breaking antioxidants or scavengers of lipid peroxyl radicals (Re et al., 1999). The average values of AAE determined in the assay ABTS was higher than those observed in the DPPH test (174.7 ± 18.0 to 78.0 ± 5.7, respectively), since DPPH reacts preferentially with lipophilic antioxidants while the ABTS reacts with both lipophilic as hydrophilic antioxidants (Prior et al., 2005). The antioxidant capacity of HEPG was also demonstrated by its significant iron reducing power (measured by FRAP assay). The iron reducing power property of HEPG indicates that the antioxidant compounds in its constitution are electron donors and can reduce oxidized intermediates during processes such as lipid peroxidation, so they can act as primary and secondary antioxidants (Yen and Chen, 1995).HEPG also showed a significant amount of phenolics. Determination of total phenolics is one of the important parameters to estimate the

amount of antioxidants. Phenolics constitute a major group of compounds acting as primary antioxidants or free radical scavengers (Kancheva and Kasaikina, 2013).

The antioxidant ability of HEPG was correlated to its protective effects against tert-butyl hydroperoxide (TBOOH)-induced mitochondrial dysfunction and ROS production. The HEPG was effective in blocking the loss of viability and ROS production elicited by the hydroperoxide *in vitro*, thus confirming its antioxidant potential. The antioxidant potential of HEPG can be attributed to its major constituents. An unpublished study by our research group determined the major compounds of HEPG to be caffeic acid>ellagic acid>isoquercitrin>quercetin (unpublished data). Previous studies also reported the antioxidant potential in several *Psidium guajava* fractions and structures (Gutiérrez et al., 2008; Vermaet al. 2013; Flores et al., 2014; Feng et al., 2015).

The increase in agricultural practices has led to indiscriminate use of agrochemicals and pesticides, which causes damage both to the environment and to human health. Chlorpyrifos (CP) is an organophosphate pesticide widely used due to its lower persistence in the environment (Soltaninejad and Shadnia, 2014). CP is known to cause neurological damage via inhibition of acetylcholinesterase enzyme and oxidative stress mechanisms (Yu et al., 2008; Goel et al., 2005). Current available treatments for CP poisoning are based primarily on the use of atropine, a symptomatic antidote and, less frequently, oximes, which are cholinesterase reactivators (Peter et al., 2014). However, whether such therapies are able to reverse the secondary damages caused by CP is unclear.

Here we show that exposure of *Drosophila melanogaster* to CP, at a concentration of 0.75 ppm for 24 hours was able to induce mortality, severe locomotor damage and inhibition of acetylcholinesterase activity. The decrease in locomotor activity as a result of inhibition of cholinesterase is well reported in literature (Moser, 2000; Nostrandt et al., 1997; Timofeeva and Gordon, 2002). AChE is responsible for the hydrolysis of the neurotransmitter acetylcholine necessary for cholinergic synaptic activity. Inhibition of AChE promotes accumulation of acetylcholine in the synaptic cleft, which results in a cholinergic overstimulation at both CNS and motor plate levels (Chakraborty et al., 2009; Xia et al., 2014). The inhibition of locomotor activity in flies exposed to the CP shown here suggests that inhibition of AChE enzyme can be related to the neurobehavioral changes noticed. The locomotor damage caused by CP has been reported in several species, including aquatic organisms (Kavitha and Rao, 2008; Yen et al., 2011; Tilton et al., 2011; Richendrfer et al., 2012). Flies treated with HEPG showed improvement in locomotor deficits caused by CP and

the AChE activity was completely restored, indicating a link between AChE inhibition and the locomotor impairment induced by CP in our model. The exact mechanisms by which HEPG restored AChE activity needs further elucidation.

The CP's ability to generate reactive oxygen species (ROS), lipid peroxidation and loss of cell viability in adult flies was also assessed. A previous report (Gupta et al., 2010) have shown a positive correlation between the ROS generation, lipid peroxidation, apoptosis and cell damage in 3rd instar *Drosophila melanogaster* larva exposed to CP. Here we have shown that in parallel to ROS and lipid peroxidation induction, the exposure of adult flies to CP was able to affect cell viability, measured as a general index of mitochondrial activity. In a previous study, isolated lymphocytes incubated for 72h with CP showed a significant decrease in viability and increase in lipid peroxidation (Navaei-Nigjeh et al., 2015). Chlorpyrifos was also able to cause a concentration-dependent reduction in cell viability in HeLa cells, HEK293 and S2 Drosophila cells (Li et al., 2015). Altogether, these results converge to demonstrate a link between oxidative stress and cell damage after exposure to CP. Co-treatment of flies with HEPG was able to reverse both ROS and lipid peroxidation, resulting in fully restored cell viability.

In order to cope with oxidative stress, organisms are constituted of a highly specialized molecular defense system aiming at shielding cells against the deleterious effects of ROS and xenobiotics (Halliwell and Gutteridge, 2007). Antioxidants can be synthesized by cells (e.g., reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) etc, or taken from the diet (Kasote et al., 2015). GSH is essential for the normal maintenance of cellular redox status. The most important physiological functions of GSH involve scavenging of free radicals, H_2O_2 and other peroxides. GSH is also involved in maintaining the –SH groups of proteins, enzymes, and other molecules in reduced form (Srikanth et al., 2013).The glutathione S-transferases (GST) are a superfamily of enzymes that catalyze the conjugation of sulfhydryl groups from GSH with electrophilic groups of xenobiotics in order to make them less reactive and facilitate the excretion of the cell (Yamuna et al. 2012). The involvement of GST in the cell response to OP compounds, including CP has been recently reported (Singh et al., 2006; Khalil, 2015). In our study, flies GST activity and GSH levels were increased after exposure to CP. Considering the key role of both GST and GSH in the detoxification of xenobiotics, one could suppose that higher levels of these antioxidants are a result of an adaptive response towards elimination of CP and its metabolites in exposed flies. Likewise, administration of HEPG resulted in restoration of GST activity and GSH content to

control levels. The protective role of *P. guajava* was previously evaluated in rats exposed to arsenic, where the treatment with the aqueous extract of *P. guajava* (100 mg / kg body weight) significantly restored oxidative stress markers such as lipid peroxidation, GSH content and activities of SOD and CAT enzymes (Tandon et al., 2012).

The antioxidant response to oxidative stress involves several signaling pathways, including the NRF2-ARE, that regulates the expression of a variety of enzymes by binding of the transcription factor NRF2 to DNA at the ARE element (antioxidant response element) (Chen et al., 2015). Under normal conditions the NRF2 is located in the cytoplasm forming an inactive complex with the Keap-1 protein that suppresses transcriptional activity of NRF2. Exposure to stressors such as ROS promotes the release of NRF2 from its suppressor Keap-1 allowing its translocation to the nucleus where the interaction occurs with the ARE, activating the transcription of antioxidant enzymes and phase II detoxification (GST, GPX, SOD, TRX and others) involved in the metabolism of xenobiotics electrophilic (Osbrurn and Kensler, 2008). The NRF2 signaling pathway has been described as major pathway of oxidative stress and cellular damage regulation. (Zang, 2006; Kobayashi and Yamamoto, 2006; Copple et al., 2008). It has been studied in both mammalian systems as well as invertebrates, including *D. melanogaster and C. elegans* (Pitoniak and Bohmann, 2015). The GST enzymes and GSH synthesis are regulated by the NRF2 pathway. Here we showed that in parallel to GST and GSH increases, CP was able to significantly increase the expression of NRF2 gene, pointing to a link between NRF2 activation and a positive modulation of GST-GSH detoxifying pathways in flies exposed to CP. Similar results were observed in JEG-3 cells, where CP significantly increased the levels of mRNA and protein NRF2 (Chiapella et al., 2013).

The nuclear translocation of NRF2 often requires the activation of signal transduction pathways, including the mitogen-activated protein kinases (MAPKs) (Shenet al., 2004). The MAPK family includes the extracellular activated protein kinase (ERK 1/2), c-Jun N-terminal kinase (JNK1/2), and 38 kDa protein kinase ($p38^{MAPK}$), proteins whose function and regulation are well conserved from unicellular to complex organisms (Paula et al., 2012). Models *in vitro* and *in vivo* have shown that the regulatory extracellular ERK and $p38^{MAPK}$ modulate the expression of antioxidant enzymes for the activation of NRF2 (Sun et al., 2008; Chen et al., 2015). Activation of these kinases may occur in response to hyperosmotic stress, cytokine exposure, and toxic injury, including oxidative stress (Paula et al., 2012). In addition to the observed increase in the expression of NRF2, the Drosophila gene (MPK2) was also significantly increased in CP exposed flies. In a previous study, exposure of human

neuroblastoma SH-SY5Y cells to CP promoted activation of the $p38^{MAPK}$ pathway in a ROS dependent manner, suggesting $p38^{MAPK}$ as a critical mediator of neuronal apoptosis induced by CP (Ki et al., 2013). Oxidative stress can act directly on the NRF2-Keap-1 complex or alternatively, via activation of protein kinases (P13K, p38, ERK, PKC, JNK) causing phosphorylation and subsequent release of NRF2 from its inhibitory protein (Son et al., 2008).Exposure of flies to HEPG abolished the effects of CP towards NRF2 and $p38^{MAPK}$ in adult Drosophila.

In this study we have shown that the CP at 0.75ppm was able to cause severe damage in adult *Drosophila melanogaster* treated during 24h, including increased mortality, locomotor deficits, inhibition of acetylcholinesterase, ROS production, decreased cell viability and changes in antioxidant defense systems. Based on the results obtained here, it is possible to point for a role of NRF2 and $p38^{MAPK}$pathways in the cell response to oxidative stress induced by CP (Scheme 1). We also suggest that oxidative stress generated by the CP may act in two ways, either by direct dissociation of NRF2-Keap1 complex or activation of MAPK pathways by increased expression of $p38^{MAPK}$ which in turn phosphorylates NRF2 promoting the translocation of NRF2 to core, where it initiates a positive modulation of phase II antioxidant pathways. As a result of this potential event, we observed GST activity and GSH levels to be increased in Drosophila exposed to CP.

Taken together, our results clearly showed, for the first time, that HEPG had high antioxidant capability and displayed a highly protective effect against toxicity induced by CP in *Drosphila melanogaster*, suggesting *Psidium guajava* as an alternative adjunt treatment for organophosphate compound poisoning.

5. Conflict of interest

Authors declare no conflict of interest.

6. Acknowledgements

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Table 1: Antioxidant Activity *in vitro* of HEPG

	DPPH (μM AAE/100mg)	ABTS (μM AAE/100mg)	Phenols (mg of GAE[a]/100 g)	FRAP (μM of Fe (II)/100 g)
HEPG	78,0 ± 5,7	174,7 ± 18,0	32,2 ± 2,8	480 ± 11,0

Data are expressed as mean ± SD.

Table 2.Genes tested by quantitative real-time RT-PCR analysis and used forward and reverse primers

Gene	Primer Sequences	
GPDH	LEFT	5'ATGGAGATGATTCGCTTCGT
	RIGHT	5'GCTCCTCAATGGTTTTTCCA
NRF2	LEFT	5'CGTGTTGTTACCCTCGGACT
	RIGHT	5'AGCGCATCTCGAACAAGTTT
P38 MPK2a	LEFT	5'GGCCACATAGCCTGTCATCT
	RIGHT	5'ACCAGATACTCCGTGGCTTG
ERK-rolled	LEFT	5'AATACGTTGCTACCCGATGG
	RIGHT	5'ACGGTGAACCCAATACTCCA
JNK-like	LEFT	5'ATGGATATGGCCACGCTAAG
	RIGHT	5'CTTTCTGTGCCTGGTGAACA
NFκB	LEFT	5'TGTGCTTTCTCTTGCCCTTT
	RIGHT	5'CCGCAGAAACCAGAGAGTTC
HO-1	LEFT	5'AAACTAAGGCGCGTTTTCAA
	RIGHT	5'GAGGGCCAGCTTCCTAAGAT
CAT	LEFT	5'ACCAGGGCATCAAGAATCTG
	RIGHT	5'AACTTCTTGGCCTGCTCGTA

Table 3: Effects of exposure to CP and HEPG on Drosophila Gene Expression.

Gene	$2^{\Delta\Delta CT}$			
	CTL	CP	Pg	Pg+CP
CAT	1,0 ± 0,1	1,2 ± 0,2	1,1 ± 0,2	1,1 ± 0,2
NFκB	1,0 ± 0,1	0,7 ± 0,2	0,6 ± 0,3	0,5 ± 0,1
JNK-like	1,2 ± 0,7	0,9 ± 0,4	2,4 ± 0,9	0,8 ± 0,3
HO-1	1,0 ± 0,5	0,5 ± 0,1	1,3 ± 0,9	0,8 ± 0,2
ERK-rolled	1,0 ± 0,4	0,7 ± 0,2	0,9 ± 0,4	0,9 ± 0,3

Data are expressed as mean ± SD.

Figure Legends

Fig.1. HEPG *in vitro* antioxidant activity. Effects of HEPG on ROS formation (A) and mitochondrial activity (B).Mitochondria enriched fractions were incubated with TBOOH and HEPG for 1h. Results are expressed as a percentage of control. Data are mean±SEM; n= 4; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to control; $^{\#\#\#}p<0.001$ compared to CP group.

Fig.2. Effects of exposure to CP and HEPG on Drosophila survival. The survival rate was computed after flies were exposed to CP 0.75 ppm and 10, 20 and 50 mg/ml concentrations of HEPG for 24h. Bars represent the mean±SEM of experiments performed individually and are expressed as percentage of survived flies in relation to control group; n=8; $^{***}p<0.001$ compared to control.

Fig.3. Effects of exposure to CP and HEPG on locomotor performance in *D. melanogaster*. (A) Collective negative geotaxis after flies were treated with CP 0.75 ppm and 10, 20 and 50 mg/ml concentrations of HEPG for 24h; n=8. (B) Negative geotaxis in flies treated with CP 0.75 ppm and 50 mg/ml of HEPG for 24h; n=8. Individual negative geotaxis after flies were treated with CP 0,75 ppm and 10, 20 and 50 mg/ml concentrations of HEPG for 24h, represents the time of climb of each fly; n=4. Data are means±SEM; $^{**}p<0.01$, $^{***}p<0.001$ compared to control; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to CP group.

Fig.4. Acetylcholinesterase activity in flies exposed to CP and HEPG. The AChE activity was measured after flies were exposed to CP 0.75 ppm and HEPG 50 mg/ml for 24h. Results are expressed as percentage of control (mean ± SEM); n=8; $^{**}p<0.01$compared to control; $^{\#\#}p<0.01$compared to CP group.

Fig.5. Mitochondrial activity in flies exposed to CP and HEPG. Resazurin reduction (A) and MTT reduction (B). Levels were determined in a mitochondrial enriched fraction prepared after flies were exposed to CP 0.75 ppm and HEPG 50 mg/ml for 24h. Results are expressed as percentage of control (mean ± SEM); n=4; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to control; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to CP group.

Fig.6. Analysis of ROS production and lipid peroxidation in *D. melanogaster* exposed to CP and HEPG. Flies were exposed to CP 0.75 ppm and HEPG 50 mg/ml for 24h. After treatments, flies were homogenized and a mitochondrial enriched supernatant was used for analysis of DCF-DA fluorescence as an index of ROS production (A) and lipid peroxidation by TBARS assay (B). Results are expressed as percentage of control (mean ± SEM); n=8; *p<0.05, **p<0.01, ***p<0.001 compared to control; $^{\#}$p<0.05, $^{\#\#\#}$p<0.001 compared to CP group.

Fig.7. Effects of exposure to CP and HEPG on thiol status in *D. melanogaster*. Flies were exposed to CP 0.75 ppm and HEPG 50 mg/ml for 24h. After treatment was finished non-protein thiols (NPSH) (A) and protein thiols (PSH) (B) were determined. Results are expressed as percentage of control (mean ± SEM); n=4; **p<0.01, ***p<0.001 compared to control; $^{\#\#\#}$p<0.001 compared to CP group.

Fig.8. Antioxidant enzyme activity in flies exposed to CP and HEPG. Flies were exposed to CP 0.75 ppm and HEPG 50 mg/ml for 24h. After treatment was finished, glutathione s-transferase (GST) (A), Catalase (CAT) (B) and superoxide dismutase (SOD) (C) were determined. Results are expressed as percentage of control (mean ± SEM); n=8 **p<0.01, ***p<0.001 compared to control; $^{\#\#}$p<0.01 compared to CP group.

Fig.9. Quantitative real time PCR (qRT-PCR) analysis of Nrf2 and p38MAPK mRNA in flies exposed to CP and HEPG. Flies were exposed to CP 0.75 ppm and HEPG 50 mg/ml for 24h. qRT-PCR was used to quantify levels of mRNA, relative to respective controls, after exposure. The data were normalized against GPDH transcript levels. Results are expressed as percentage of control (mean ± SEM); n=3; *p<0.05, **p<0.01, ***p<0.001 compared to control; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 compared to CP group.

Scheme 1. Potential mechanisms involved during CP exposure of adult *Drosophila melanogaster* and the protective effects of HEPG. Based on the results, CP induces ROS, which in turn lead to several deleterious effects (as demonstrated in the results section). As a response to CP induced oxidative stress, NRF2 protein is dissociated from its inhibitory

protein Keap1 via a direct action of ROS on NRF2 containing thiol groups or via a potential phosphorylation by $p38^{MAPK}$. The subsequent migration of NRF2 to the nuclei initiates transcription of ROS/xenobiotics detoxifying pathways, which may involve both GST and GSH.

Figure 1.

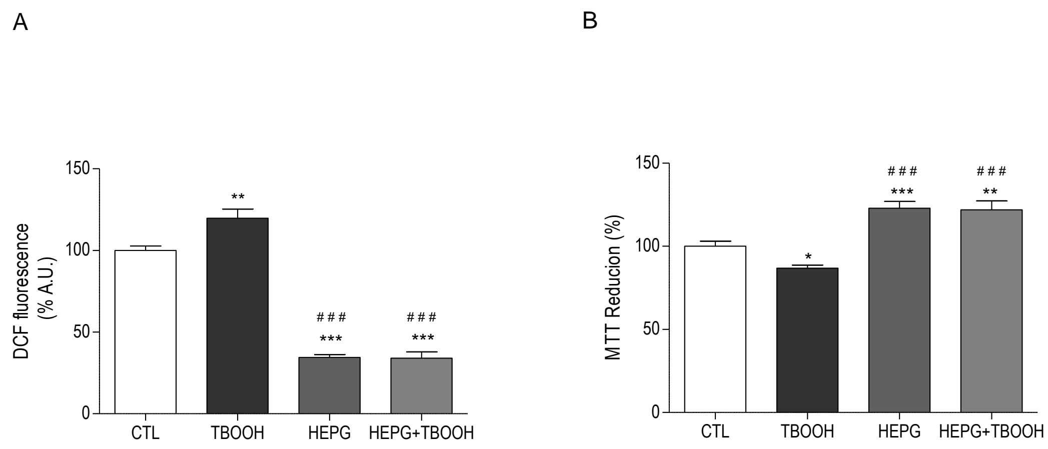


Figure 2.

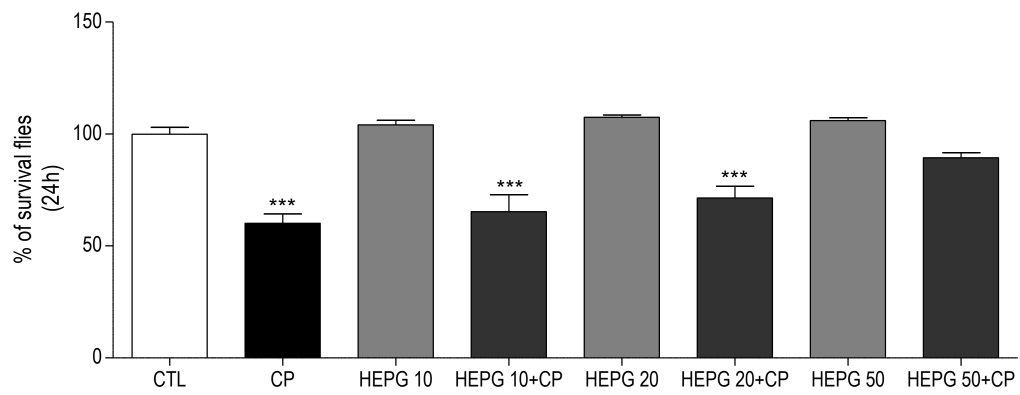
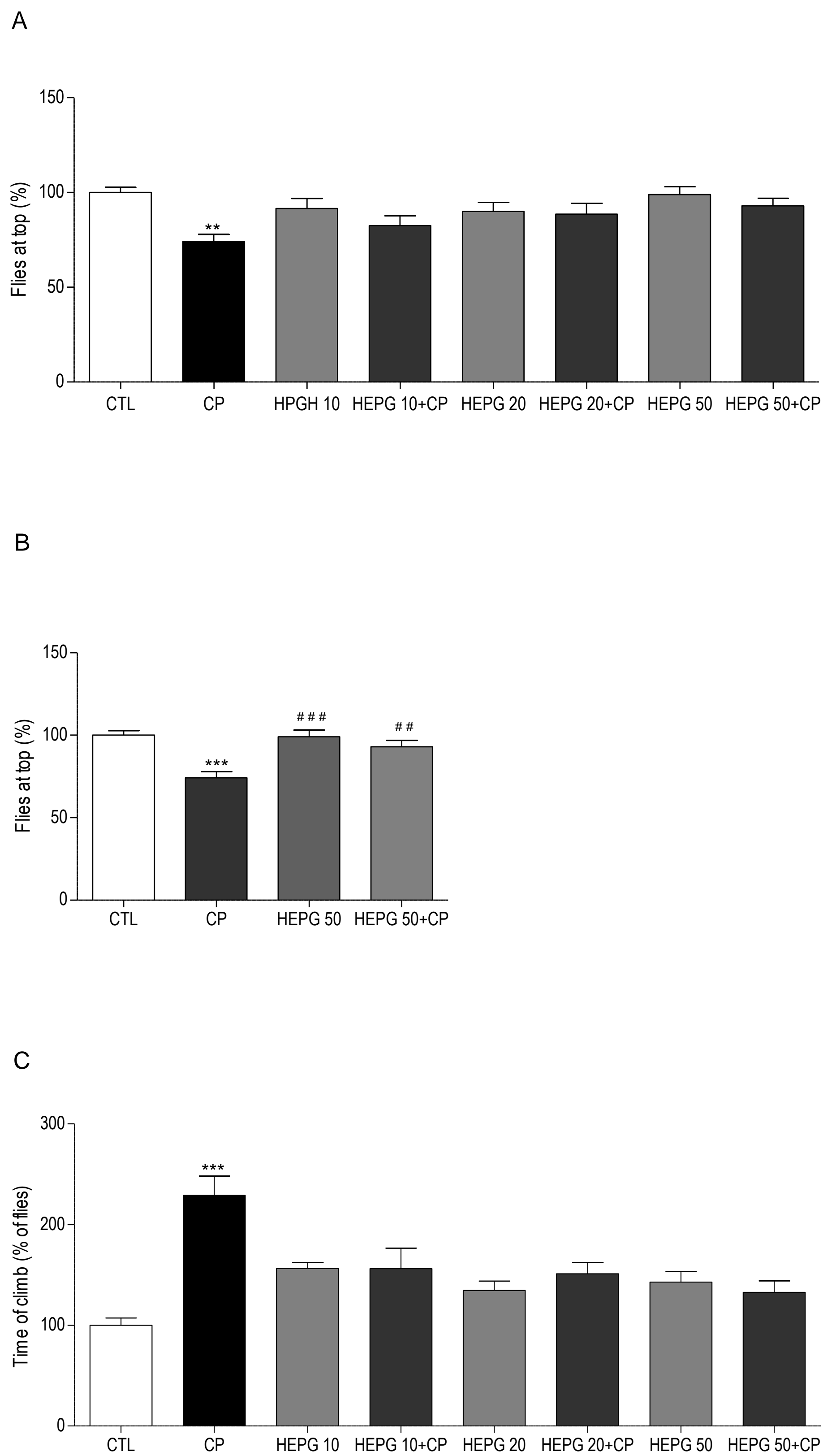


Figure 3.



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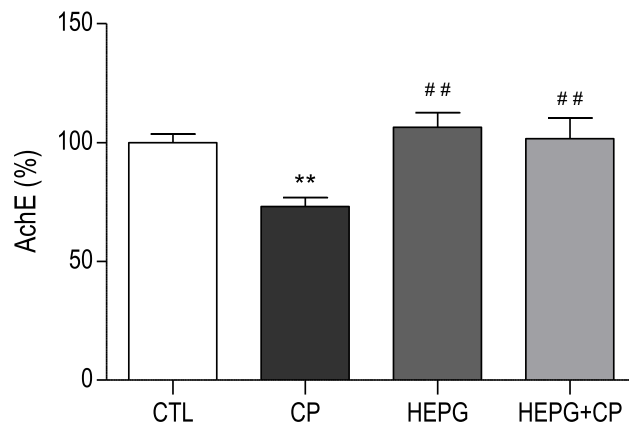
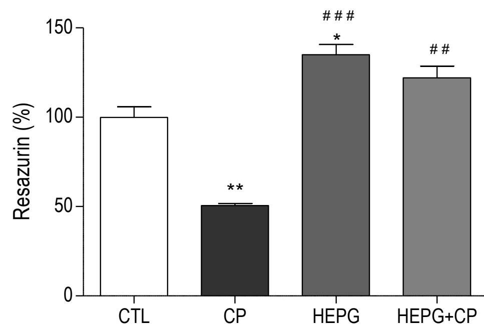


Figure 5.

A



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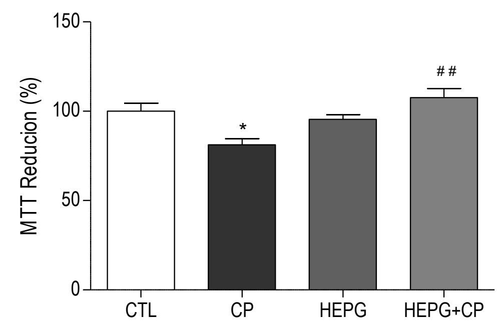

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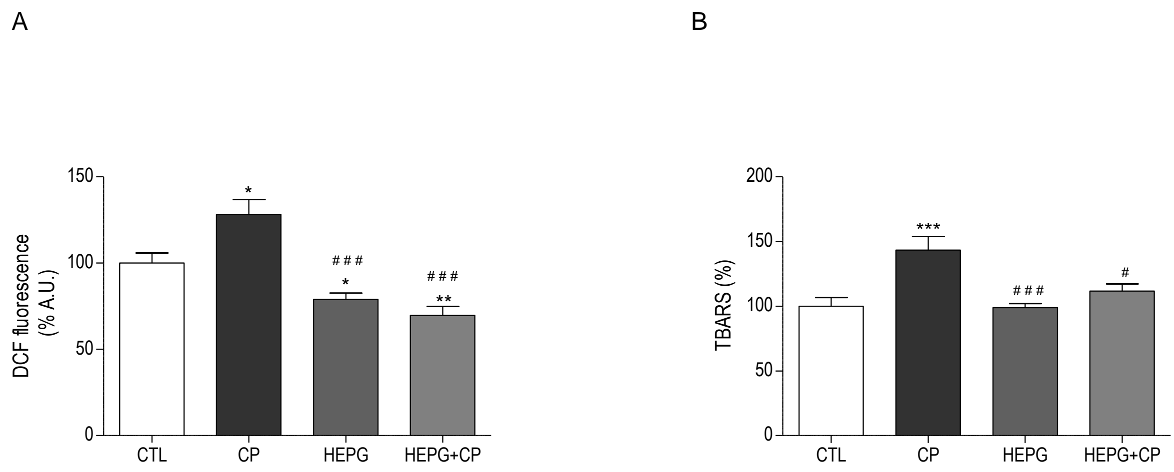

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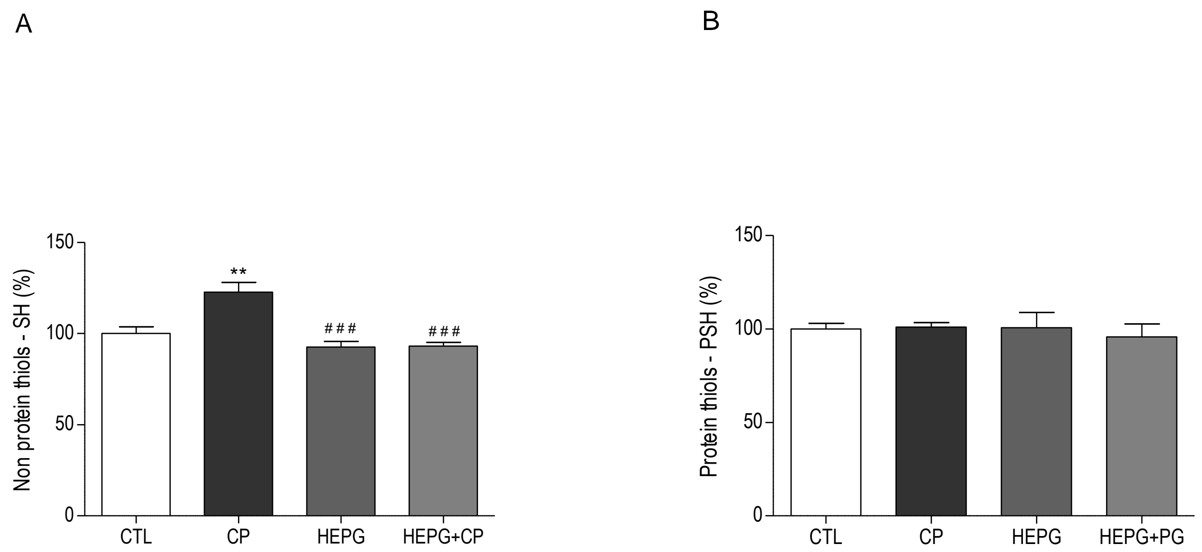
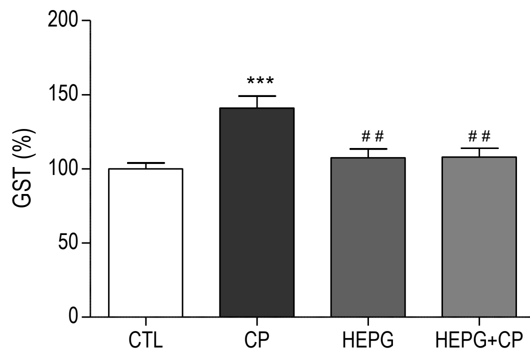


Figure 8.

A



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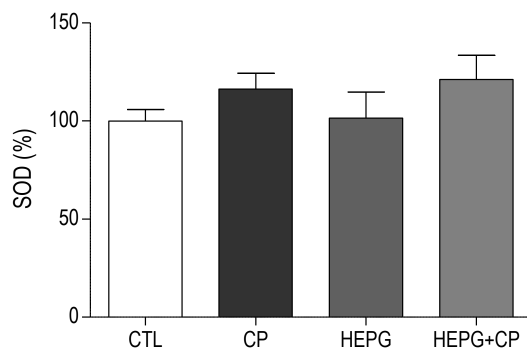


C

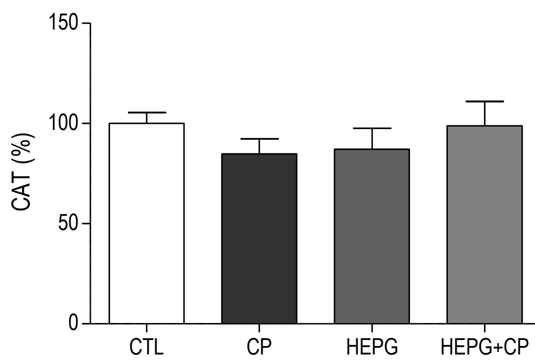
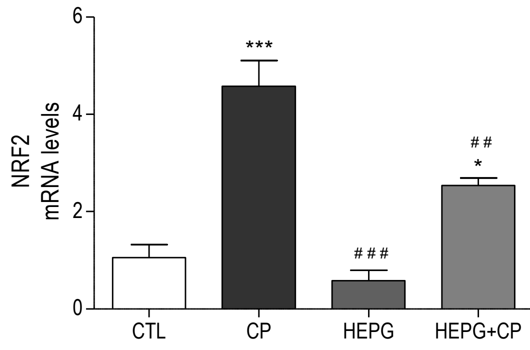
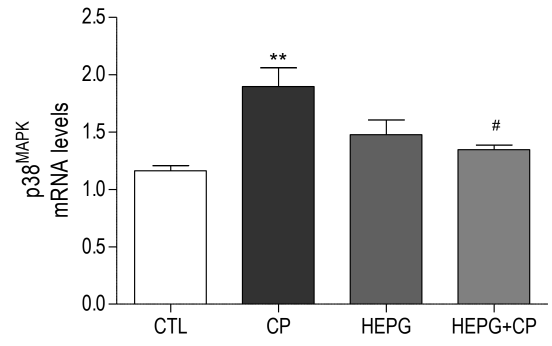


Figure 9.

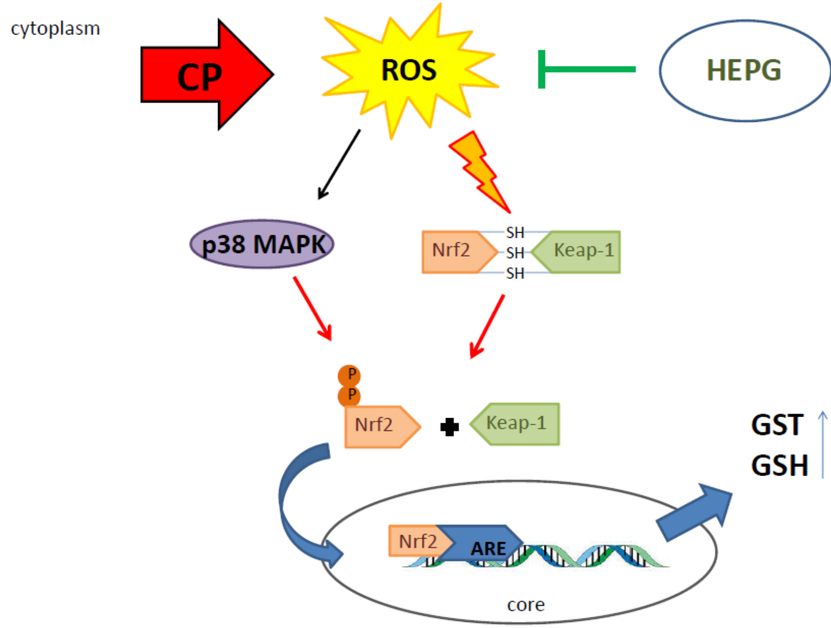
A



B



Scheme 1



4. CONCLUSÕES

A avaliação do extrato hidroalcoólico de *P. guajava* apresentou potencial antioxidante significativo,

A exposição de *Drosophila melanogaster* ao clorpirifós demonstrou:

- Aumento na mortalidade, déficit locomotor e inibição da enzima acetilcolinesterase;
- A viabilidade celular foi afetada;
- Aumento na produção de ROS e peroxidação lipídica;
- Aumento nos níveis de GSH e atividade de GST;
- Aumento na expressão nos genes NRF2 e $p38^{MAPK}$.

O co-tratamento de drosophilas com o HEPG foi capaz de reverter todos os danos causados pelo CP.

Tomados em conjunto, nossos resultados mostraram claramente, pela primeira vez, que HEPG tem elevada capacidade antioxidante e exibiu um efeito altamente protetor contra a toxicidade induzida por CP em *Drosphila melanogaster*, sugerindo *Psidium guajava* como um tratamento alternativo adjunto para o envenenamento por compostos organofosforados.

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