UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS NATURAIS E EXATAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:
BIOQUÍMICA TOXICOLÓGICA

CARACTERIZAÇÃO FÍSICO-QUÍMICA DO MEL DO BIOMA PAMPA BRASILEIRO E ESTUDO DO SEU POTENCIAL ANTIOXIDANTE *in vitro E in vivo* EM MODELO DE *Drosophila melanogaster*

DISSERTAÇÃO DE MESTRADO

Litiele Cezar da Cruz

Santa Maria, RS, Brasil

2014

CARACTERIZAÇÃO FÍSICO-QUÍMICA DO MEL DO BIOMA PAMPA BRASILEIRO E ESTUDO DO SEU POTENCIAL ANTIOXIDANTE *in vitro E in vivo* EM MODELO DE *Drosophila melanogaster*

Litiele Cezar da Cruz

Dissertação de mestrado apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Bioquímica Toxicológica**.

Orientador: Prof. Dr. Jeferson Luis Franco

Santa Maria, RS, Brasil

2014

Universidade Federal de Santa Maria

Centro de Ciências Naturais e Exatas

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica

A Comissão Examinadora, abaixo assinada,

Aprova a Dissertação de Mestrado

CARACTERIZAÇÃO FÍSICO-QUÍMICA DO MEL DO BIOMA PAMPA BRASILEIRO E ESTUDO DO SEU POTENCIAL ANTIOXIDANTE *in vitro E in vivo* EM MODELO DE *Drosophila melanogaster*

Elaborada por

Litiele Cezar da Cruz

Como requisito parcial para obtenção do grau de **Mestre em Bioquímica Toxicológica**

Comissão Examinadora:

Prof. Dr. Jeferson Luis Franco (UFSM) - Presidente/Orientador

Dr. Gabriel da Luz Wallau (UNIPAMPA) - Membro efetivo

Profª. Drª. Nilda Berenice de Vargas Barbosa (UFSM) - Membro efetivo

Santa Maria, 14 de março de 2014.

“Happiness is only real when shared.”

Christopher McCandless

AGRADECIMENTOS

Agradeço primeiramente ao meu orientador Prof. Jeferson Franco, pela oportunidade de fazer parte de seu grupo de pesquisa, pela confiança, paciência, compreensão e ensinamentos, lições importantes que aprendi, no qual me trouxeram até aqui. A todos os meus colegas e amigos do laboratório na Unipampa - São Gabriel, em especial, Ana Paula Zemolin, Jéssica Eduarda e Mauro E. Nunes, assim como também a Profª. Thaís Posser, no qual estiveram comigo o tempo todo e contribuíram muitíssimo para desenvolver este trabalho.

Agradeço aos apicultores João Morrudo e Aldo em São Gabriel/RS pela confiança e fornecimento das amostras para este estudo, assim como também a Profª. Maria da Graça e ao Departamento de Ciência e Tecnologia dos Alimentos da UFSM pelas informações e ajuda para o desenvolvimento inicial desse trabalho.

Agradeço ao Prof. João Batista por abrir as portas de seu laboratório e me receber no início do mestrado, lugar onde conheci pessoas maravilhosas, em especial Bruna K. Occai (*in memorian*) na qual além dos ensinamentos, foi uma verdadeira colega e grandíssima amiga.

Aos meus pais e irmã pelo amor e apoio incondicional; a minha prima Marciele, querida! No qual foi a inspiração para tudo que comecei de importante na vida, meu amuleto! Ao Felipe C. Luzzi, pelos anos de confiança e amizade, acreditando mais que ninguém no meu potencial e me incentivando sempre.

A minha amiga de todas as horas Jamille Scapin, sou grata por sua amizade, pelos conselhos, me colocando para frente quando pensava em recuar, tenho profunda admiração pela sua maneira de ser, sua alegria e simplicidade!

Ao CAPES pela bolsa de estudos, CNPq e FAPERGS por recursos financeiros concedidos.

Enfim, agradeço à Universidade Federal do Pampa, Universidade Federal de Santa Maria e ao Programa de Pós- Graduação em Ciências Biológicas (Bioquímica Toxicológica) pela possibilidade de realização deste curso.

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria

CARACTERIZAÇÃO FÍSICO-QUÍMICA DO MEL DO BIOMA PAMPA BRASILEIRO E ESTUDO DO SEU POTENCIAL ANTIOXIDANTE *in vitro E in vivo* EM MODELO DE *Drosophila melanogaster*

Autora: Litiele Cezar da Cruz
Orientador: Prof. Dr. Jeferson Luis Franco
Data e local da defesa: Santa Maria, 14 de março de 2014.

O mel é uma mistura complexa produzida por abelhas melíferas a partir do néctar, sendo um produto bastante utilizado pelas suas propriedades edulcorantes, bem como pelos seus benefícios para a saúde humana. O objetivo deste estudo foi caracterizar, pela primeira vez na literatura, o mel Bioma Pampa Brasileiro em termos de parâmetros de qualidade e determinação de suas propriedades antioxidantes *in vitro* e *in vivo*. Um total de 10 amostras de méis foram testadas quanto os parâmetros físico-químicos, como a umidade, acidez livre, açúcares redutores, níveis de hidroximetilfurfural (HMF), entre outros. Na atividade antioxidante (*in vitro*), foram avaliados os compostos fenólicos totais, teor de flavonóides, FRAP e atividade sequestradora de radicais (DPPH e ABTS). *Drosophila melanogaster* (*in vivo*) foram expostas a estresse oxidativo induzido por Ferro (Fe) e Paraquat (PQ) em diferentes protocolos de tratamento, na presença ou ausência de mel. Foram analisadas a sobrevivência e a atividade locomotora (geotaxia negativa). Possíveis alterações glicêmicas na dieta de mel também foram avaliadas. Os resultados das análises físico-químicas indicaram que todos os méis estavam de acordo com os padrões estabelecidos pela legislação Brasileira, que segue os padrões internacionais. Todas as amostras de mel mostraram atividade antioxidante significativa *in vitro*. Moscas tratadas com mel mostraram aumento da expectativa de vida e foram protegidos contra o estresse oxidativo induzido por Fe e PQ nos diferentes protocolos de tratamento (48 horas e 7 dias) em relação a déficit locomotores e mortalidade. Apesar do alto teor de açúcar do mel, moscas alimentadas com este não tiveram seus níveis de glicose alterados, quando comparado com moscas alimentadas com quantidades de açúcares em quantidades equivalentes ao mel. Este estudo demonstra que o mel do Bioma Pampa Brasileiro tem um alto nível de qualidade, uma atividade antioxidante *in vitro* significativa e um potencial de proteção contra o estresse oxidativo (*in vivo*). Também está demonstrado que a *Drosophila melanogaster* pode ser um modelo válido para estudos com mel, bem como a utilização deste produto natural, como uma alternativa para a terapia de doenças associadas ao estresse oxidativo.

Palavras-chave: mel, qualidade, antioxidante, *Drosophila melanogaster,* estresse oxidativo.

ABSTRACT

Dissertation of Master's Degree

Graduate Course in Toxicological Biochemistry Federal University of Santa Maria, RS, Brazil

PHYSICOCHEMICAL CHARACTERIZATION OF BRAZILIAN PAMPA BIOME HONEY AND STUDY OF ITS ANTIOXIDANT POTENTIAL *in vitro* AND *in vivo* IN *Drosophila melanogaster* MODEL

Author: Litiele Cezar da Cruz

Advisor: Prof. Dr. Jeferson Luis Franco

Place and Date of the Defense: Santa Maria, March 14th 2014.

Honey is a complex mixture produced by honey bees from the nectar, which is used by its sweetening properties, as well as by its human health benefits. The aim of this study was to characterize, for the first time in literature, the Brazilian Pampa Biome honey in terms of quality parameters and determination of its antioxidant properties *in vitro* and *in vivo*. A total of 10 honey samples were tested for physicalchemical parameters, as moisture, free acidity, reducing sugars, hydroxymethylfurfural (HMF) levels, among others. The antioxidant activity (*in vitro*), as evaluated by total phenolics, flavonoid content, FRAP and DPPH-ABTS scavenging activity. *Drosophila melanogaster* (*in vivo*) were exposed to oxidative stress induced by Iron (Fe) and Paraquat (PQ) in different treatment protocols, in the presence or absence of honey. The survivorship and locomotor activity (negative geotaxis) were analyzed. Possible glycemic alterations on honey diet were also evaluated. The results of physicochemical analysis indicated that all honeys were in accordance with the standards established by the Brazilian law, in which follows international standards. All honey samples showed significant antioxidant activity *in vitro*. Flies treated with honey showed increased lifespan and were protected against oxidative stress induced by Fe and PQ in the different treatment protocols (48h and 7 days), in regards of mortality and locomotor deficits. Despite the high sugar content of honey, glucose content were unchanged in honey fed flies, when compared to flies fed on honey-equivalent amounts of sugars. This study demonstrates that Brazilian Pampa Biome honey has a high quality level, a significant antioxidant activity *in vitro* and a protective potential against oxidative stress (*in vivo*). It has been also demonstrated that *Drosophila melanogaster* could be a valid model for studies with honey, as well as the usage of this natural product as an alternative in the therapy of oxidative stress-associated diseases.

Keywords: honey, quality, antioxidant, *Drosophila melanogaster,* oxidative stress.

APRESENTAÇÃO

No item **INTRODUÇÃO**, consta uma breve revisão bibliográfica 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 e Discussões e Referências Bibliográficas.

O item **CONCLUSÕES**, encontrado no final desta dissertação, apresenta interpretações e comentários gerais sobre os resultados dos manuscritos presentes neste trabalho.

As **REFERÊNCIAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO** e **CONCLUSÕES** desta dissertação.

SUMÁRIO

1. INTRODUÇÃO

1.1. Bioma Pampa Brasileiro

O Brasil possui seis biomas terrestres: Amazônia, Mata Atlântica, Caatinga, Cerrado, Pantanal e Pampa (Roesch et al., 2009). O Bioma Pampa ocupa uma área compartilhada por Brasil, Argentina e Uruguai no sul da América do Sul (figura 1) que apresenta características distintas de vegetação, clima e solo, tornando-se um ecossistema único no planeta. Caracteriza-se por uma vegetação típica de campo nativo, com formações arbustivas e arbóreas dispersas (Lupatini et al., 2013). O Bioma Pampa Brasileiro, ocupa uma área de 176,496 km², representando cerca de 2,07% do território nacional. Este está localizado entre as latitudes 28º 00' S e 34º 00' S e longitudes 49º 30' W e 58º 00' W, ocupando uma área de 63% do estado do Rio Grande do Sul (Lupatini et al., 2013).

O Brasil é um dos maiores países produtores de mel, com exportação para quase todos os continentes, incluindo a Europa. O estado do Rio Grande do Sul é o maior produtor de mel do Brasil (IBGE 2011), responsável por 6,985 toneladas da produção total do país (41,578 t). Mas, até agora, nenhum estudo de caracterização de méis do Bioma Pampa estão disponíveis.

Figura 1: Bioma Pampa. *Fonte:* Martino, 2004.

1.2. Aspectos gerais do mel

O mel é uma mistura complexa produzida por abelhas melíferas a partir do néctar e exsudados de plantas (Crane, 1975). Este produto é consumido mundialmente por suas propriedades adoçantes e terapêuticas (Aljadi & Kamaruddin, 2004). As abelhas melíferas acompanham a humanidade durante toda sua história e ao longo da evolução vem se especializando para produzir um produto cada vez mais complexo, e isto reflete a uma variada composição e um efeito sinérgico entre os compostos. O mel é constituído principalmente por açúcares, como monossacarídeos e dissacarídeos, bem como ácidos orgânicos, vitaminas, compostos derivados de carotenóides, aminoácidos, proteínas, oligo-elementos, flavonóides e outros compostos fenólicos. Também contêm enzimas, tais como glicose oxidase, diastase, invertase, catalase e peroxidase (Bogdanov et al., 2008).

A composição de cada mel é influenciada por uma série de fatores, incluindo a origem geográfica, fontes botânicas de néctar, ambiente e as condições climáticas, bem como manuseio e processamento de técnicas apícolas (Wang & Li, 2011). Neste sentido, existem limites determinados pela legislação para padrões físico-químicos do mel (Brasil, 2000). A legislação Brasileira possui um regulamento técnico para fixação de identidade e qualidade do mel, no qual os limites e métodos são preconizados pela *Códex Alimentarius Commission* (CAC) e o *Association of Official Analytical Chemists* (AOAC).

Apesar de o mel ser um produto natural, a produção inadequada, como também adulteração da sua composição pode torná-lo um produto de baixa qualidade, assim podendo também comprometer suas propriedades terapêuticas (Couto & Couto, 2006; Ananias, 2010). Dessa forma, torna-se importante caracterizar a identidade e qualidade de méis de diferentes regiões em todo o mundo. O mesmo serve para conteúdo fenólico do mel, o qual tem sido correlacionado com a capacidade antioxidante de amostras de mel de diferentes regiões (Ferreira et al., 2009 ; Zalibera et al., 2008).

1.2.1. Benefícios do mel

Evidências indicam que o mel pode exercer diversos efeitos benéficos à saúde humana, como gastroprotetor (Gharzouli et al., 2002), hepatoprotetor (Al-Waili et al., 2006), reprodutivo (Mohamed et al., 2012), anti-hipertensivo (Al-Waili, 2003), anti-inflamatório

(Kassim et al., 2010), antifúngico, antibacteriano (Erejuwa et al., 2012), hipoglicêmico (Erejuwa et al., 2010 a; Abdulrhman et al., 2013), e efeitos antioxidantes (Erejuwa et al., 2010 a e b).

Apontando alguns benefícios descritos, Abdulrhman et al. (2013), desenvolveu um estudo com indivíduos com diabetes tipo 1, onde se comparou a ingestão de mel, glicose e sacarose. Os resultados deste estudo demonstrara que tanto os controles (não diabéticos) como os doentes diabéticos possuíram uma redução significativa nos níveis de glicose no sangue na dieta com mel. Erejuwa et al. (2010 a), também obteve resultados similares em ratos anos antes. Abdulrhman et al. (2013) aborda em seu estudo sobre a possível capacidade do mel em estimular as células beta produtoras de insulina em humanos.

Jubri et al. (2013) em um recente estudo com ratos mostrou que suplementação com mel de Manuka (uma espécie de planta presente na Nova Zelândia e Austrália) reduziu o nível de danos no DNA, o nível de formação de malondialdeído (marcador de dano oxidativo) e atividade das enzimas antioxidantes glutationa peroxidase e catalase no fígado quando comparado com ratos controles (sem suplementação de mel). Os mesmos resultados foram encontrados para mel de Gelam (espécie de planta) na Malásia (Yao et al., 2011). Estes efeitos antioxidantes foram positivamente relacionados com teores de compostos fenólicos presentes nas amostras utilizadas.

Nas pesquisas de Erejuwa et al. (2010 a e b) associadas com danos oxidativos em modelo de diabetes em ratos, foi demonstrado que a suplementação de mel reduziu os níveis de malondialdeído e restaurou a atividade das enzimas superoxido dismutase e catase, comparado com o controle (não receberam mel). Nestes estudos o autor sugere que o efeito hipoglicêmico do mel poderia estar relacionado ao seu efeito antioxidante sobre o pâncreas. Em outro trabalho, com cultura de células pancreáticas de hamster em condições de hiperglicemia, com estresse oxidativo induzido por glicose (glicotoxicidade), a suplementação com mel diminuiu a produção de espécies reativas de oxigênio (Batumalaie et al., 2013).

Dentre as várias aplicações terapêuticas dos antioxidantes, ressalta-se a ação neuroprotetora, uma vez que, o Sistema Nervoso Central (SNC) exibe uma maior vulnerabilidade e susceptibilidade aos insultos oxidativos. Dados da literatura mostram que alguns produtos naturais ou polifenóis isolados apresentam atividade antioxidante e revertem os sinais de parkinsonismo (Hosamani, 2009).

Entretanto, até o momento, não existem estudos abordando os possíveis efeitos benéficos do mel em modelos de Doença de Parkinson, porém sabe-se que a administração de mel sozinho ou em combinação com terapias convencionais apresenta efeitos terapêuticos benéficos contra doenças crônicas que envolvem estresse oxidativo (Erejuwa et al., 2012).

1.3. Estresse oxidativo

Nas últimas décadas, têm se evidenciado um aumento na prevalência mundial de doenças degenerativas ou crônicas, como diabetes mellitus, hipertensão, câncer, Doença de Alzheimer e Doença de Parkinson (Albright, 2008). Evidências apontam no papel do estresse oxidativo na patogênese e/ou complicações destas doenças (Kadenbach et al., 2009). O estresse oxidativo é definido como um desequilíbrio entre agentes oxidantes (espécies reativas) e antioxidantes, levando a danos irreversíveis à biomoléculas, como lipídios, proteínas e DNA, comprometendo assim funções fisiológicas vitais (Sies, 1991).

Espécies reativas podem ser espécies reativas de oxigênio (ERO) ou espécies reativas de nitrogênio (ERN). ERO incluem o radical ânion superóxido ($O_2^{\bullet-}$), radical hidroxila ($OH^{\bullet}$) e peróxido de hidrogênio (H_2O_2). Entre as ERN estão o óxido nítrico (NO), dióxido de nitrogênio ($NO_2^{\bullet-}$) e peroxinitrito ($OONO^-$) (Sies, 1991; Halliwell & Gutteridge, 2007). Estas substâncias podem ser produzidas de diversas maneiras, como por exemplo, durante a "respiração mitocondrial", resultado de reações de auto-oxidação de biomoléculas instáveis, resposta à inflamação induzida por bactérias invasoras, exposição à radiação UV, contaminação por metais pesados, pesticidas, entre outros (Sies, 1991; Halliwell, 2011).

A capacidade das células em neutralizar as espécies reativas é em grande parte atribuída à eficiência do sistema de defesa antioxidante celular (Halliwell & Gutteridge, 2007; Halliwell, 2011). Esta rede de defesa antioxidante é constituída de antioxidantes endógenos e exógenos. Os antioxidantes endógenos compreendem uma linha de defesa enzimática, como as enzimas superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx) e não-enzimática, incluindo a glutationa (GSH), vitaminas C e E (Halliwell & Gutteridge, 2007). Os antioxidantes exógenos compreendem os micronutrientes e outros antioxidantes provenientes principalmente da dieta (Sies, 1991; Halliwell & Gutteridge, 2007).

1.3.1. Ferro, Paraquat e Parkinsonismo

Parkinsonismo é uma síndrome caracterizada por tremor, rigidez e instabilidade postural. A causa mais comum de parkinsonismo é a condição neurodegenerativa conhecida como Doença de Parkinson, entretanto, o parkinsonismo pode ocorrer por diversas causas, como doenças metabólicas, outras condições neurológicas, como também, induzido por algumas toxinas (Christine & Aminoff, 2004).

Ferro (Fe) é um metal essencial para muitos processos biológicos, no entanto, em excesso pode levar a deficiências metabólicas e neurológicas associadas com distúrbios do movimento tais como a Doença de Parkinson (DP) (Peran et al., 2010) . Inquestionavelmente, quantidades cada vez maiores de dados sugerem que o ferro pode estar envolvido na patogênese da DP (Berg & Youdim, 2006). No entanto, ainda permanece desconhecido se o ferro pode ser um fator causal ou secundário no processo da doença. Porém a intoxicação por este metal provou induzir efeitos semelhantes ao parkinsonismo em modelos animais (Jimenez-Del-Rio et al., 2010). Assim como, foi encontrado acumulo deste metal na substância nigra de pacientes com DP (Wallis et al., 2008).

Paraquat (PQ) é um herbicida não seletivo de alta eficiência cujo nome comercial é Gramoxone 200 e a nomenclatura conforme a IUPAC é 1,1'-dimetil-4,4'-bipiridina-dicloreto (Ballard et al., 1985). Recentemente, demonstrou-se que o paraquat é absorvido através da membrana interna mitocondrial, e uma vez na matriz mitocondrial o PQ^{2+} é reduzido a um radical monocation (PQ^{+}) pelo complexo I, este reage rapidamente com o oxigênio gerando o radical ânion superóxido ($O_2^{\bullet -}$). Isso, então, desencadeia a formação de outras EROs como o peróxido de hidrogênio (H2O2), que na presença de ferro ferroso (Fe^{2+}) é capaz de formar o radical hidroxila ($OH^{\bullet}$) (Jimenez-Del-Rio et al., 2010). Desta forma, o PQ pode atuar através da indução de estresse oxidativo, e como consequência originando diminuição de sistemas antioxidantes como superoxido dismutase (SOD), catalase, glutationa peroxidase (GPx), vitaminas C e E (Botella & Belenguer, 2000).

Em estudos com mosca da fruta (Bonilla et al., 2008) ratos e camundongos o PQ tem se mostrado especificamente prejudicial a células neuronais dopaminérgicas (Zhou et al., 2011). Assim, esse composto pode ser empregado para induzir parkinsonismo nesses animais, tornando-se um modelo de grande valia para o entendimento dos mecanismos bioquímicos, moleculares e comportamentais da doença (Jimenez-Del-Rio et al., 2010).

1.4. *Drosophila melanogaster*

A mosca da fruta, *Drosophila melanogaster* (figura 2), destaca-se como um organismo de grande importância para estudos de doenças humanas e pesquisas toxicológicas (Siddique et al., 2005). O modelo tem sido utilizado devido às vantagens advindas de seu ciclo biológico, rápido desenvolvimento, fácil manipulação, assim como a inexistência de restrições éticas para o uso deste animal em pesquisas científicas. Outra vantagem é a ausência de mitose celular nas moscas em fase adulta. Isso torna possível a determinação dos danos causados por um xenobiótico ao longo do tempo e a viabilidade celular (Jimenez-Del-Rio et al., 2010).

Embora os seres humanos e *D. melanogaster* estejam distantemente relacionado evolutivamente, quase 75% de genes relacionados com doenças em humanos, têm ortólogos funcionais na mosca, para tanto, tornando-se um sistema modelo razoável no estudo de doenças humanas. As Drosophilas são consideradas também um sistema modelo poderoso para estudos do desenvolvimento e funcionamento do sistema nervoso e vias celulares fundamentais envolvidas nos efeitos tóxicos provocados por metal e inseticidas (Paula et al., 2012).

Figura 2: *Drosophila melanogaster. Fonte:* modificado - Klok & Harrison, 2009.

Entre diversas pesquisas recentes utilizando este modelo animal, destaca-se o modelo de Doença de Parkinson em *D. melanogaster* (Jimenez-Del-Rio et al., 2010; Bonilla et al., 2008), o qual reproduz respostas comportamentais e bioquímicas correspondentes aos modelos animais tradicionais, usando ratos e camundongos. Musselman et al. (2011) em sua

pesquisa aponta a *Drosophila melanogaster* como um organismo válido para estudos de Diabetes, devido a existência de consistentes mecanismos de resistência à insulina semelhantes aos conhecidos em humanos. Entretanto, não há relatos de estudos do potencial hipoglicemiante e/ou antioxidante do mel com este modelo animal tanto para doenças neurológicas quanto possíveis alterações sobre o metabolismo.

Desta forma, considerando a falta de estudos sobre a qualidade e identidade do mel do Bioma Pampa Brasileiro, bem como o seu potencial terapêutico, torna-se relevante caracterizar os parâmetros físico-químicos deste produto natural, bem como avaliar suas propriedades antioxidantes e protetoras. Assim como, avaliar e validar um modelo, usando *Drosophila melanogaster* para estudos com o mel.

2. OBJETIVOS

2.1. Objetivos gerais

Caracterizar o mel do Bioma Pampa Brasileiro quanto a parâmetros de identidade e qualidade bem como avaliar o potencial antioxidante/protetor *in vitro* e *in vivo* deste mel em *Drosophila melanogaster* como organismo alvo.

2.2. Objetivos Específicos

2.2.1. Determinar as características físico-químicas de méis produzidos no Bioma Pampa Brasileiro;

2.2.2. Analisar o conteúdo fenólico total, concentrações de flavonóides e atividade antioxidante *in vitro* dos diferentes méis do Pampa;

2.2.3. Avaliar o possível efeito protetor do mel, contra toxicidade induzida por Ferro e Paraquat em *D. melanogaster* através da avaliação da mortalidade e análises comportamentais;

2.2.4. Avaliar possíveis alterações glicêmicas da *D. melanogaster* tratadas com mel.

3. MANUSCRITO

Submetido à **Food Research International (ISSN 0963-9969)**

Brazilian Pampa Biome honey protects *Drosophila melanogaster* against oxidative stress stimuli: a new model for studying the antioxidant properties of honey

L.C. Cruz[a,b], J.E.S. Batista[b], A.P.P. Zemolin[a,b], M.E.M. Nunes[b], D.B. Lippert[b], L. F. F. Royes[b], F. A. Soares[b], A.B. Pereira[a], T. Posser[b], J.L. Franco[b,]*

[a]*Interdisciplinary Center for Biotechnology Research, CIPBIOTEC, Universidade Federal do Pampa, Campus São Gabriel, 97.300-000 São Gabriel, RS, Brazil*

[b]*Department of Chemistry, Universidade Federal de Santa Maria, 97.105-900 Santa Maria, RS, Brazil*

*To whom correspondence should be addressed: Tel.: +55 55 32326075 (4614). E-mail addresses: jefersonfranco@unipampa.edu.br, jeferson.franco@pesquisador.cnpq.br (J.L. Franco).

ABSTRACT

We characterized, for the first time, the quality and identity of Brazilian Pampa biome honeys and its antioxidant properties *in vitro* (FRAP, DDPH and ABTS). It was also tested the potential protective effect of honeys against oxidative stress induced by Iron (Fe) and Paraquat (PQ) in a *Drosophila melanogaster* model (*in vivo*). The results indicated that all honey samples tested showed antioxidant activity *in vitro*. Flies treated with honey showed increased lifespan and were protected against oxidative stress induced by Fe and PQ. Despite the high concentration of sugars in honey (approximately 70-80%), our results demonstrate a hypoglycemic-like effect of honey in *Drosophila*. Thus, this study shows the high quality of Brazilian Pampa biome honeys as well as its significant antioxidant activity *in vitro* and *in vivo*, pointing to the potential use of this natural product as an alternative in the therapy of oxidative stress-associated diseases.

Keywords: honey, quality, antioxidant, *Drosophila melanogaster,* oxidative stress

1. Introduction

Honey is a complex mixture produced by honeybees from the nectar and is consumed by its sweetener and therapeutic properties (Rodriguez et al., 2012). Honey is constituted primarily by sugars such as monosaccharides, disaccharide as well as organic acids, vitamins, carotenoid-derived compounds, amino acids, proteins, trace elements, phenolic compounds and flavonoids. It also contains enzymes such as glucose oxidase, diastase, invertase, catalase and peroxidase (Bogdanov et al., 2008). The composition of each honey is influenced by a number of factors including geographical origin, botanical sources of nectar, environmental and climatic conditions as well as handling and processing techniques (Wang and Li, 2011). In addition, honey phenolic content has been correlated with the antioxidant capacity of honey samples from different regions (Ferreira et al., 2009; Zalibera et al., 2008). Thus it becomes important to characterize the identity and quality of honeys from distinct regions worldwide.

The Brazilian Pampa biome is one of the six biomes existing in Brasil and covers an area shared by Argentina and Uruguay in the southern of South America which presents distinctive characteristics of vegetation, climate and soil types, making it a unique ecosystem on the planet (Lupatini et al., 2013). Brazil is one of the largest honey producing countries, exporting this natural product to almost all continents, including Europe. The Rio Grande do Sul state (where the Pampa biome is located) is the largest honey producer in Brazil (IBGE 2011). But so far, no characterization studies of honeys from the Pampa biome are available.

Studies have shown that honey can deliver health benefits such as antihypertensive, reproductive, anti-inflammatory, anti-fungal, antibacterial (Erejuwa et al., 2012), hypoglycemic (Erejuwa et al., 2010; Abdulrhman et al., 2013), and antioxidant effects (Erejuwa et al., 2010; Rodriguez et al., 2012). In these studies with honey, the researchers

commonly use rodent based animal models such as rats and mice (Erejuwa et al., 2012). However, so far there is no registry of the use of alternative animals, which enables comparable responses having advantages over traditional models, such as the non existence of ethical constraints (Paula et al., 2012).

The fruit fly, *Drosophila melanogaster*, is a model-organism that has been used due to the advantages arising from its life cycle, as the rapid development and easy handling making them ideal organisms for the use on *in vivo* bioassays (Paula et al., 2012). Another advantage is the absence of cellular mitosis in flies in adulthood. This makes it possible to determine the damage caused by xenobiotic over time and cell viability (Jimenez-Del Rio et al., 2010). Although humans and *D. melanogaster* are only distantly evolutionarily related, almost 75% of disease-related genes in humans have functional orthologs in the fly, making it a reasonable model system for human diseases. Drosophila have been also proving to be a powerful model system for the study the development and functioning of nervous system and for the study of fundamental cellular pathways responsible for metal and insecticide toxicity (Paula et al., 2012).

Recent studies have shown that natural products and isolated polyphenols have antioxidant activity and are able to block oxidative stress induced by redox-active chemicals in *Drosophila* models of human diseases, including Parkinson's Disease (Jimenez-Del Rio et al., 2010; Ortega-Arellano et al., 2011). However, to date, there are no studies regarding the beneficial effects of honey in these models.

In this way, considering the lack of studies on the quality and identity of the Brazilian Pampa biome honey, as well as its therapeutic potential, it is relevant to characterize the physicochemical parameters of this natural product as well as to evaluate its antioxidant/protective properties. Thus, the present study as focused on the characterization of

Brazilian Pampa Biome honey, as well as its antioxidant properties *in vitro* and *in vivo*, using an Iron/Paraquat model of oxidative stress in *Drosophila melanogaster*, thus, developing an alternative model for studying the antioxidant and protective effects of honey.

2. Material and methods

2.1. Chemicals

Sodium bisulfate, copper (ii) sulfate, potassium sodium tartrate tetrahydrate, tannic acid, Folin-Ciocalteu, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt, sodium acetate, HEPES, minimum 99,5% titration, 2,4,6 –Tris (2-pyridyl) -5-triazine (TPTZ), methyl viologen dichloride hydrate (Paraquat), amyloglucosidase from aspergillus niger and 2, 2-di(4-tert-octylphenyl)-1-picrylhydrazyl free radical (DPPH) were purchased from Sigma–Aldrich (São Paulo, SP, Brazil). Glucose PAP liquiform (Kit) was purchased from Labtest (Belo Horizonte, MG, BRA), potassium ferrocyanide trihydrate, zinc acetate dihydrate, iron(II) ammonium sulfate hexahydrate, gallic acid monohydrate, aluminum chloride hexahydrate, potassium persulfate and iron (III) chloride, were purchased from Vetec Química Fina LTDA (Rio de Janeiro, RJ, BRA). All the other chemicals used in this work were of analytical grade.

2.2. Honey sampling

We used ten (n = 10) honey samples from *Apis mellifera* from different areas of the Brazilian Pampa Biome (Supplementary Figure 1) for physico-chemical and *in vitro* analysis. In *in vivo* analyzes was used a random sample. They honeys were identified according to floral origin and harvest date (Supplementary Table 1). The samples were provided by the Cooperativa Apícola do Pampa Gaúcho LTDA (COAPAMPA), located in the city of São

Gabriel in the state of Rio Grande do Sul - Brazil. All honey samples were stored at room temperature (20–25 °C) in plastic containers until analysis.

2.3. Physico-chemical analysis

The methods used for determination of quality parameters were according to Brazilian regulation, which determines the technical procedures for attachment of identity and quality of honey (Brazil, 2000), following international standards. The methods advocated by this legislation follow the *Codex Alimentarius* Commission (CAC, 2001) and the Association of Official Analytical Chemists (AOAC, 1990). Qualitative analysis, according to described by Instituto Adolfo Lutz (IAL) were also made to determine possible adulteration of honeys, complementing those provided by the Brazilian law.

2.3.1. Moisture

The moisture was determined using refractometric method of Chataway (AOAC, 1990). All measurements were taken using an Abbe refractometer (Carl Zeiss, Germany) with honey at 20 °C. The value of refractive index indicated was converted to the moisture content using a reference table (Table Chataway), which provides the percentage of moisture content in honey samples.

2.3.2. Free acidity and pH

Honey was diluted with distilled water and pH was measured using a pH meter. Then, the free acidity of the sample was determined by titration with NaOH. The total volume required in titration procedure was used for the calculation of the free acidity, represented as milliequivalents per kg of honey (AOAC, 1990).

2.3.3. Water insoluble solids

We used a gravimetric method in which honey was diluted with the least possible water at temperature of 80 ºC. The solution was filtered using a glass crucible (pore 15.40 µM) until free sugar was remained. The crucible was dried at 135 °C for one hour. The insoluble solids concentration is calculated from the weight difference before and after the crucible honey passage and expressed in percent of insoluble solids present in sample honey (CAC, 1990).

2.3.4. Hydroxymethylfurfural (HMF) levels

HMF was determined by the method described by White (1979). Honey was diluted in water and added both Carrez I (15 % potassium ferrocyanide) and Carrez II (30 % zinc acetate) reagents. After filtration, a reference sample was obtained by addition of 0.2 % (w/v) sodium bisulfite. Absorbance was determined at 284 and 336 nm in a quartz cuvette in a Thermo Scientific® Evolution 60s UV–Visible spectrophotometer. HMF contents are expressed as mg/kg.

2.3.5. Reducing sugars and Apparent sucrose

The determination of reducing sugars and apparent sucrose was conducted by titration in which the colorimetric change of the reaction indicated the percentage concentration of reducing sugars in the sample (CAC, 1990). The reaction occurs with the honey sample (0.5 %) as titrant, which reduces copper (blue color) at boiling point of Fehling's reagent (44.21 mg/ml copper sulphate and 346 mg/ml sodium potassium tartrate + 100 mg/ml sodium hydroxide) in cuprous oxide (red color) using 0.2 % methylene blue as indicator. The apparent sucrose content was determined after inversion by acid hydrolysis (1ml hydrochloric acid) and the value subtracted from the value obtained for total reducing sugars.

2.3.6. Lugol’s reaction

Lugol's reagent is composed of iodine (20 mg/ml) and potassium iodide (60 mg/ml). 10 g honey were dissolved in 20 mL of distilled water and kept in a water bath for 1h. Then, an aliquot of 0.5 ml Lugol's reagent was added to the solution. This is a qualitative test used to investigate the presence of starch and dextrin in honey. It is considered positive when the final color varies from violet to blue, indicating adulteration (Instituto Adolfo Lutz, 1985).

2.3.7. Lund's reaction

Two grams of each sample were weighted and diluted in 20 mL of water. Then, 5 mL of tannic acid (0.5 %) was added and the final volume completed to 40 mL. The mixture was shaken and allowed to rest for 24 h. In the presence of pure honey an albuminoid precipitate (0.6-3.0 mL) is developed (Instituto Adolfo Lutz, 1985).

2.3.8. Total Protein

The total protein content in honey (0.1g/ml) was estimated as described by Bradford (1976) using bovine serum albumin (BSA) as standard. The absorbance was measured at 595nm with the aid of an EnSpire® multimode plate reader (PerkinElmer, USA). Total protein contents present in honey sample is expressed as mg/g.

2.4. Analysis of Antioxidant Properties

All spectrophotometric assay of the analysis of antioxidant properties were performed in 96 well plates using the EnSpire® multimode plate reader (PerkinElmer, USA).

2.4.1. Total phenolics

Phenolic compounds from honey samples were detected by the Folin-Ciocalteu method (Singleton et al., 1999) with minor modifications. Briefly, 4 µL honey solution (0.1 g/ml) was mixed with 35 µL 1N Folin-Ciocalteu's reagent. After 3 min, 70 µL 15% Na2CO3

solution was added to the mixture and adjusted to 284 µL with distilled water. The reaction was kept in the dark for 2 h, after which the absorbance was read at 760 nm. Gallic acid was used as standard (10 - 300 µg/mL). The results were expressed as mg of Gallic acid equivalents (GAEs) per 100g honey.

2.4.2. Flavonoid Content

The total flavonoid content was determined using the Dowd method as adapted by Arvouet-Grand et al. (1994). Briefly, 150 µL of 2% aluminium trichloride (AlCl3) was mixed with the same volume of a honey solution (0.01g/ml). The blank consisted of 150 µL honey solution with 150 µL methanol without AlCl3 and after 10 min the absorbance was read at 415 nm. Quercetin was used as standard (0 - 25 µg/mL). The results were expressed as mg of quercetin equivalents (QE) per 100g honey.

2.4.3. 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. 30 µL of DPPH· (900 µM) in methanol were mixed with 50 µL of honey (0.1g/ml) and completing the volume to 300 µl with methanol. Ascorbic acid was used as a positive control. The absorbance was determined after 45 min. The radical scavenging activity (RSA) was calculated by the formula: % RSA = [(AB - AA)/AB] x 100; where RSA = DPPH· scavenging; AB = absorption of a blank sample (only DPPH·); AA = absorption of a tested honey sample.

2.4.4. ABTS·+ radical scavenging assay

The antioxidant activity of honey sample in the reaction with ABTS·+ 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 1 ml of 7 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt stock solution with 17.5 µL of 140 mM potassium persulfate (K2S2O8). 200 µL of ABTS·+ solution were mixed with 10 µL of honey solution (0.1g/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 radical scavenging activity (RSA) was calculated by the formula of % RSA, same described for DPPH• radical.

2.4.5. Ferric Reducing Antioxidant Power (FRAP)

The reducing capacity of honey samples was assayed with the original method of Benzie and Strain (1996), adjusted to analysis of honey samples. 9 µL of honey sample (0.1g/ml) were mixed with of 270 µL of freshly prepared FRAP reagent. The FRAP reagent was prepared by mixing 2.5 ml of 0.3 M acetate buffer pH 3.6, with 250 µL of 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) solution and 250 µL of FeCl3.6H2O. The mixture was shaken and left in a water bath for 30 min and the absorbance readings were taken at 595 nm. Ammonium iron (II) sulfate hexahydrate was used to calculate the standard curve (100 - 2000µM). The reducing ability of honey was expressed as µM of Fe (II) equivalent/100g honey.

2.4.6. Color intensity (ABS_{450})

Color intensity was determined by the method of Piljac-Žegarac et al. (2009). Honey was diluted to 50% (w/v) in warmed (45–50 ◦C) milli-Q water and filtered (0.45 µm pore size) to remove large particles. The absorbance was measured in plate reader at 450 and 720 nm, and the difference in absorbance was expressed as mAU.

2.5. Drosophila Stock

D. melanogaster (Harwich strain) was obtained from the National Species Stock Center, Bowling Green, OH. The flies were reared in glass vials containing Bloomington standard cornmeal Drosophila medium (Paula et al., 2012) in a constant temperature and humidity (20°C ± 1; 60% relative humidity). All experiments were performed with the same strain, using adult flies at 0-4 days old.

2.6. *D. melanogaster* treatments

For all in vivo experiments, honey sample number ten was used due to its high in vitro antioxidant capacity.

2.6.1. Paraquat and Iron Exposure

In order to check for the protective effects of honey, flies were exposed to Iron (Fe; 10 or 15mM) and paraquat (PQ; 10 or 20mM) separately, based on the methodology described by Jimenez-Del-Rio et al. (2010). These compounds are known to induce oxidative stress in several animal models (Zhou et al. 2011; Bonilla et al., 2011). Different treatments were performed (see Supplementary Figure 2). In a first treatment schedule (co-treatment), flies were acutely exposed to Fe or PQ in the presence or absence of honey for 48h. In a pre-treatment protocol, flies were firstly supplied with honey for 48h, then, Fe or PQ during 48h. In a post-treatment schedule, 20 flies per group were treated with Fe or PQ for 48h and subsequently fed with honey for 48h. In a chronic honey exposure protocol, flies were fed on honey or sugars during 7 days and then exposed to the pro-oxidants for 48h. After treatments were finished, survivorship and locomotor activity were evaluated.

2.6.2. Lifespan

For lifespan experiments, 20 male flies were assayed for each experimental group (n=200). In the control group flies were kept in standard medium; the honey group was

treated with 10% honey (w/w) in standard medium and the glucose+sucrose group received equivalent amounts of sugars (as to the amount present in 10% honey) in standard medium. The honey used for Drosophila treatment contained 76.8 ± 0.9 % reducing sugars and 3.0 ± 0.5 % sucrose (sample 10). The flies were evaluated during 40 days, in which the food was replaced every 10 days. The percentage of survival was estimated every five days.

2.6.3. Total glucose and glycogen

Ten females flies from each group: Control - 1% sucrose; honey – 10% solution and glucose+ sucrose were treated for 7 days on filter paper, replacing the solutions every 24 h. Flies were anesthetized in ice, weighed (to reach 10 mg of flies) and transferred to chilled microcentrifuge tubes containing 1 ml buffer 20 mM HEPES buffer (pH 7.0). The whole fly bodies were homogenized in a PowerLyzer™ 24 Bench Top Bead-Based Homogenizer (MOBIO, Carlsbad, CA EUA). After centrifugation (20,000g for 30min.), 10 µL of the supernatants was used for determination of glucose by using a glucose detection kit (Labtest) in a multimode plate reader (EnSpire® - PerkinElmer, USA). Glycogen was determined by was conversion into glucose after addition of 0.1 U/ml amyloglucosidase. Following incubation for 15 min at 60ºC, glucose was determined and values subtracted from total glucose (Morris et al., 2012; Musselman et al., 2011). Results were expressed as percent of control.

2.7. Locomotor Assay

The locomotor deficits were evaluated by negative geotaxis assay according to Jimenez-Del-Rio et al. (2010) with minor modifications. After treatments flies were transferred to test tubes marked at 5cm height. The flies were gently tapped to the bottom of the tube and the number of flies able to climb 5 cm after 6 seconds was recorded at 1 minute intervals. Each experiment was repeated twice. The climbing performance index (PI) was

calculated according to the following equation: 1/2[(ntot ntop - nbot)/ntot] where: ntop = numbers of flies at the top, nbot= at the bottom, ntot= total number of flies. Results were expressed as percentage of control.

2.8. Statistical analysis

Statistical analysis was performed using a “one way” ANOVA followed by Tukey’s post hoc test. Differences were considered to be significant at the $p < 0.05$ level.

3. Results and Discussion

3.1. Physicochemical parameters

The results from the analysis of identity and quality parameters of Brazilian Pampa biome honeys are summarized in Table 1. In the Supplementary Table 2 are summarized standards international, preconized by the Codex Alimentarius Commission (CAC) and the Association of Official Analytical Chemists (AOAC) required for Brazilian honeys. Moisture, reducing sugars and apparent sucrose are quality parameters related to honey maturity; for example, immature honeys may present higher humidity, which can lead to undesirable fermentation. Insoluble solids are related to honey purity, while pH, free acidity and HMF are related to honey deterioration. HMF, lugol/lund's reactions are also related to potential adulterations of honey (Rodriguez et al., 2012; Almeida-Muradian et al., 2013).

HMF is an aldehyde resulting from degradation of fructose in honey. The determination of its content is one of the most important indicator of honey quality. HMF is naturally produced during aging of the honey; however its formation can be accelerated when adulterations such as, overheating, addition of plain sugars and/or acidification takes place (Fallico et al., 2004). Lund's and Lugol's reaction are qualitative analysis to determine possible adulteration of honey. Lugol's reaction is based on the reaction between iodine and potassium iodide in the presence of commercial glucose, sugar syrups, or dextrin in honey, resulting in a stained solution (red-purple to blue). Lund's reaction is based on the precipitation of honey's proteins by the tannic acid. The reaction is considered positive, indicating the presence of pure honey, when the precipitate varies from 0.6 -3.0 mL (Almeida-Muradian et al., 2013). There is no any regulation or legislation imposing limits for the protein content and pH in honey, but it is known from literature that the honey is naturally

acidic irrespective of its geographical origin, which may be due to the presence of organic acids that contribute to its stability against growth of microorganisms (Khalil et al., 2012). The protein levels can be attributed mainly to the presence of different types of enzymes and other products that were introduced by the bees from the pollen and flower nectar. Protein content in honey depends on their botanical or geographical origin and storage time (Islam et al., 2012). According to Bogdanov et al. (2008) the honey contains roughly 0.5% proteins.

The results of the 10 honey samples regarding quality parameters shown in table 1, demonstrated that all honeys tested are in accordance to the international official required limits (Supplementary Table 2). Compared with other studies using honeys from other regions of Brazil, the Brazilian Pampa biome honeys achieves better quality parameters, regarding, for example to HMF levels (Lage et al., 2012; Paulino and Marcucci, 2009; Marchini et al., 2005). Confirming for the first time that Brazilian Pampa biome honeys present an acceptable quality with respect to the physicochemical parameters tested, as well as an identity when compared to other Brazilian honeys. This quality can be related to the unique ecosystem characteristics found in the Pampa biome, as mentioned before.

3.2. Antioxidant Properties *in vitro*

The antioxidant activity of honey is directly related to its chemical composition, especially to the presence/concentrations of phenolic compounds and flavonoids. The concentrations of these compounds vary depending on the floral origin of the honey samples (Khalil et al., 2012; Islam et al., 2012). Table 2 summarizes Brazilian Pampa biome honey *in vitro* antioxidant properties. The total phenolic content (mg of GAE/100g of honey) had a mean of 54.2 ± 12.9 between samples. The flavonoids content (mg of QE/100 g of honey) a mean of 2.3 ± 1.0 and the color intensity (mAU) had a mean of 351.2 ± 136.3 mAU between samples. Comparing to other studies that employed the same method, the phenolic content of the honeys we analyzed are similar to the values found for Burkina Fasan (Meda et al., 2005), Croatian (Piljac-Žegarac et al., 2009), Mexican (Rodriguez et al., 2012) honeys and higher levels than Algerian (Khalil et al., 2012), Bangladeshi (Islam et al., 2012), Malaysian (Mohamed et al., 2010) honeys. The flavonoid content also matches those found in literature reports (Meda et al., 2005). It was also observed that light colored honeys presented a lower content of phenolic compounds when compared to darker ones. This can be explained by the fact that polyphenols contribute to the color of honeys (Jasicka-Misiak et al., 2012). This correlation between color and phenolic compounds also was observed in other studies (Khalil et al., 2012; Islam et al., 2012; Piljac-Žegarac et al., 2009). Thus, our results indicate that honey samples from Brazilian Pampa biome are of equivalent, and in some cases, of superior quality in terms of antioxidant properties when compared to honeys tested worldwide.

The Ferric Reducing Antioxidant Power (FRAP) of Pampa biome honeys was tested. The principle of this method is based on the reduction of complex Fe^{3+}-TPTZ to form Fe^{2+}-TPTZ in the presence of antioxidants (Islam et al., 2012). The results on Table 2 summarize

FRAP values obtained for the tested honeys. The results showed a mean between the honey samples of 320.8 ± 125.0 μM Fe [II]/100g of honey. The FRAP values of the honey samples analyzed are similar found to those found in literature (Piljac-Žegarac et al., 2009; Rodriguez et al., 2012; Khalil et al., 2012; Islam et al., 2012; Mohamed et al., 2010). In order to complement the evaluation of antioxidant activity of Brazilian Pampa biome honeys, the DPPH· and ABTS·+ radical scavenging capacity were also tested. All honeys showed scavenging potential for both radicals. The percent scavenging capacity for DPPH· assay had a mean of 29.5 ± 8.0 between the honey samples and % scavenging of ABTS ·+ with a mean of 23.7 ± 6.8 between samples. The *in vitro* antioxidant activity found for Brazilian Pampa biome honeys are in the range of those found in literature (Baltrušaitytė et al., 2007; Alvarez-Suarez et al., 2012)

A positive correlation between the presence of phenolic compounds and honey color and the antioxidant capacity *in vitro* was observed (Supplementary Table 3). A significant correlation ($p < 0.05$) among FRAP, ABTS·+ radical scavenging and DPPH· radical scavenging, was found, however, a lower ($R=0.615$) correlation between phenolic compounds and DPPH· radical scavenging test. This can be justified by the fact that DPPH· radical reacts mainly with lipophilic antioxidants while ABTS·+ radical reacts with both hydrophilic and lipophilic antioxidants (Piljac-Žegarac et al., 2009). The color of Brazilian Pampa Biome honeys was positively correlated ($p < 0.05$) with phenolic compounds content and antioxidant capacity tests. This results show that honey's color can, at least in part, reflect the antioxidant capacity, as already observed elsewhere (Jasicka-Misiak et al., 2012). The lack of correlation between flavonoids content with color intensity, phenolic compounds and consequently antioxidant capacity tests needs further elucidation, however, at least in part, it may be justified by the low sensitivety of the method used in this study. According of the Meda et al. (2005), flavonoid content determined by the aluminum chloride method is specific only for

flavones and flavonols. This means that this method alone, underestimates the real content of total flavonoids presented in samples.

3. 3. Lifespan

The antioxidant capacity of several compounds and natural products has been correlated with their potential protective effects against oxidative stress-related comorbidities (Alvarez-Suarez et al., 2012; Erejuwa et al., 2010; Ortega-Arellano et al., 2011). In order to test the potential beneficial effects of Brazilian Pampa biome honey, we evaluate the lifespan of flies treated with honey (10%) and their sugar equivalents in order to respond whether honey and or its sugars would prolong the lifespan of flies. The experiment lasted 40 days. As seen in Figure 1, honey significantly increased lifespan. Flies that were administered sugars, at the same amount as present in honey, had an increased lifespan when compared to control, however, did not match to the honey treatment. This indicates that sugars present in honey may contribute in part for the observed prolongation of survivorship, however, additional non-sugar compounds, may contribute as main factors by which Brazilian Pampa biome honeys exerts its effects on flies lifespan. Studies have shown that low concentrations of sugars, due to its high energy content, decreases mortality of *Drosophila* (Ortega-Arellano et al., 2011). However, the honey complex composition in which besides sugars also contains vitamins, amino acids, proteins (such as enzymes), phenolic compounds, flavonoids, mineral salts, among others (Bogdanov et al., 2008) ended up contributing with a greater survivability of treated flies.

3.4. Antioxidant Properties *in vivo*: Paraquat and Iron exposure

Since honey induced a prolongation of flies lifespan and the sugar content of honey is only partially related to its protective effect, we tested whether the antioxidant capacity of Brazilian Pampa biome honeys present a role on the protection against oxidative stress *in vivo*. Paraquat (PQ) and Iron (Fe) have been widely used to induce oxidative stress in animal models including *Drosophila melanogaster* and are also claimed as chemical-induced models for parkinsonism (Bove et al., 2005; Jimenez-Del-Rio et al., 2010). Paraquat (1,1' dimethyl-4',4'-bipyridilium dimethylsulphate) is a nonselective herbicide which causes neurotoxicity via oxidative stress-mediated cell death of dopaminergic neurons (Jimenez-Del-Rio et al., 2010). Iron (Fe) is an essential metal for many biological processes, however in excess might lead to metabolic and neurological impairments associated with movement disorders such as Parkinson's disease (Peran et al., 2010). Therefore, Fe and PQ were used to induce oxidative stress in flies, to evaluate whether honey has the ability to reverse oxidative damage. The Figure 2a shows that honey, in a co-treatment schedule fully protected flies against Fe and partially protected against PQ exposure. It was also shown that honey completely blocked Fe and PQ induced locomotor deficits (Figure 2b). In order to discard the possibility that the observed protective effect of honey against Fe and PQ is solely related to a chelating effect, it was designed a pre- and post-treatment schedule, in which honey and oxidative stressors are not concomitantly administered. In a pre-treatment condition, honey was administered to flies during 48 hours; then honey was removed and Fe and PQ were delivered to the experimental animals. In the post-treatment exposure, flies were first treated with Fe or PQ during 48h then received a honey solution for a period of 48h as outlined in Supplementary Figure 2. The pre-treatment of flies with honey completely protected against Fe and PQ induced mortality

(Figure 2c). It was also observed a significant protection against Fe and PQ induced locomotor impairments (Figure 2d). It was also evaluated whether honey was able to rescue *D. melanogaster* against Fe/PQ induced mortality and locomotor deficits. As illustrated in Figure 2 (e and f), the post-treatment with honey fully recovers the survivorship of flies exposed to Fe and PQ. Honey was also able to significantly recover locomotor changes induced by Fe and PQ (Figure 2f).

Honey has about 70-80% of its composition of reducing sugars and 2-6% sucrose, so we asked whether the protection against Fe / PQ could be due to the presence of these sugars, since it was shown previously that reducing sugars can afford some degree of protection in a Drosophila model (Ortega-Arellano et al., 2011). In order to answer this question, a prolonged treatment (7 days) with the same amount of sugars existing in honey was administered to flies. This would give an insight regarding whether the sugar or non-sugar components of honey are responsible for the observed protection of flies against Fe/PQ toxicity. The results showed that glucose and sucrose equivalents did not protect against Fe / PQ, while in turn, honey protected the flies against Fe/ PQ toxicity (Figure 3).

It is shown for the first time that Brazilian Pampa biome honeys afford protection against toxicity induced by PQ and Fe in a Drosophila model. It was also shown that the non-sugars components of honey are more likely to participate in this phenomenon. The observed protection can be attributed to the presence of phenolic components in the honey since they are directly related to the antioxidant activity *in vitro* (supplementary table 3). Jimenez-Del-Rio et al. (2010) showed that pre-treatment with polyphenols might be helpful in reducing iron and paraquat-induced toxicity in Drosophila. Our results corroborate with this fact and suggest that the *in vivo* antioxidant effects of honey may be due to the presence of phenols in honey composition. More studies are needed, to identify and isolate these phenolic

compounds to determine if the antioxidant activity is due to some specific phenol or due to complex mixture of natural compounds present in honey.

Nevertheless, it is known that Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the degeneration of dopaminergic neurons in the substantia nigra pars compacta (Bove et al., 2005). The mechanisms that lead to death of dopaminergic neurons remain unclear, but studies have shown a central role for oxidative stress in this process (Jimenez-Del-Rio, 2010). Thus, current research seeks different neuroprotection strategies, including antioxidant therapies, thus highlighting the potential of natural products in such a context. The protective effect of honey against Fe and PQ observed here provides an initial evidence for the potential use of honey as an adjuvant in the therapeutics of Parkinson's disease.

3.5. Total glucose and glycogen

It was observed a protective effect of honey against Fe / PQ toxicity after 7 days of treatment (Figure 3). However, as honey has high sugar content, we inquired that maybe this characteristic can produce an increase in glucose levels, characterizing a hyperglycemic effect, which would be detrimental to honey's antioxidant and protective effects in our model. Thus, in order to answer this query, flies were fed during 7 days in honey (10% solution), glucose + sucrose (equivalent amounts as present in honey) and a low sugar control (1 % sucrose). No changes on glucose and glycogen levels were observed after 48 and 96h (data not shown). However, after 7 days treatment, a significant increase in total glucose was observed on the glucose + sucrose treated group ($p<0,0001$) when compared to the control and honey diet. Honey diet did not differ from the control, indicating a hypoglycemic-like activity (Figure 4a), since honey equivalent amounts of sugars were able to raise glucose levels in flies. The glycogen levels remained constant on all diets tested (Figure 4b).

Despite the high sugar content of honey, our results showed that this natural product did not change glucose levels in treated flies, when substituted equivalently by common sugars, even after a prolonged administration schedule. In fact, a hypoglycemic effect of honey has been shown in humans (Abdulrhman et al., 2013), both normal as well as type I diabetes patients, pointing out the beneficial effects of honey also as an anti-hyperglycemic and anti-diabetic agent. Further studies are needed to elucidate the role of honey in the glucose metabolism in Drosophila as well as its potential use as a therapeutic agent against metabolic diseases.

4. Conclusion

At least in our knowledge, this is the first report on the quality and identity of the Brazilian Pampa biome honeys. The results demonstrate its great value in terms of quality parameters, overcoming National and International standards. It was also demonstrated a prominent antioxidant activity *in vitro* and *in vivo*, indicating the potential use of this natural product as an alternative supplement on the therapy of important human disease-conditions, such as Parkinson's Disease and Diabetes. Our study also highlights the use of *Drosophila melanogaster* as a viable model for preliminary studies on the antioxidant aspects of honey and its constituents. Additional studies are needed to assess potential therapeutic properties of honey in the management of chronic diseases associated with oxidative stress.

5. Acknowledgements

Authors acknowledge the financial support provided by Brazilian Research-funding Agencies: CNPq, CAPES, FAPERGS, INCTAPA, and FINEP. Authors also are in debt with Professor Peter Robert Dunkley, from The University of Newcastle, NSW, Australia for his invaluable scientific collaborations to our research group.

6. Conflict of interest statement

All authors declare no conflict of interest.

7. References

Abdulrhman, M., El Hefnawy, M., Ali, R., Abdel Hamid, I., Abou El-Goud, A., & Refai, D. (2013). Effects of honey, sucrose and glucose on blood glucose and C-peptide in patients with type 1 diabetes mellitus. *Complementary therapies in clinical practice, 19* (1), 15-19.

Almeida-Muradian, L. B., Stramm, K. M., Horita, A., Barth, O. M., Freitas, A. S., & Estevinho, L. M. (2013). Comparative study of the physicochemical and palynological characteristics of honey from *Melipona subnitida* and *Apis mellifera. International Journal of Food Science and Technology, 48* (8), 1698-1706.

Alvarez-Suarez, J. M., Giampieri, F., Gonzalez-Paramas, A. M., Damiani, E., Astolfi, P., Martinez-Sanchez, G., Bompadre, S., Quiles, J. L., Santos-Buelga, C., & Battino, M. (2012). Phenolics from monofloral honeys protect human erythrocyte membranes against oxidative damage. *Food and chemical toxicology: an international journal published for the British Industrial Biological Research Association, 50* (5), 1508-1516.

AOAC. (1990). In: K. Helrich (Ed.), Official methods of analysis (15th ed.). Arlington VA. USA: Association of Official Analytical Chemists.

Arvouet-Grand, A., Vennat, B., Pourrat, A., & Legret, P. (1994). [Standardization of propolis extract and identification of principal constituents]. *Journal de pharmacie de Belgique, 49* (6), 462-468.

Baltrušaitytė, V., Venskutonis, P. R., & Ceksteryt, V. (2007). Radical scavenging activity of different floral origin honey and beebread phenolic extracts. *Food Chemistry, 101* (2), 502-514.

Benzie, I. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical biochemistry, 239* (1), 70-76.

Bogdanov, S., Jurendic, T., Sieber, R., & Gallmann, P. (2008). Honey for nutrition and health: a review. *Journal of the American College of Nutrition, 27* (6), 677-689.

Bove, J., Prou, D., Perier, C., & Przedborski, S. (2005). Toxin-induced models of Parkinson's disease. *NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics, 2* (3), 484-494.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry, 72*, 248-254.

Brazil. Ministry of Agriculture, Livestock and Supply. Legislation of bee products and derivatives. Normative Instruction n. 11, 20 October, 2000. Technical regulation of identity and quality of honey, 2000. URL http://www.agricultura.gov.br/. Accessed 25.09.12.

Codex Alimentarius. (2001). Revised Codex Standard for Honey, Codex STAN 12–1981, Rev. 1 (1987), Rev. 2.

Erejuwa, O. O., Gurtu, S., Sulaiman, S. A., Ab Wahab, M. S., Sirajudeen, K. N., & Salleh, M. S. (2010). Hypoglycemic and antioxidant effects of honey supplementation in streptozotocin-induced diabetic rats. *International journal for vitamin and nutrition*

research. Internationale Zeitschrift fur Vitamin- und Ernahrungsforschung. Journal international de vitaminologie et de nutrition, 80 (1), 74-82.

Erejuwa, O. O., Sulaiman, S. A., & Ab Wahab, M. S. (2012). Honey: a novel antioxidant. *Molecules, 17* (4), 4400-4423.

Fallico, B., Zappalà, M., Arena, E., & Verzera, A. (2004). Effects of conditioning on HMF content in unifloral honeys. *Food Chemistry, 85* (2), 305-313.

Ferreira, I. C. F. R., Aires, E., Barreira, J. C. M., & Estevinho, L. M. (2009). Antioxidant activity of Portuguese honey samples: different contributions of the entire honey and phenolic extract. *Food Chemistry, 114* (4), 1438-43.

IAL. (1985). Analytical standards of the Instituto Adolfo Lutz: Chemical and physical methods for food analysis, 3th ed. São Paulo BRA: IMESP.

IBGE. Brazilian Institute of Geography and Statistics. Municipal Livestock Production, 2011. URL http://www.ibge.gov.br. Accessed 20.04.13.

Islam, A., Khalil, I., Islam, N., Moniruzzaman, M., Mottalib, A., Sulaiman, S. A., & Gan, S. H. (2012). Physicochemical and antioxidant properties of Bangladeshi honeys stored for more than one year. *BMC complementary and alternative medicine, 12*, 177.

Jasicka-Misiak, I., Poliwoda, A., Deren, M., & Kafarski, P. (2012). Phenolic compounds and abscisic acid as potential markers for the floral origin of two polish unifloral honeys. *Food Chemistry, 131* (4), 1149–1156.

Jimenez-Del-Rio, M., Guzman-Martinez, C., & Velez-Pardo, C. (2010). The effects of polyphenols on survival and locomotor activity in Drosophila melanogaster exposed to iron and paraquat. *Neurochemical research, 35* (2), 227-238.

Khalil, I., Moniruzzaman, M., Boukraa, L., Benhanifia, M., Islam, A., Islam, N., Sulaiman, S. A., & Gan, S. H. (2012). Physicochemical and antioxidant properties of Algerian honey. *Molecules, 17* (9), 11199-11215.

Lage, L. G., Coelho, L. L., Resende, H. C., Tavares, M. G., Campos, L. A., & Fernandes-Salomao, T. M. (2012). Honey physicochemical properties of three species of the Brazilian Melipona. *Anais da Academia Brasileira de Ciencias, 84* (3), 605-608.

Lupatini, M., Jacques, R. J., Antoniolli, Z. I., Suleiman, A. K., Fulthorpe, R. R., & Roesch, L. F. (2013). Land-use change and soil type are drivers of fungal and archaeal communities in the Pampa biome. *World journal of microbiology & biotechnology, 29* (2), 223-233.

Marchini, L. C., Moreti, A. C. C. C., & Otsuk, I. P. (2005). Análise de agrupamento, com base na composição físico-química, de amostras de méis produzidos por Apis mellifera L. no Estado de São Paulo. *Ciência e Tecnologia de Alimentos, 25* (1), 8-17.

Meda, A., Lamien, C. E., Romito, M., Millogo, J., & Nacoulma, O. G. (2005). Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. *Food Chemistry, 91* (3), 571-577.

Mohamed, M., Sirajudeen, K., Swamy, M., Yaacob, N. S., & Sulaiman, S. A. (2010). Studies on the antioxidant properties of Tualang honey of Malaysia. *African journal of traditional, complementary, and alternative medicines : AJTCAM / African Networks on Ethnomedicines, 7* (1), 59-63.

Morris, S. N., Coogan, C., Chamseddin, K., Fernandez-Kim, S. O., Kolli, S., Keller, J. N., & Bauer, J. H. (2012). Development of diet-induced insulin resistance in adult Drosophila melanogaster. *Biochimica et biophysica acta, 1822* (8), 1230-1237.

Musselman, L. P., Fink, J. L., Narzinski, K., Ramachandran, P. V., Hathiramani, S. S., Cagan, R. L., & Baranski, T. J. (2011). A high-sugar diet produces obesity and insulin resistance in wild-type Drosophila. *Disease models & mechanisms, 4* (6), 842-849.

Ortega-Arellano, H. F., Jimenez-Del-Rio, M., & Velez-Pardo, C. (2011). Life span and locomotor activity modification by glucose and polyphenols in Drosophila melanogaster chronically exposed to oxidative stress-stimuli: implications in Parkinson's disease. *Neurochemical research, 36* (6), 1073-1086.

Paula, M. T., Zemolin, A. P., Vargas, A. P., Golombieski, R. M., Loreto, E. L., Saidelles, A. P., Picoloto, R. S., Flores, E. M., Pereira, A. B., Rocha, J. B., Merritt, T. J., Franco, J. L., & Posser, T. (2012). Effects of Hg(II) Exposure on MAPK Phosphorylation and Antioxidant System in D. melanogaster. *Environmental toxicology*.

Paulino, R. S., & Marcucci, M. C. (2009). Análises físico químicas de méis do Ceará. *Revista de Pesquisa e Inovação Farmacêutica, 1* (1), 63-78.

Peran, P., Cherubini, A., Assogna, F., Piras, F., Quattrocchi, C., Peppe, A., Celsis, P., Rascol, O., Demonet, J. F., Stefani, A., Pierantozzi, M., Pontieri, F. E., Caltagirone, C., Spalletta, G., & Sabatini, U. (2010). Magnetic resonance imaging markers of Parkinson's disease nigrostriatal signature. *Brain : a journal of neurology, 133* (11), 3423-3433.

Piljac-Žegarac, J., Stipčević, T., & Belščak, A. (2009). Antioxidant properties and phenolic content of different floral origin honeys. J*ournal of ApiProduct and ApiMedical Science, 1* (2), 43-50.

Rodriguez, B. A., Mendoza, S., Iturriga, M. H., & Castano-Tostado, E. (2012). Quality parameters and antioxidant and antibacterial properties of some Mexican honeys. *Journal of food science, 77* (1), C121-127.

Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin- Ciocalteu reagent. *Methods in Enzymology, 299*, 152-178.

Wang, J., & Li, Q. X. (2011). Chemical composition, characterization, and differentiation of honey botanical and geographical origins. *Advances in food and nutrition research, 62*, 89-137.

White, J. W. (1979). Spectrophotometric method for hydroxymethyfurfural in honey. *Journal of the Association of Official Analytical Chemists, 62* (3), 509-514.

Zalibera, M., Stasko, A., Slebodavá, A., Jancovicová, V., Cermáková, T., & Brezová, V. (2008). Antioxidant and radical-scavenging activities of Slovak honeys: an electron paramagnetic resonance study. *Food Chemistry, 110* (2), 512-21.

Zhou, H., Huang, C., Tong, J., & Xia, X. G. (2011). Early exposure to paraquat sensitizes dopaminergic neurons to subsequent silencing of PINK1 gene expression in mice. *International journal of biological sciences, 7* (8), 1180-1187.

Figure legends

Supplementary Figure 1. Brazilian Pampa biome representative map showing the locations of honey sample collection. 1 – VIAMÃO; 2 - SÃO BORJA; 3 – URUGUAIANA; 4 – ALEGRETE; 5 – CACEQUI; 6 - SÃO GABRIEL; 7 - VILA NOVA; 8 – QUARAÍ; 9 - ROSÁRIO DO SUL; 10 - SANTANA DO LIVRAMENTO.

Supplementary Figure 2. Schematic representation of *Drosophila melanogaster* treatment schedule. (A) Co-treatment with honey and Fe or PQ during 48h; (B and D) Pre-treatment with honey during 48h (B) or 7 days (D) respectively following Fe or PQ exposure for 48h; (C) Post-treatment with honey for 48h after Fe or PQ exposure for 48h.

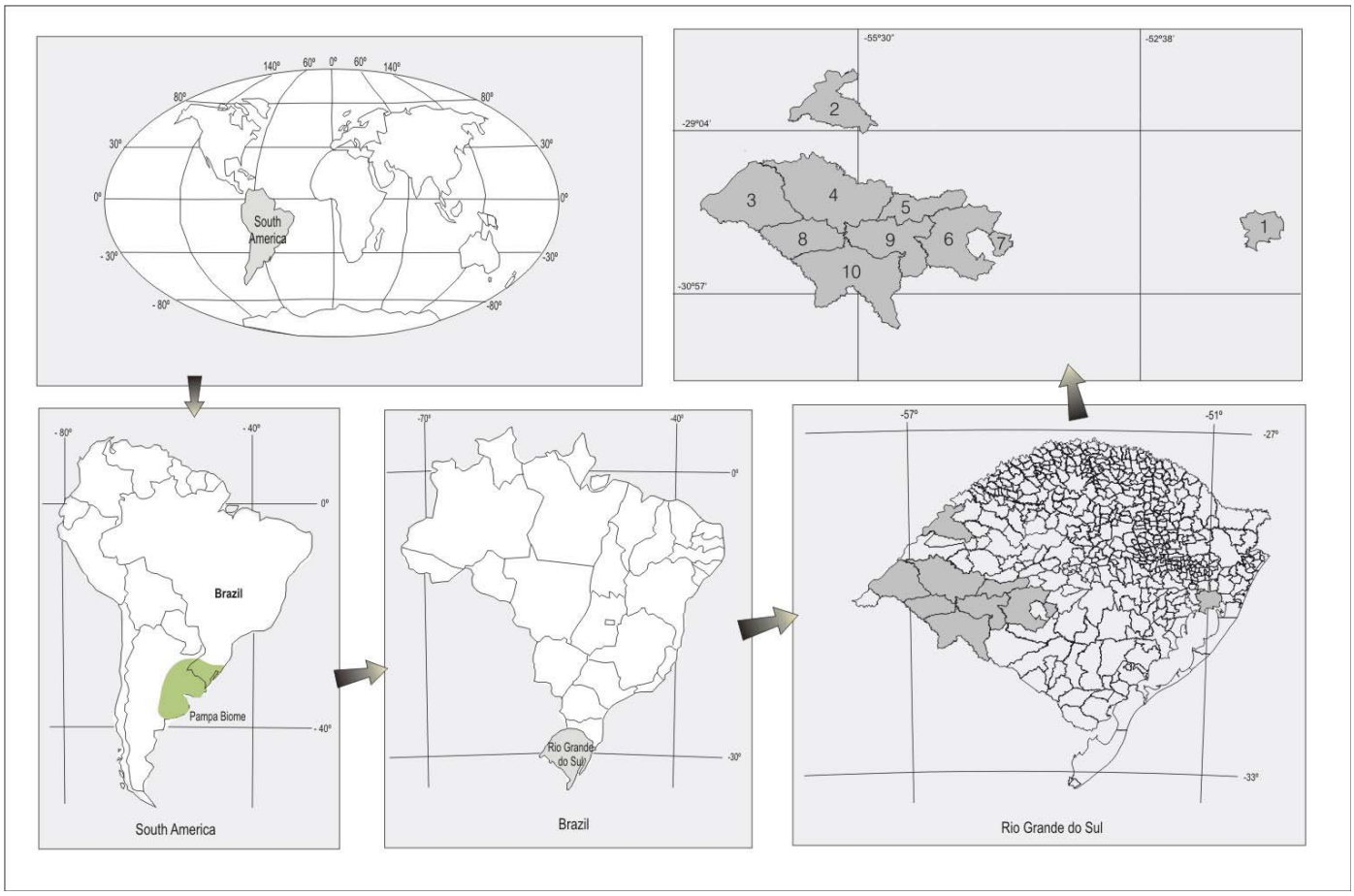
Figure 1. Effect of honey and sugars on *Drosophila melanogaster* lifespan. Flies were treated in standard medium over the course of the treatment schedule. The control group received standard medium only; honey group was administered a 10% honey solution (w/w) and the glucose+sucrose (glu+suc) group received equivalent amounts of sugars (as to the amount present in 10% honey). Lifespan was followed during 40 days. Statistically significant differences were observed between groups starting after the 10th day of treatment ($p < 0.05$).

Figure 2. Flies treated with honey were protected against the toxicity induced by Fe and PQ in different treatments. Co-treatment of flies with honey and Fe or PQ for 48 hours. (A) Percentage of surviving flies after treatment; (B) Locomotor activity test. Pre-treatment of flies with honey and Fe or PQ for 48 hours. (C) Percentage of surviving flies after treatment; (D) Locomotor activity test. Post-treatment of flies with honey and Fe or PQ for 48 hours. (E) Percentage of surviving flies after treatment; (F) Locomotor activity test. Results were

expressed as percent of control (Mean±SD). Statistical comparisons revealed $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) between groups.

Figure 3. Chronic pre-treatment of flies with honey or sugars (7 days) followed by Fe or PQ for 48 hours. (A) Percentage of surviving flies after treatment; (B) Locomotor activity test. Results were expressed as percent of control (Mean±SD). Statistical comparisons revealed $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) between groups.

Figure 4. Glucose and glycogen levels of flies treated for 7 days with honey or sugars. (A) Total glucose. (B) Glycogen content. Results were expressed as percent of control (Mean±SD). Statistical comparisons revealed $p < 0.001$ (***) between groups.



Supplementary Figure 1. Cruz et al

Supplementary Figure 2. Cruz et al

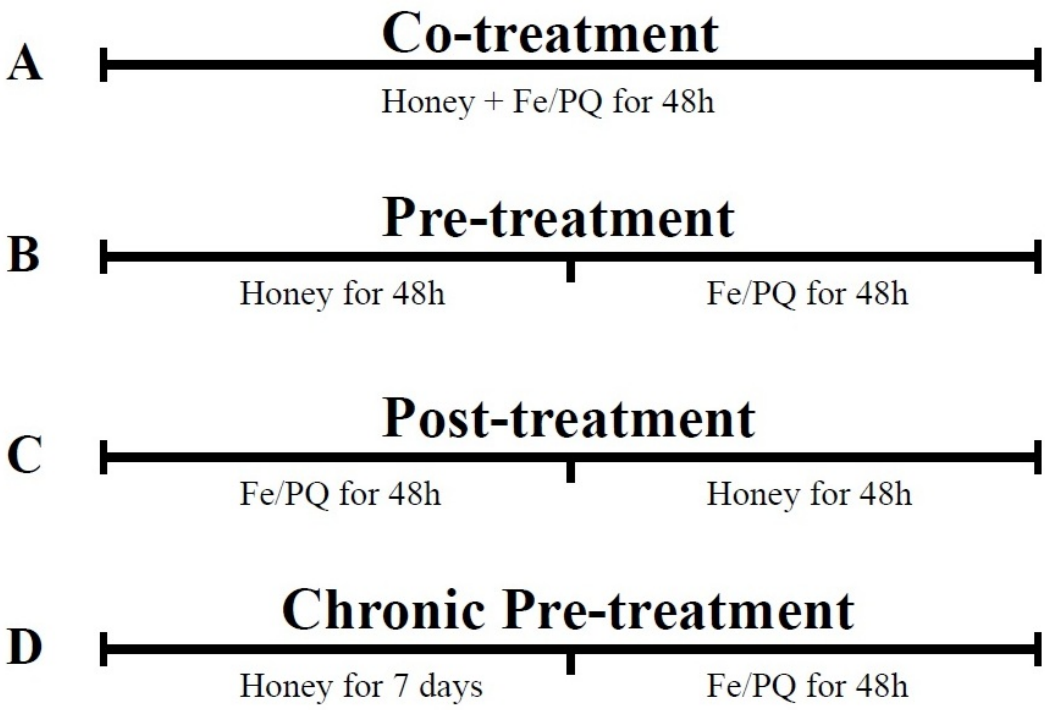
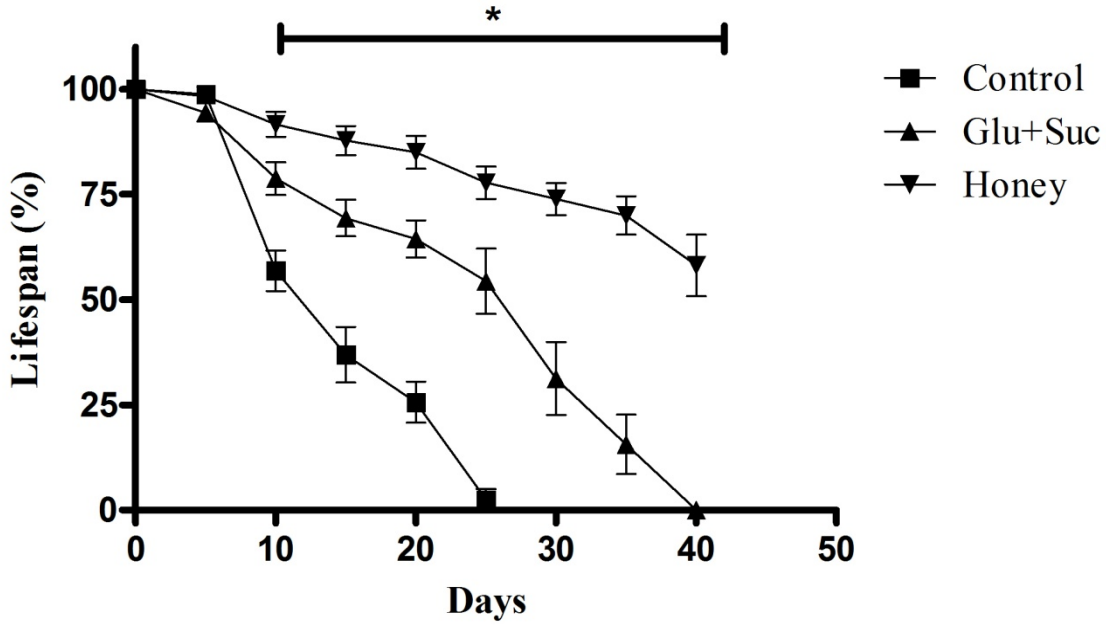


Figure 1. Cruz et al



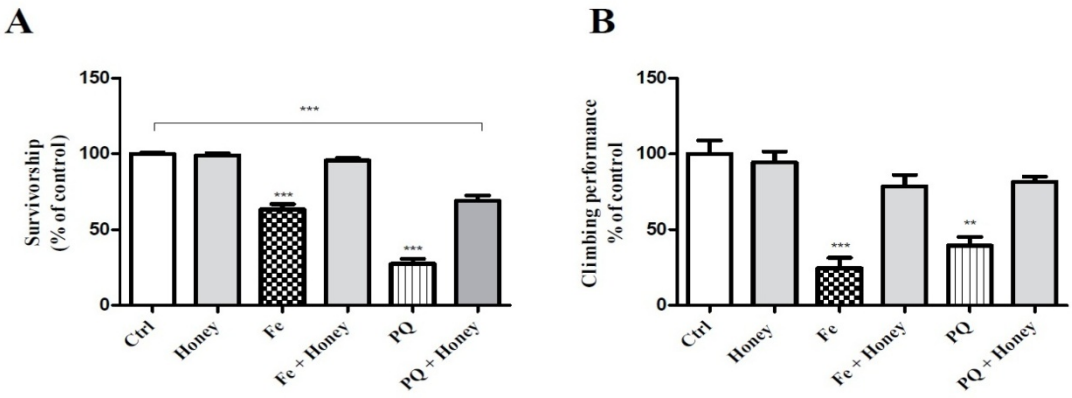
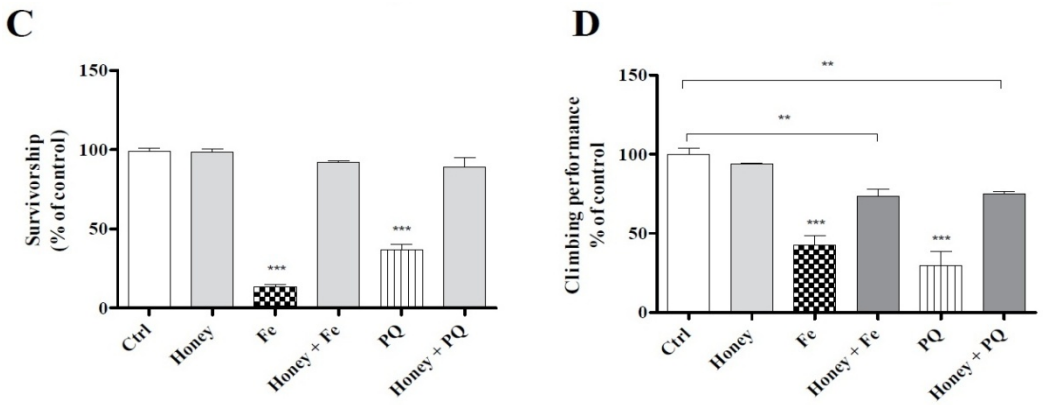


Figure 2. Cruz et al

CO -

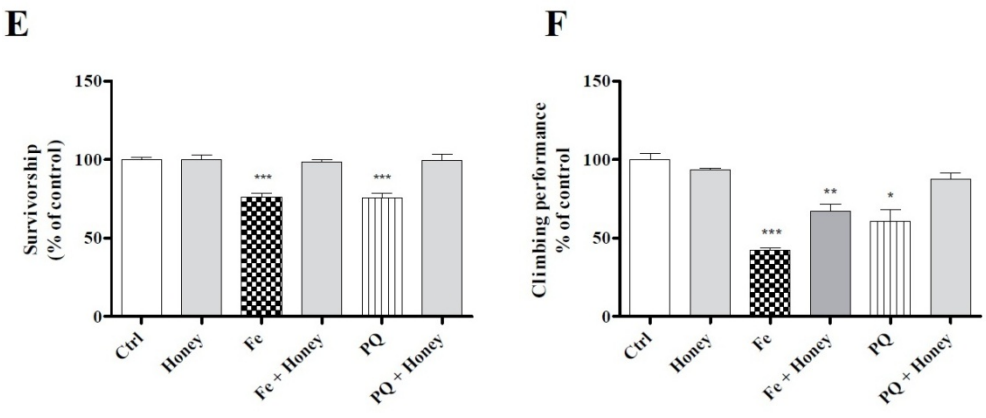


TREATMENT

PRE - TREATMENT

POST - TREATMENT

Figure 3. Cruz et al



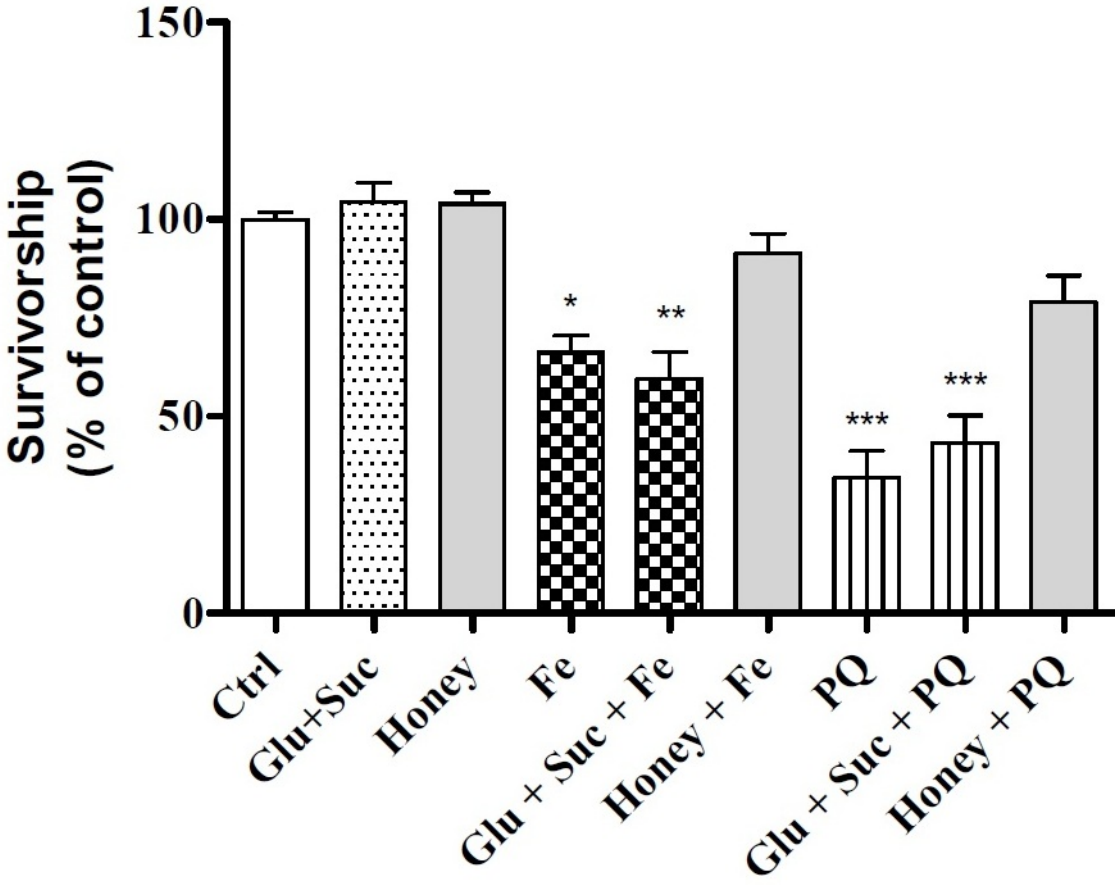
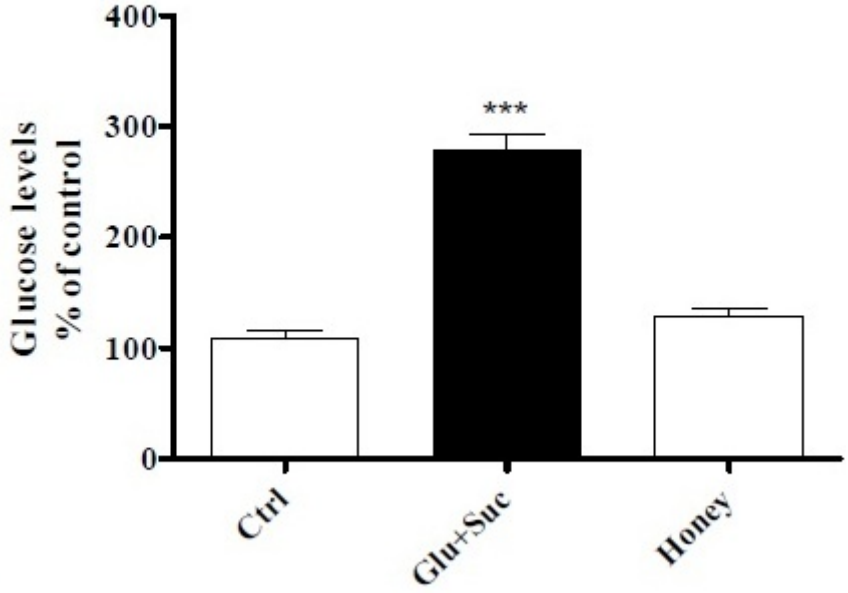


Figure 4. Cruz et al

A


400
300
200
100
0
Glucose levels
% of control
***
Ctrl
Glu+Suc
Honey

B

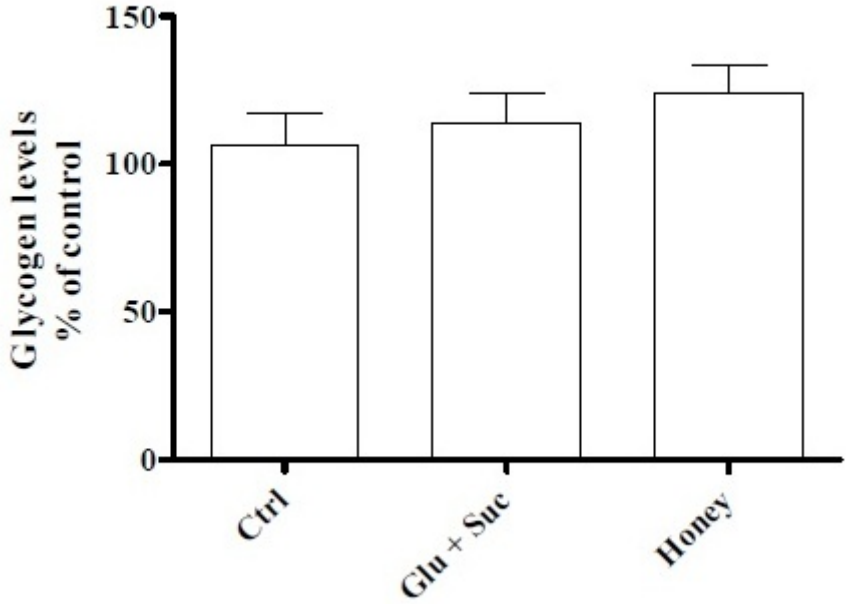

150
100
50
0
Glycogen levels
% of control
Ctrl
Glu + Suc
Honey

Table 1. Physicochemical analysis of Brazilian Pampa biome honey samples.

	Moisture %	Insoluble solids (%)	pH	Free Acidity (mEq/Kg)	Reducing Sugars (%)	Apparent Sucrose (%)	Total Protein (mg/g)	HMF (mg/Kg)	Lugol's reaction	Lund's reaction (ml)
1	20.0 ± 0.0	< 0.00	4.3 ± 0.0	39.2 ± 0.9	72.2 ± 0.3	5.9 ± 1.3	10.9 ± 0.2	8.7 ± 1.0	Negative	0.7 ± 0.0
2	18.6 ± 0.0	< 0.0	4.0 ± 0.0	44.2 ± 0.4	84.6 ± 1.9	2.3 ± 0.3	12.6 ± 0.5	17.8 ± 2.1	Negative	0.7 ± 0.0
3	19.4 ± 0.0	< 0.0	3.9 ± 0.0	32.7 ± 0.1	75.8 ± 2.7	4.3 ± 0.4	15.0 ± 0.4	14.4 ± 0.8	Negative	0.8 ± 0.0
4	19.0 ± 0.0	< 0.00	3.9 ± 0.0	33.3 ± 0.9	83.5 ± 0.9	4.9 ± 0.3	15.2 ± 0.5	15.7 ± 0.0	Negative	0.8 ± 0.0
5	19.0 ± 0.0	< 0.0	4.3 ± 0.0	25.8 ± 0.0	79.7 ± 2.7	4.2 ± 0.8	8.0 ± 0.4	ND*	Negative	0.7 ± 0.0
6	18.6 ± 0.0	< 0.00	5.0 ± 0.0	13.0 ± 0.6	78.5 ± 2.4	5.3 ± 1.2	7.1 ± 0.6	1.6 ± 0.1	Negative	0.7 ± 0.0
7	19.8 ± 0.0	< 0.00	3.7 ± 0.0	24.6 ± 0.5	76.9 ± 0.1	5.1 ± 0.8	10.5 ± 0.8	3.7 ± 0.3	Negative	0.7 ± 0.0
8	19.0 ± 0.0	< 0.00	4.3 ± 0.0	26.3 ± 2.1	78.1 ± 2.1	2.9 ± 0.8	10.2 ± 0.2	2.3 ± 0.1	Negative	0.7 ± 0.0
9	18.8 ± 0.0	< 0.00	3.9 ± 0.0	22.2 ± 0.4	82.5 ± 0.9	2.3 ± 0.9	7.9 ± 0.4	8.9 ± 0.2	Negative	0.7 ± 0.0
10	18.2 ± 0.0	< 0.00	4.4 ± 0.0	32.2 ± 0.6	76.8 ± 0.9	3.0 ± 0.5	11.4 ± 0.7	3.2 ± 0.1	Negative	1.0 ± 0.0

Data are expressed as mean ± SD. *ND: not detected.

Table 2. Brazilian Pampa biome honeys *in vitro* antioxidant properties.

	Phenols (mg of GAE[a] / 100g)	**Flavonoids (mg of QE[b] / 100g)**	**FRAP (µM of Fe (II) / 100g)**	**% scavenging of DPPH**	**% scavenging of ABTS**	**ABS 450 (mAU)**
1	64.5 ± 2.0	2.4 ± 0.2	461.3 ± 1.2	37.6 ± 4.2	31.3 ± 2.9	463.0 ± 0.0
2	69.0 ± 5.1	ND	358.0 ± 13.0	33.1 ± 0.5	27.1 ± 0.4	490.0 ± 8.5
3	63.7 ± 2.8	1.8 ± 0.4	318.0 ± 34.2	21.9 ± 3.3	25.5 ± 0.5	432.0 ± 11.3
4	63.5 ± 6.7	2.5 ± 0.3	314.7 ± 8.2	29.5 ± 2.8	24.0 ± 1.7	438.5 ± 16.3
5	41.0 ± 4.3	2.5 ± 0.0	271.3 ± 24.7	22.4 ± 1.3	15.9 ± 5.0	308.5 ± 10.6
6	46.5 ± 6.5	4.2 ± 0.5	290.5 ± 18.9	27.5 ± 0.9	21.0 ± 0.5	209.0 ± 0.0
7	47.0 ± 4.3	2.3 ± 0.5	133.0 ± 5.9	22.3 ± 1.9	14.0 ± 0.2	120.5 ± 4.9
8	56.2 ± 8.3	2.3 ± 0.4	378.8 ± 18.9	35.9 ± 0.2	27.7 ± 0.1	374.0 ± 7.1
9	28.9 ± 1.6	1.8 ± 0.4	145.5 ± 30.6	20.9 ± 2.0	16.5 ± 0.4	188.0 ± 0.0
10	61.9 ± 3.7	3.4 ± 0.5	537.2 ± 4.7	44.2 ± 3.7	34.3 ± 2.8	488.5 ± 19.1

Data are expressed as mean ± SD. [a] GAE, gallic acid equivalent; [b] QE, quercetin equivalent. DPPH / ABTS radical scavenging refers to 10% honey solution. ABS450 refers to honey color intensity; *ND: not detected.

Supplementary Table 1. Harvest period and floral origin of ten different honeys from Brazilian Pampa Biome

	Harvest	Floral origin
1	October 2012	Multifloral – wildflowers
2	November 2012	Multifloral - wildflowers
3	January 2013	Multifloral – wildflowers
4	January 2013	Multifloral – wildflowers
5	September 2012	Multifloral – bushland
6	January 2013	Multifloral – wildflowers
7	September 2012	Multifloral – bushland
8	March 2012	Multifloral – wildflowers
9	August 2012	Monofloral - *Brassica napus*
10	May 2012	Monofloral - *Eucalyptus grandis*

Supplementary Table 2. Brazilian legislation requirements for Physicochemical parameters

Parameter	Limits
Moisture (%)	Maximum 20
Insoluble solids (%)	Maximum 0.1
pH	#
Free Acidity (mEq/Kg)	Maximum 50
Reducing Sugars (%)	Minimum 65
Apparent Sucrose (%)	Maximum 6
Total Protein (mg/g)	#
HMF (mg/Kg)	Maximum 60
Lugol's reaction	Qualitative - negative
Lund's reaction (ml)	Maximum 0.6 -3.0

#: There is no regulation or legislation imposing limits

Supplementary Table 3. Correlation analysis of Brazilian Pampa biome honey *in vitro* antioxidant activity. total phenolics (TP), flavonoids content (FC) and color intensity (ABS_{450})

Variable[a]	TP	FC	FRAP	DPPH	ABTS	ABS_{450}
TP						
FC	0.258					
FRAP	**0.715***	0.147				
DPPH	0.615	0,136	**0,903***			
ABTS	**0.782***	0.001	**0.950***	**0.888***		
ABS_{450}	**0.844***	-0.280	**0.844***	**0.675***	**0.862***	

[a] Statistically significant Pearson correlation coefficients are indicated by: $p < 0.05$ (*)

4. CONCLUSÕES

De acordo com os resultados apresentados nesta dissertação pode-se concluir que:

- Os resultados demonstram que o mel do Bioma Pampa Brasileiro esta de acordo com termos de parâmetros de qualidade exigidos pela legislação, mostrando que méis desta região possui uma ótima qualidade frente aos testes;

- Foi demonstrado que os méis do Bioma Pampa possuem atividade antioxidante significativa *in vitro*;

- Méis do Bioma Pampa aumentaram a perspectiva de vida das moscas da fruta quando comparado com seus açúcares equivalentes;

- Mel do Bioma Pampa protegeu *D. melanogaster* contra estresse oxidativo induzido por Ferro e Paraquat, quanto à mortalidade e déficits locomotores;

- Foi mostrado que apesar do alto teor de açúcar dos méis, estes não são responsáveis pela proteção contra o estresse oxidativo nas moscas, cabendo a compostos não-açúcares esta proteção;

- A administração de mel em moscas mostrou um potencial anti-hiperglicêmico quando comparado com seus açúcares em quantidades equivalentes;

- Este estudo mostra pela primeira vez na literatura a utilização e validação do modelo de *Drosophila melanogaster* para estudos com o mel;

- Este trabalho indica o uso potencial de um produto natural em alternativa à terapia de doenças humanas importantes associadas ao estresse oxidativo, como a doença de Parkinson.

5. PERSPECTIVAS

- A partir dos resultados encontrados nesta dissertação, o trabalho futuro será identificar e isolar compostos fenólicos presentes no mel, de forma que possamos descobrir se estes tem a mesma resposta isoladamente, ou os resultados cabem à complexidade da composição existente no mel;

- Entre as perspectivas deste trabalho, é viabilizada uma pesquisa futura apontando o uso do mel como um adoçante substituinte utilizando o modelo de diabetes mellitus em *Drosophila melanogaster*, dessa forma estudando ambos os benefícios, hipoglicemiante e antioxidante do mel.

6. REFERÊNCIAS

Abdulrhman, M., El Hefnawy, M., Ali, R., Abdel Hamid, I., Abou El-Goud, A., & Refai, D. (2013). Effects of honey, sucrose and glucose on blood glucose and C-peptide in patients with type 1 diabetes mellitus. *Complementary therapies in clinical practice, 19* (1), 15-19.

Albright, A. (2008). Biological and social exposures in youth set the stage for premature chronic diseases. *Journal of the American Dietetic Association, 108* (11), 1843-1845.

Aljadi, A. M. & Kamaruddin, M. Y. (2004). Evaluation of the phenolic contents and antioxidant capacities of two malaysian floral honeys. *Food Chemistry 85* (4), 513-518.

Al-Waili, N. (2003). Intrapulmonary administration of natural honey solution, hyperosmolar dextrose or hypoosmolar distill water to normal individuals and to patients with type-2 diabetes mellitus or hypertension: their effects on blood glucose level, plasma insulin and C-peptide, blood pressure and peaked expiratory flow rate. *European journal of medical research, 8* (7), 295-303.

Al-Waili, N. S., Saloom, K. Y., Al-Waili, T. N., Al-Waili, A. N., Akmal, M., Al-Waili, F. S., & Al-Waili, H. N. (2006). Influence of various diet regimens on deterioration of hepatic function and hematological parameters following carbon tetrachloride: a potential protective role of natural honey. *Natural product research, 20* (13), 1258-1264.

Ananias, K. R. (2010). Avaliação das condições de produção e qualidade de mel de abelhas (Apis mellifera L.) produzido na microrregião do Pires do Rio, no Estado de Goiás. 2010. 70f. Dissertação (Mestrado em Ciência e Tecnologia de Alimentos) – Escola de Agronomia e Engenharia de Alimentos, Universidade Federal de Goiás. Goiânia.

AOAC. (1990). In: K. Helrich (Ed.), Official methods of analysis (15th ed.). Arlington VA. USA: Association of Official Analytical Chemists.

Ballard, P. A., Tetrud, J. W. & Langston, J. W. (1985). Permanent human parkinsonism due to 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP): seven cases. *Neurology, 35* (7), 949-956.

Batumalaie, K., Qvist, R., Yusof, K. M., Ismail, I. S., & Sekaran, S. D. (2013). The antioxidant effect of the Malaysian Gelam honey on pancreatic hamster cells cultured under hyperglycemic conditions. *Clinical and experimental medicine*.

Berg, D., & Youdim, M. B. (2006). Role of iron in neurodegenerative disorders. *Topics in magnetic resonance imaging : TMRI, 17* (1), 5-17.

Bogdanov, S., Jurendic, T., Sieber, R., & Gallmann, P. (2008). Honey for nutrition and health: a review. *Journal of the American College of Nutrition, 27* (6), 677-689.

Bonilla-Ramirez, L., Jimenez-Del-Rio, M., & Velez-Pardo, C. (2011). Acute and chronic metal exposure impairs locomotion activity in Drosophila melanogaster: a model to study Parkinsonism. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine, 24* (6), 1045-1057.

Botella, M. J. & Belenguer, T. J. E. (2000). Paraquat poisoning. A study of 29 cases and evaluation of the effectiveness of the "Caribbean scheme". *Medicina Clínica (Barc), 115* (14), 530-533.

Brazil. Ministry of Agriculture, Livestock and Supply. Legislation of bee products and derivatives. Normative Instruction n. 11, 20 October, 2000. Technical regulation of identity and quality of honey, 2000. URL http://www.agricultura.gov.br/. Accessed 25.09.12.

Christine, C. W., & Aminoff, M. J. (2004). Clinical differentiation of parkinsonian syndromes: prognostic and therapeutic relevance. *The American journal of medicine, 117* (6), 412-419.

Cicchetti, F., Drouin-Ouellet, J., & Gross, R. E. (2009). Environmental toxins and Parkinson's disease: what have we learned from pesticide-induced animal models? *Trends in pharmacological sciences, 30* (9), 475-483.

Codex Alimentarius. (2001). Revised Codex Standard for Honey, Codex STAN 12–1981, Rev. 1 (1987), Rev. 2.

Couto, L. A. & Couto, R. H. N. (2006). Apicultura: manejo e produtos. *Funep.*

Crane, E. (1975). Honey. *Morrison and Gibb*, 608.

Erejuwa, O. O., Gurtu, S., Sulaiman, S. A., Ab Wahab, M. S., Sirajudeen, K. N., & Salleh, M. S. (2010 a). Hypoglycemic and antioxidant effects of honey supplementation in streptozotocin-induced diabetic rats. *International journal for vitamin and nutrition research. Internationale Zeitschrift fur Vitamin- und Ernahrungsforschung. Journal international de vitaminologie et de nutrition, 80* (1), 74-82.

Erejuwa, O. O., Sulaiman, S. A., & Ab Wahab, M. S. (2012). Honey: a novel antioxidant. *Molecules, 17* (4), 4400-4423.

Erejuwa, O. O., Sulaiman, S. A., Wahab, M. S., Sirajudeen, K. N., Salleh, M. S., & Gurtu, S. (2010 b). Antioxidant protection of Malaysian tualang honey in pancreas of normal and streptozotocin-induced diabetic rats. *Annales d'endocrinologie, 71* (4), 291-296.

Ferreira, I. C. F. R., Aires, E., Barreira, J. C. M., & Estevinho, L. M. (2009). Antioxidant activity of Portuguese honey samples: different contributions of the entire honey and phenolic extract. *Food Chemistry, 114* (4), 1438-43.

Gharzouli, K., Amira, S., Gharzouli, A., & Khennouf, S. (2002). Gastroprotective effects of honey and glucose-fructose-sucrose-maltose mixture against ethanol-, indomethacin-, and acidified aspirin-induced lesions in the rat. *Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie, 54* (3), 217-221.

Halliwell, B. & Gutteridge, J. M. C. (2007). Free Radicals in Biology and Medicine. Clarendon Press, Oxford.

Halliwell, B. (2011). Free radicals and antioxidants - quo vadis? *Trends in pharmacological sciences, 32* (3), 125-130.

Hosamani, R. (2009). Neuroprotective efficacy of Bacopa monnieri against rotenone induced oxidative stress and neurotoxicity in Drosophila melanogaster. *Neuro toxicology, 30* (6), 977-985.

IBGE. Brazilian Institute of Geography and Statistics. Municipal Livestock Production, 2011. URL http://www.ibge.gov.br. Accessed 20.04.13.

Jimenez-Del-Rio, M., Guzman-Martinez, C., & Velez-Pardo, C. (2010). The effects of polyphenols on survival and locomotor activity in Drosophila melanogaster exposed to iron and paraquat. *Neurochemical research, 35* (2), 227-238.

Jubri, Z., Rahim, N. B., & Aan, G. J. (2013). Manuka honey protects middle-aged rats from oxidative damage. *Clinics, 68* (11), 1446-1454.

Kadenbach, B., Ramzan, R., & Vogt, S. (2009). Degenerative diseases, oxidative stress and cytochrome c oxidase function. *Trends in molecular medicine, 15* (4), 139-147.

Kassim, M., Achoui, M., Mustafa, M. R., Mohd, M. A., & Yusoff, K. M. (2010). Ellagic acid, phenolic acids, and flavonoids in Malaysian honey extracts demonstrate in vitro anti-inflammatory activity. *Nutrition research, 30* (9), 650-659.

Klok, C. J., & Harrison, J. F. (2009). Atmospheric hypoxia limits selection for large body size in insects. *PLoS ONE, 4* (1), e3876.

Lupatini, M., Jacques, R. J., Antoniolli, Z. I., Suleiman, A. K., Fulthorpe, R. R., & Roesch, L. F. (2013). Land-use change and soil type are drivers of fungal and archaeal communities in the Pampa biome. *World journal of microbiology & biotechnology, 29* (2), 223-233.

Martino, D. (2004). Conservación de praderas en el conosur: valoración de las áreas protegidas existentes. *Ecosistemas, 13* (2), 114-123.

Mohamed, M., Sulaiman, S. A., Jaafar, H., & Sirajudeen, K. N. (2012). Effect of different doses of Malaysian honey on reproductive parameters in adult male rats. *Andrologia, 44 Suppl 1*, 182-186.

Musselman, L. P., Fink, J. L., Narzinski, K., Ramachandran, P. V., Hathiramani, S. S., Cagan, R. L., & Baranski, T. J. (2011). A high-sugar diet produces obesity and

insulin resistance in wild-type Drosophila. *Disease models & mechanisms, 4* (6), 842-849.

Paula, M. T., Zemolin, A. P., Vargas, A. P., Golombieski, R. M., Loreto, E. L., Saidelles, A. P., Picoloto, R. S., Flores, E. M., Pereira, A. B., Rocha, J. B., Merritt, T. J., Franco, J. L., & Posser, T. (2012). Effects of Hg(II) Exposure on MAPK Phosphorylation and Antioxidant System in D. melanogaster. *Environmental toxicology*.

Peran, P., Cherubini, A., Assogna, F., Piras, F., Quattrocchi, C., Peppe, A., Celsis, P., Rascol, O., Demonet, J. F., Stefani, A., Pierantozzi, M., Pontieri, F. E., Caltagirone, C., Spalletta, G., & Sabatini, U. (2010). Magnetic resonance imaging markers of Parkinson's disease nigrostriatal signature. *Brain : a journal of neurology, 133* (11), 3423-3433.

Roesch, L. F. W., Vieira, F. C. B., Pereira, V. A., Schünemann, A. L., Teixeira, I. F., Senna, A. J. T., & Stefenon, V. M. (2009). The Brazilian Pampa: A Fragile Biome. *Diversity, 1, 182*-198.

Siddique, H. R., Gupta, S. C., Dhawan, A., Murthy, R. C., Saxena, D. K., & Chowdhuri, D. K. (2005). Genotoxicity of industrial solid waste leachates in Drosophila melanogaster. *Environmental and molecular mutagenesis, 46* (3), 189-197.

Sies, H. (1991). Oxidative stress: Introduction. In Oxidative Stress: Oxidants and Antioxidants. Academic Press, London, UK.

Wallis, L. I., Paley, M. N., Graham, J. M., Grunewald, R. A., Wignall, E. L., Joy, H. M., & Griffiths, P. D. (2008). MRI assessment of basal ganglia iron deposition in Parkinson's disease. *Journal of magnetic resonance imaging : JMRI, 28* (5), 1061-1067.

Wang, J., & Li, Q. X. (2011). Chemical composition, characterization, and differentiation of honey botanical and geographical origins. *Advances in food and nutrition research, 62*, 89-137.

Yao, L. K., Razak, S. L. A., Ismail, N., Fai, N. C., Asyraf, M. H., Sharif, G. J. A. N. M., & Jubri, Z. (2011). Malaysian gelam honey reduces oxidative damage and modulates antioxidant enzyme activities in young and middle aged rats. *Journal of medicinal plant research, 5* (23), 5618-5625.

Zalibera, M., Stasko, A., Slebodavá, A., Jancovicová, V., Cermáková, T., & Brezová, V. (2008). Antioxidant and radical-scavenging activities of Slovak honeys: an electron paramagnetic resonance study. *Food Chemistry, 110* (2), 512-21.

Zhou, H., Huang, C., Tong, J., & Xia, X. G. (2011). Early exposure to paraquat sensitizes dopaminergic neurons to subsequent silencing of PINK1 gene expression in mice. *International journal of biological sciences, 7* (8), 1180-1187.