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AVALIAÇÃO DO EFEITO DO PÓ LIOFILIZADO DA CASCA DE JABUTICABA (*Myrciaria jaboticaba (Vell.) Berg*) SOBRE O ESTRESSE OXIDATIVO, TRATAMENTO DA OBESIDADE E PARÂMETROS DE SAÚDE *IN VIVO*.

Dissertação apresentada a Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas para obtenção do Titulo de Mestre em Alimentos e Nutrição - Área de Nutrição Básica Experimental Aplicada a Tecnologia de Alimentos.

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Albert Einstein

SUMÁRIO

RESUMO GERAL

O objetivo do presente estudo foi avaliar o efeito da casca de jabuticaba liofilizada (CJL) adicionada à dieta hiperlipídica no desenvolvimento da obesidade, perfil lipídico e hormonal e resistência à insulina, bem como sobre a peroxidação lipídica e modulação das enzimas com atividade antioxidante. Para tanto, ratos Sprague-Dawley foram alimentados com dieta Controle, dieta hiperlipídica (HF) e dietas HF adicionadas de 1, 2 e 4% de CJL. Ingestão energética, ganho de peso e composição corporal, além de glicose sérica e perfil lipídico e hormonal foram determinados. A resistência à insulina (RI) foi determinada pelo Teste de Tolerância à Glicose (GTT), Teste de Tolerância à Insulina (ITT) e cálculo do indicador de HOMA-IR. Para avaliar o status antioxidante, os níveis de TBARS e isoprostanos, bem como as concentrações de glutationa total, glutationa redutase, glutationa peroxidase, catalase e superóxido dismutase foram mensuradas no plasma dos animais. No tecido hepático, foram mensurados os níveis de TBARS e hidroperóxidos. Todos os resultados foram submetidos ao teste estatístico de ANOVA e teste de Tukey ($p<0,05$). A CJL adicionada à dieta HF não reverteu o ganho de peso e não alterou o colesterol total, perfil hormonal e glicose sérica. Contudo, o consumo de 1, 2 e 4% de CJL reduziu significativamente a insulina sérica e o indicador de HOMA-IR nos animais experimentais. Além disso, os animais alimentados com 2% de CJL mostraram níveis de HDL-colesterol similares ao grupo Controle (aumento de 41,65% comparado com o grupo HF). No status antioxidante, a CJL adicionada à dieta HF não reverteu a peroxidação lipídica no plasma e fígado dos animais. Porém, os níveis das enzimas glutationa redutase, catalase e superóxido dismutase foram aumentadas no plasma dos animais. Assim, o consumo de CJL pode promover aumento do HDL-colesterol, melhorar a RI e modular positivamente a atividade dessas enzimas, promovendo aumento no potencial antioxidante e prevenindo o desenvolvimento de doenças cardiovasculares, diabetes melitos tipo 2 e outras doenças relacionadas com o estresse oxidativo.

ABSTRACT

The objective of this study was to evaluate the effect of freeze-dried jaboticaba peel (FJP) added to high-fat diet on the development of obesity, lipid and hormonal profile and insulin resistance, as on lipid peroxidation and modulation of enzymes with antioxidant activity. For this purpose, Sprague-Dawley rats were fed a control diet, high-fat control diet and high-fat diets added to 1, 2 and 4% of FJP. Energy intake, weight gain and body composition, as serum glucose, lipid and hormonal profile were determined. The Insulin Resistance (IR) was determined by glucose tolerance test (GTT), insulin tolerance test (ITT) and calculation of HOMA-IR index. To evaluate the antioxidant status, the levels of TBARS and isoprostane, well as the concentrations of total glutathione, glutathione reductase, glutathione peroxidase, catalase and superoxide dismutase were measured in plasma of animals. Levels of TBARS and hydroperoxides were measured in liver tissue. All results were submitted to statistical analysis ANOVA and Tukey's test ($p<0.05$). The FJP added to the diet did not reverse the weight gain and did not alter the total cholesterol, hormonal profile and serum glucose. However, consumption of 1, 2 and 4% FJP reduced serum insulin and HOMA-IR index of the experimental animals. In addition, animals fed with 2% FJP showed HDL-cholesterol levels similar to control group (increase 41.65% compared to HF group). In antioxidant status, the FJP added to HF diet did not reverse the lipid peroxidation in plasma and liver of animals. However, the levels of enzymes glutathione reductase, catalase and superoxide dismutase were increased in plasma of animals. Thus, the consumption of FJP can increases HDL-cholesterol, improvement insulin resistance and modulate positively the activity these enzymes, promoting an increase in antioxidant potential and preventing the development of cardiovascular diseases, type 2 diabetes mellitus and other diseases related with oxidative stress.

1 INTRODUÇÃO

O elevado consumo de frutas e vegetais têm sido freqüentemente associado à proteção do organismo contra diversas doenças, incluindo câncer, doenças cardiovasculares e neurodegenerativas, como doença de Alzheimer (CROZIER et al, 2009). O efeito benéfico desses alimentos é, em grande parte, atribuído aos antioxidantes que fazem parte de sua composição, como as vitaminas C e E, os carotenóides, o licopeno, e os flavonóides, que possuem a propriedade de prevenir e amenizar os danos celulares causados pelos radicais livres. Os compostos fenólicos, aos quais pertencem os flavonóides, constituem a principal fonte de antioxidantes da dieta humana, podendo ser encontrados em elevadas concentrações em diferentes frutas e vegetais (DEGÁSPARI & WASZCZYNSKYJ, 2004).

As antocianinas, compostos da classe dos flavonóides, são os pigmentos naturais mais abundantes no reino vegetal. Além da potente ação antioxidante, associa-se a esses compostos a redução da obesidade, melhora do sistema imune, inibição de processos inflamatórios e prevenção de doenças cardiovasculares e câncer (BOBBIO et al, 2000; BRITO et al, 2007). O consumo de antocianinas têm sido associado a redução da inflamação do tecido adiposo, presente na obesidade, que por sua vez, se associa a resistência à insulina e outras complicações. Dessa forma, as pesquisas com antocianinas têm mostrado significativos efeitos benéficos à saúde, como melhora da obesidade, da glicemia, da insulinemia e do perfil lipídico (DORNAS et al, 2007).

Tendo em vista que o estresse oxidativo é responsável pelo aparecimento de diversas doenças de significante morbidade e mortalidade, associando a crescente epidemia de obesidade, diabetes melitos tipo 2 e doenças cardiovasculares e neurodegenerativas, as quais estão intimamente relacionadas com um padrão alimentar pouco saudável, pesquisas

para identificar e promover o consumo de alimentos que possam intervir no aparecimento e progressão destes agravos à saúde são urgentemente necessárias. Neste sentido, o presente trabalho avaliou o efeito do consumo da casca de jabuticaba liofilizada, rica em antocianinas, na prevenção do dano oxidativo, no combate à obesidade e na melhora de parâmetros de saúde *in vivo.*

2 REVISÃO BIBLIOGRÁFICA

2.1 Antocianinas: características químicas e propriedades biológicas

Os compostos fenólicos representam uma importante classe de substâncias orgânicas presentes nos vegetais, dividida em diversos subgrupos, dentre os quais se destacam os flavonóides. Os flavonóides são os fotoquímicos mais abundantes em plantas, resultantes do metabolismo da via dos fenilpropanóides dos tecidos vegetais, sendo encontrados em folhas, frutos e flores. Fatores ambientais, como radiação solar, nutrição, pluviosidade, estação do ano, e ainda a presença de poluentes, podem influenciar o metabolismo e a produção desses compostos e, conseqüentemente, sua concentração final no vegetal (DEGÁSPARI & WASZCZYNSKYJ, 2004).

As antocianinas são classificadas quimicamente como flavonóides devido a sua estrutura carbônica característica (C_3-C_6-C_3). De origem grega, a palavra antocianina deriva de *anthos* (flor) e *kyanos* (azul). Essas substâncias são responsáveis pelas cores azul, violeta, vermelho, roxo, magenta e laranja de diversos vegetais. Pigmentos antocianínicos estão presentes em muitos alimentos da dieta humana, como uva, maçã, feijão preto, morango, amora, jabuticaba, cerejas, açaí e jambolão, bem como os sucos, geléias, licores e vinhos preparados a partir desses alimentos (BOBBIO et al, 2000; BRITO et al, 2007). Essas substâncias são derivadas do cátion 2-fenilbenzopirona, também conhecido como cátion *flavylium* (ilustrado na figura 1) e ocorrem na natureza como glicosídeos derivados desta estrutura. A identificação de determinada antocianina se dá pela diferença no número de hidroxilas e/ou grupos metoxi presentes, o tipo, número e sítios de ligação dos açúcares, e o tipo e número de ácidos alifáticos ligados à molécula. A hidrólise do açúcar da

antocianina produz a fração aglicona, chamada de antocianidina (McGUIE & WALSON, 2007).

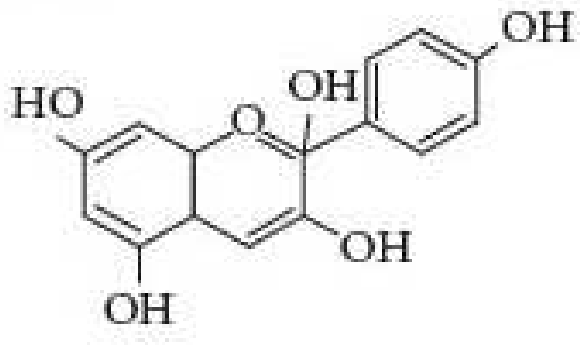

Figura 1 – Estrutura básica das antocianinas: cátion *flavylium*

Nos últimos anos o interesse da comunidade científica pelos efeitos fisiológicos das antocianinas se intensificou, tendo em vista que diversos estudos demonstraram um grande potencial dessas substâncias como compostos bioativos benéficos à saúde humana (CROZIER et al, 2009). A propriedade mais comumente atribuída às antocianinas é a sua capacidade de redução do dano oxidativo, a qual está intimamente relacionada com inúmeros efeitos benéficos ao organismo, como proteção contra o aparecimento e a progressão de certos tipos de cânceres, proteção contra as doenças cardiovasculares e diabetes, atividade antiinflamatória e de modulação da resposta imune, além da proteção contra distúrbios neuronais e doenças neurodegenerativas relacionadas ao envelhecimento (DORNAS et al, 2007).

2.2 Estresse oxidativo

O estresse oxidativo é definido como um desequilíbrio entre a quantidade formada e a quantidade inativada de espécies reativas de oxigênio e nitrogênio (ERONs) e parece ser

o mecanismo chave no envelhecimento e no desenvolvimento de uma variedade de doenças relacionadas à idade como a aterosclerose, diversos tipos de câncer, diabetes e doença de Alzheimer. O radical livre é um átomo ou molécula que possui elétrons desemparelhados em sua última camada, o que lhe confere alta reatividade. Espécies reativas de oxigênio são encontradas em todos os sistemas biológicos, formadas a partir do metabolismo celular aeróbio, no qual ocorrem inúmeras reações de oxidação e redução. Durante essas reações são formados intermediários reativos, como o radical superóxido (O_{-2}), hidroperoxila (HO_2), hidroxila (OH) e o peróxido de hidrogênio (H_2O_2) (FERREIRA & MATSUBARA, 1997).

As ERONs representam parte fundamental de processos biológicos indispensáveis, como a fagocitose e apoptose. Sendo assim, a eliminação total dos radicais livres seria tão prejudicial quanto o excesso destes. Quando em excesso, os radicais livres possuem a capacidade de causar danos muitas vezes irreversíveis as macromoléculas, como proteínas, lipídios, DNA e RNA. Dentre os prejuízos causados pelas ERONs, destaca-se a peroxidação lipídica, que ocorre geralmente a partir do ataque do radical livre a lipídios poliinsaturados, que são parte estrutural das membranas celulares. A peroxidação lipídica leva a uma modificação das características físico-químicas das membranas celulares, com alteração de sua fluidez e permeabilidade, provocando risco de ruptura e conseqüente morte celular (VASCONCELOS et al, 2007).

O combate ao dano oxidativo é mediado endogenamente pelo sistema antioxidante enzimático, composto basicamente pelas enzimas superóxido dismutase (SOD), glutationa redutase, glutationa peroxidase e catalase, que são responsáveis pela remoção e inativação das ERONs produzidas durante o metabolismo. Além dessa proteção endógena, o

organismo conta com os antioxidantes alimentares, como as vitaminas C e E, os carotenóides, o licopeno, e os flavonóides, que possuem a propriedade de prevenir e amenizar os danos celulares causados pelos radicais livres. Os compostos fenólicos, aos quais pertencem os flavonóides, constituem a principal fonte de antioxidantes da dieta humana, podendo ser encontrados em elevadas concentrações em diferentes frutas e vegetais (CHAGAS, 2002).

Alimentos e bebidas ricos em flavonóides têm sido associados a redução de risco de doenças relacionadas ao envelhecimento, devido a sua potente ação antioxidante, sendo importantes na remoção de ERONs. Além disso, eles são capazes de formar quelatos com íons metálicos, diminuindo a atividade pró-oxidante de metais como ferro e cobre (HALLIWELL, RAFTER & JENNER, 2005).

Estudos *in vitro* mostram que compostos polifenólicos, dentre os quais estão as antocianinas, possuem efetiva capacidade antioxidante. Essa propriedade é influenciada pelo número e posição dos grupos OH, assim como pelas posições de glicosilação. Os compostos polifenólicos se diferenciam de outros antioxidantes, como ácido ascórbico e α-tocoferol, que agem em meio aquoso e na camada fosfolipídica, respectivamente, por sua capacidade de agir em ambas as fases. Os polifenóis são capazes de captar diversos radicais como a alcoxila (RO•), alquilperoxila (ROO•), superóxido (O2 •-), radical hidroxila (HO•), óxido nítrico (NO•). A eficácia antioxidante de polifenóis *in vivo* ainda precisa ser mais bem avaliada, pois se sabe que a biodisponibilidade desses compostos pode ser influenciada por diferentes situações fisiológicas (CERQUEIRA; MEDEIROS; AUGUSTO, 2007)

A produção excessiva de espécies reativas de oxigênio é apontada como um dos mecanismos chave no desenvolvimento da obesidade e outros distúrbios metabólicos, como a resistência à insulina (RI). Segundo Feillet-Coudray (2009) em animais obesos há uma super produção de espécies reativas de oxigênio, e esse dano oxidativo excessivo é um importante iniciador da patogênese do diabetes e outras complicações. De acordo com Ando & Fujita (2009), a indução da obesidade por meio de dieta hiperlipídica em ratos aumenta o estresse oxidativo e o mecanismo ao qual se atribui tal efeito é a produção de adipocinas, como o Fator de Necrose Tumoral Alfa (TNF-α), pelos adipócitos. A elevada produção de ERONs pode também induzir um quadro de resistência à insulina e o consumo de drogas antioxidantes demonstram melhorar a RI.

2.3 Obesidade e Resistência à Insulina: implicações para a saúde humana

Nas últimas décadas a obesidade atingiu dimensões epidêmicas, especialmente nos países ocidentais e industrializados. Além dos aspectos psicossociais envolvidos neste processo patológico, a freqüente associação da obesidade com outras doenças como diabetes, hipertensão e doenças cardiovasculares preocupa pelo alto grau de morbidade e mortalidade (OGLESBY, 2006).

Define-se por obesidade o excessivo acúmulo de gordura nos adipócitos, geralmente associado à presença de um estado inflamatório e a resistência periférica à insulina. O aumento do tecido adiposo é acompanhado de uma maior expressão de citocinas inflamatórias, como o TNF-α. Essas citocinas possuem a capacidade de inibir a sinalização celular da insulina, além de prejudicar a funcionalidade das células β-pancreáticas,

responsáveis pela produção e liberação deste hormônio, levando a um quadro de resistência à insulina e, posteriormente, diabetes melitos tipo 2 (BRAY, 2006; DeFURIA *et al,* 2009).

A predisposição genética é vista como um dos fatores promotores para o desenvolvimento da obesidade, porém a dieta e o estilo de vida são apontados como a principal causa da elevada prevalência do excesso de peso entre a população. Hoje se sabe que muito além da elevada ingestão energética, o consumo de uma dieta rica em gorduras, especialmente as saturadas, é um importante desencadeante da obesidade. Segundo Willett (2006) o uso de gorduras como fonte de energia é menos eficiente que o uso dos outros macronutrientes, o que faz com que seu consumo elevado não estimule sua maior utilização, como ocorre com carboidratos e proteínas, e sim seu acúmulo nos adipócitos. Além disso, o aumento da massa dos adipócitos traz consigo uma maior hidrólise dos triacilgliceróis devido ao aumento da atividade da enzima lipase sensível ao hormônio (LHS), contribuindo para uma elevação dos níveis de ácidos graxos livres circulantes (ANGULO, 2002).

A resistência à insulina (RI) pode ser definida como um fenômeno biológico no qual o organismo necessita de uma quantidade maior de insulina para estimular as células a captarem uma mesma quantidade de glicose e, assim manterem as respostas celulares a este hormônio. Distúrbios transducionais na sinalização da insulina podem ser responsáveis pelo aparecimento da RI e também pelo estado de hiperinsulinemia compensatório (DIAS et al, 2009).

A insulina é um hormônio de extrema importância para o organismo, sendo responsável por efeitos metabólicos imediatos que incluem: aumento da captação de glicose, principalmente em tecido muscular e adiposo, aumento da síntese de proteínas, ácidos

graxos e glicogênio por esses tecidos, bloqueio da produção hepática de glicose, da lipólise e proteólise. Este hormônio age diretamente no fígado, tecidos periféricos e sistema nervoso central (hipotálamo), estimulado por substratos energéticos metabolizáveis pelas células β pancreáticas, sendo a glicose o secretagogo mais importante. No hipotálamo, este hormônio desempenha função importante no controle da ingestão alimentar, atuando de maneira anorexigênica (XU et al, 2005; TILG et al, 2006).

A hipertrofia do tecido adiposo, como ocorre na obesidade, particularmente na região abdominal, é associado à RI, hiperglicemia, dislipidemias, hipertensão e estados pró-trombóticos e inflamatórios. O estado inflamatório sub-clínico, comumente encontrado em indivíduos obesos associado ao aumento na secreção de adipocinas interferem negativamente na via de sinalização da insulina, ocasionando e instalando o quadro de RI (WELLEN et al, 2005).

2.3.1 Obesidade e resistência à insulina: o papel do tecido adiposo

Anteriormente, pensava-se que o tecido adiposo tinha como única função, a manutenção de uma reserva energética, contudo as descobertas recentes têm rompido com esse conceito. Os adipócitos são responsáveis pela síntese e secreção de substâncias com atividades biológicas importantes, conhecidas como adipocinas, que incluem o TNF-α, interleucinas, leptina, adiponectina, resistina, entre outras, o que dá a esse tecido importante função endócrina. Tais substâncias atuam na regulação do metabolismo energético, funções neuroendrócrinas, resposta inflamatória e sistema imune e, com exceção da adiponectina, tem sua produção aumentada na presença da obesidade (HAUNER, 2004)

Na obesidade, o tecido adiposo está infiltrado por macrófagos ativados, os quais também liberam quantidade excessiva de citocinas pró-inflamatórias, tais como o TNF-α, o inibidor 1 de ativador de plasminogênio (PAI-1), interleucina-6 (IL-6), proteína 4 ligadora de retinol, proteína 1 quimioatrativa de macrófagos (MCP-1) e proteínas de fase aguda. A produção excessiva desses fatores perpetua a inflamação local no tecido adiposo e induz à resistência à insulina e a disfunções vasculares e cardíacas (PERMANA, 2006).

O excesso de tecido adiposo é acompanhado por importantes modificações de perfil lipídico que estão intimamente associadas ao desenvolvimento de doenças cardiovasculares, como aterosclerose. O aumento da adiposidade resulta em uma maior liberação de ácidos graxos livres (AGL), devido ao aumento na taxa de lipólise. Esses AGL estimulam a deposição de triglicerídeos em tecidos periféricos, sendo a causa mais provável para o desenvolvimento de RI em fígado e músculos. A resistência à insulina presente nesses tecidos desencadeia diversas alterações metabólicas, como hiperglicemia, hiperinsulinemia, elevação dos níveis de triglicerídeos e LDL colesterol, além de diminuição nos níveis de HDL colesterol (MESHKANI & ADELI, 2009).

Algumas adipocinas produzidas no tecido adiposo têm sido foco nas pesquisas sobre obesidade e RI. A leptina, cujo nome deriva do grego *leptos* (magro) é hormônio fundamental na regulação e sinalização do apetite, produto do gene *Ob*, inicialmente identificado e seqüenciado em camundongos obesos geneticamente modificados da linhagem ob/ob (LAGO et al, 2009). A síntese de leptina ocorre no tecido adiposo e é regulada pela massa de gordura corporal e estado nutricional, apresentando níveis elevados em indivíduos obesos. No hipotálamo a leptina regula a expressão de neuropeptídeos ligados aos mecanismos de inibição da ingestão alimentar e aumento do gasto energético total. Seus

efeitos também se estendem ao metabolismo lipídico, com aumento da oxidação lipídica no músculo esquelético e redução da síntese de triglicerídeos a partir de ácidos graxos monoinsaturados no fígado (FONSECA-ALANIZ et al, 2006).

Ao contrário da demais adipocinas, a adiponectina, possui ação protetora contra doenças cardiovasculares e RI. Também conhecida como Acrp-30 (30-kDa adipocyte complement-related protein), ou adipoQ, é uma proteína expressa exclusivamente em adipócitos diferenciados. Os níveis circulantes de adiponectina apresentam correlação negativa com o índice de massa corporal (IMC), estando diminuída em indivíduos obesos. Os efeitos metabólicos desta adipocina incluem o aumento da sensibilidade à insulina, aumento da captação de glicose e da oxidação de ácidos graxos no músculo esquelético, além de ação antiinflamatória e anti-aterogênica (VÁZQUEZ-VELA et al, 2008; LAGO et al, 2009).

2.4 Perspectivas das pesquisas sobre o papel das antocianinas no combate ao dano oxidativo, obesidade e resistência à insulina.

Impulsionadas pelo expressivo crescimento nos índices de obesidade, diabetes tipo 2, câncer e doenças cardiovasculares e neurodegenerativas, as pesquisas na busca de alimentos e compostos bioativos que possam ter efeitos benéficos sobre a saúde humana têm ganho importante destaque nos últimos anos. Tendo em vista a íntima relação entre esses processos patológicos e a presença excessiva de radicais livres, as propriedades antioxidantes são as mais exploradas.

Nesse contexto, Tsuda et al (1994) utilizaram diferentes sistemas *in vitro* para avaliar o potencial antioxidante das antocianinas extraídas do feijão vermelho,

prioritariamente a cianidina-3-glicosideo (C3G), na sua forma natural e aglicona (cianidina), comparando-as com a atividade do α-tocoferol. Nos diferentes sistemas todos os compostos mostraram efetiva ação antioxidante, sendo que o mais potente foi a cianidina, seguido pela C3G e pelo α-tocoferol, com significativo decréscimo na produção de malondialdeido (MDA), produto da peroxidação lipídica. Esses resultados sugerem que C3G, bem como sua aglicona, cianidina, possuem potente efeito inibidor da peroxidação lipídica *in vitro.*

Resultados bastante similares foram encontrados também por Tsuda et al (1996), em estudo subseqüente, no qual utilizaram antocianinas isoladas de feijão (*Phaseulos vulgaris*), prioritariamente C3G, e sua aglicona, para avaliar o potencial dessas substâncias na inibição da peroxidação lipídica e sua atividade na eliminação do radical oxigênio, em diferentes sistemas *in vitro*, usando como padrão de comparação a atividade α-tocoferol. Nos sistemas estudados, tanto C3G, como sua aglicona, mostraram ação antioxidante superior ao α-tocoferol, por meio da inibição da formação de malondialdeido (MDA).

Han et al (2007) avaliaram os efeitos de *red potato flakes* (RPF), ricos em antocianinas, sobre a peroxidação lipídica e os níveis de RNAm da enzima superóxido dismutase hepática (SOD) em ratos machos da linhagem F344/DuCrj, que receberam dieta AIN-93G modificada, enriquecida com 25% de RPF por 4 semanas. Os resultados mostraram diminuição nos níveis de TBARS séricos, mas não houve alteração na concentração dessas substâncias no fígado, e maior expressão hepática da enzima SOD. Dessa forma, a dieta rica em RPF teve efeito positivo sobre a peroxidação lipídica sérica e no sistema antioxidante enzimático, sugerindo que as antocianinas da dieta podem aumentar o potencial antioxidante pós-prandial.

Em outro estudo de Han et al (2007), foram avaliados os efeitos de *purple potato flakes*, também ricos em antocianinas, no status antioxidante de ratos alimentados com dieta rica em colesterol. Ratos machos, da linhagem F344/DuCrj, receberam dietas enriquecidas com 0,5% de colesterol (controle e suplementadas) e dietas suplementadas com diferentes tipos de flocos de batata, que continham variadas concentrações de antocianinas. O estudo mostrou que a suplementação com *purple potato flakes* diminuiu a quantidade de TBARS no soro e no fígado dos animais, houve também diminuição dos níveis séricos de urato, importante marcador endógeno do dano oxidativo. Os níveis de glutationa hepática foram significantemente maiores nos animais que receberam a suplementação. Quanto às enzimas antioxidantes hepáticas, houve aumento na atividade da glutationa redutase e glutationa-s-transferase. Segundo os autores, tais resultados indicam que as antocianinas de *purple potato flakes* podem inibir o dano oxidativo e modular a atividade das enzimas antioxidantes hepáticas, o que dá a esses compostos relevante papel na proteção contra os efeitos danosos da peroxidação lipídica.

Em estudo que avaliou o efeito do *tart cherry juice*, rico em C3G, sobre o estresse oxidativo em homens e mulheres adultos saudáveis, o potencial antioxidante dessas substâncias foi novamente comprovado. Participaram da pesquisa 12 voluntários, sendo 6 homens e 6 mulheres, com média de idade de 69 anos, que receberam 240ml do suco testado duas vezes ao dia por 14 dias. A intervenção com o suco de torta de cereja reduziu os níveis de F2-isoprostana, indicando redução da peroxidação lipídica. Houve também redução na excreção urinária basal de ácidos nucléicos oxidados, mas não na excreção urinária de isoprostanas. Sendo assim, o consumo dessa bebida foi capaz de promover

maior proteção contra o dano oxidativo nos indivíduos participantes (TRAUSTADÓTTIR et al, 2009).

Há evidências de que o dano oxidativo seja um importante desencadeante do processo aterosclerótico, o que tem motivado pesquisas na busca de antioxidantes que inibam esse processo. Seguindo essa vertente, Rouanet et al (2010) em estudo recente avaliaram o potencial anti-aterogênico dos compostos fenólicos presentes no suco de diversas frutas da família das berries e nos chás preto e verde. Para isso, utilizaram 60 hamsters machos, divididos em 6 grupos de 10, que receberam uma dieta aterogênica, com 0,5% de colesterol durante 12 semanas de experimento. Durante esse período apenas o grupo controle teve acesso livre a água, enquanto os demais grupos receberam apenas sucos de berries ou chá preto e verde por meio de gavagem. O volume recebido pelos animais foi ajustado diariamente ao peso, baseado no cálculo de 275 ml/dia para um adulto humano de 70 kg. Os resultados obtidos mostraram que a deposição de gorduras na parede da aorta foi significantemente menor ($p< 0.001$) nos grupos suplementados com berries ou chás quando comparados ao controle. Contudo, os animais suplementados não apresentaram menores níveis circulantes de colesterol, o que indica que a hipercolesterolemia não é o principal determinante na aterogênese e sim a oxidação do LDL-colesterol. Os animais suplementados apresentaram uma redução não esperada na atividade das enzimas antioxidantes hepáticas, sendo esta mais pronunciada no consumo dos chás que dos sucos de berries. O autor justifica tal fato pela efetividade dos antioxidantes dietéticos em remover os radicais livres, reduzindo assim necessidade de ativação do sistema enzimático.

Além da atividade antioxidante, atribuí-se as antocianinas a capacidade de redução da obesidade, da resistência à insulina (RI) e melhora do perfil lipídico (PRIOR et al,

2008). Em estudo de Tsuda et al (2003), camundongos machos da linhagem C57BL/6J foram submetidos à dieta high-fat (HF), com 30% de banha suína e 5% de óleo de milho, suplementada com 2% de C3G de *purple corn color* (PCC). Os animais suplementados com C3G mostraram menor ganho de peso e diminuição dos níveis séricos de glicose, insulina e leptina, quando comparados ao padrão HF. Além disso, as antocianinas do PCC diminuíram os lipídios totais e triglicerídeos hepáticos desses animais.

Jayaprakasam e colaboradores (2006) avaliaram o efeito de C3G, de *Cornelian Cherry*, sobre a obesidade e resistência à insulina. Os camundongos C57BL/6 alimentados com dieta hiperlipidica suplementada com C3G (1g/Kg de dieta) apresentaram diminuição no ganho de peso, diminuição nos níveis séricos de insulina, normalização da intolerância à glicose e diminuição dos lipídios hepáticos, quando comparados aos não tratados. Esses animais também mostraram uma discreta diminuição nos níveis plasmáticos de colesterol e na deposição hepática de gordura. Portanto a dieta rica em antocianinas parece reduzir o risco de obesidade e diabetes tipo 2.

Em recente trabalho, Takikawa et al (2010), suplementou a dieta de camundongos machos diabéticos com extrato de *bilberry*, contendo 375g de antocianinas/Kg, equivalente a 1% de antocianinas na dieta, e mostrou que houve a diminuição da glicose sérica e aumento da sensibilidade à insulina, por meio da ativação da proteína quinase ativadora do AMP no tecido adiposo branco, músculo esquelético e fígado dos animais. A ativação da AMP quinase foi acompanhada de diminuição nos transportadores de glicose no tecido adiposo branco e músculo esquelético e supressão da produção hepática de glicose, além de menor deposição de lipídios nesse órgão.

Os resultados encontrados com a suplementação dietética de antocianinas sugerem que essas substâncias exercem importantes efeitos terapêuticos na inibição do estresse oxidativo, redução da obesidade e RI e melhora do perfil lipídico.

2.5 Jabuticaba (Myrciaria ssp): características e potencial terapêutico.

Nativa do Brasil, a jabuticabeira (*Myrciaria spp.)* é uma árvore frutífera que pode ser encontrada desde o Pará até o Rio Grande do Sul, tendo maior ocorrência e produtividade nos estados da região Sudeste. Seu cultivo é basicamente doméstico, em pequenas propriedades rurais, como chácaras, sítios e fazendas (SATO & CUNHA 2007). Dentre as espécies atualmente conhecidas, destacam-se a *Myrciaria cauliflora (DC) Berg* (jabuticaba Paulista ou Açu) e a *Myrciaria jabuticaba (Vell) Berg* (jabuticaba Sabará), com a última ocupando a maior área cultivada no Brasil. O fruto da jabuticabeira possui forma globosa, tamanho pequeno e polpa macia, esbranquiçada, mucilaginosa e de sabor sub-ácido e intensamente doce (DONADIO, 2000; MATTOS, 1983).

Do ponto de vista nutricional, a jabuticaba é um fruto bastante interessante. Sua polpa apresenta quantidades consideráveis de vitamina C e minerais, com destaque para ferro, cálcio, fósforo e potássio (OLIVEIRA et al., 2003). Quanto à casca, embora esta seja comumente desprezada, possui elevados teores de minerais e fibras solúveis e insolúveis. Além disso, a casca de jabuticaba apresenta altos níveis de compostos fenólicos totais, cerca de 2,70 g/100g na variedade Paulista e 1,89 g/100 g na Sabará, de acordo com Lima et al (2008). As antocianinas presentes nesta fração da fruta também aparecem em quantidades consideráveis. Segundo Terci (2004) o teor de antocianinas da casca, obtido por três diferentes métodos espectrofotométricos, variou entre 310 e 315 mg de

antocianinas por 100 g de fruta. Favaro (2008) também determinou o teor de antocianinas da casca de jabuticaba, utilizando métodos espectrofotométricos e CLAE, encontrando valores de 29,1 a 40,0 mg de antocianinas por 100 g de fruta.

A jabuticaba possui extensa aplicabilidade culinária e medicinal. O consumo desse fruto se dá geralmente na forma in natura ou como geléias e licores. Os efeitos medicinais atribuídos a esta fruta incluem ação antiasmática da polpa, sendo sua casca útil contra diarréia e irritações cutâneas (LIMA et al., 2008). Embora bastante popular em todo o País, a jabuticaba tem seu comércio limitado devido à alta perecibilidade, provocada principalmente pelo elevado teor de água e açúcares na polpa. Essa limitação comercial, além de reduzir a quantidade produzida, compromete a qualidade e valor comercial do fruto (DONADIO, 1993; MAGALHÃES, 1996).

Apesar do pronunciado consumo de jabuticaba, especialmente na região sudeste, a literatura traz poucos trabalhos que tenham avaliado seus constituintes químicos, propriedades físicas e funcionais. Diante desse déficit de estudos, se faz necessário o conhecimento mais aprofundado dos compostos bioativos presentes nas frações da fruta, bem como seus possíveis efeitos promotores de saúde, na busca de um melhor aproveitamento e maior valorização econômica desta fruta tipicamente brasileira.

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CAPÍTULO 1

FREEZE-DRIED JABOTICABA PEEL ADDED TO HIGH-FAT DIET INCREASES HDL-CHOLESTEROL AND IMPROVES INSULIN RESISTANCE IN SPRAGUE-DAWLEY RATS

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ABSTRACT: The objective of this study was to evaluate the effect of freeze-dried jaboticaba peel (FJP) added to high-fat diet on the development of obesity and associated parameters. For this purpose, Sprague-Dawley rats were fed a control diet, high-fat diet (HF) and HF diets added to 1, 2 and 4% of FJP. Energy intake, weight gain and body composition, well as serum glucose, lipid and hormonal profile were determined. The Insulin Resistance (IR) was determined by glucose tolerance test (GTT), insulin tolerance test (ITT) and calculation of HOMA-IR index. The results were submitted to ANOVA and Tukey's test, $p < 0.05$. The FJP added to the diet did not reverse the weight gain and did not alter the total cholesterol, hormonal profile and serum glucose. However, consumption of 1, 2 and 4% FJP reduced serum insulin and HOMA-IR index of the experimental animals. In addition, animals feed with 2% FJP showed HDL-cholesterol levels similar to control group (increase 41.65% compared to HF control group). Thus, the consumption of FJP can increases HDL-cholesterol and improvement insulin resistance, preventing the development of cardiovascular diseases and type 2 diabetes mellitus.

Keywords: *Myrciaria Jaboticaba* (Vell.) Berg., obesity, insulin resistance, HDL-cholesterol

1 INTRODUCTION

Obesity is reaching epidemic proportions in recent decades as the number of obese individuals worldwide increased considerably [1]. Obesity is defined by the excessive accumulation of fat in adipocytes, usually associated with the presence of an inflammatory state and insulin resistance. It is known that, in addition to insulin resistance, several complications are attributed to obesity, like cardiovascular disease, which is the major cause of deaths in the World [2].

In this context, the search for novel foods and bioactive compounds that may help prevent and control obesity and its complications has increased. Among the compounds currently studied anthocyanins and phenolic compounds should be cited. They belong to the family of flavonoids, are present in many fruits and flowers, and are responsible for red, purple and blue pigments in the vegetables [3]. The most important property of anthocyanins is its ability to reduce oxidative damage, which is associated with inhibition of the appearance and /or progression of certain types of cancers, well as protection against cardiovascular disease and diabetes [4].

Beyond the antioxidant effects, the consumption of foods rich in anthocyanins (such as blueberries, strawberries, black soybeans, purple corn and cherries, as purified anthocyanins) has been associated with reduced weight gain, regulation of hormones associated with obesity, improved glucose and insulin resistance in experimental animals [5, 6, 7]. Jaboticaba (*Myrciaria* spp.) is a typical fruit from Brazilian Cerrado. Jaboticaba peel has high levels of minerals, soluble and insoluble fiber and high levels of total phenolics compounds [8]. Anthocyanins are found in great concentrations on the fruit peel.

Several authors compare jaboticaba with berries (blackberry specifically) due to the high concentrations of cyanidin-3-glucoside and delphinidin-3-glucoside found on the peel; in this jaboticaba is currently regarded as a Brazilian berry [9].

Besides the chemical characteristics of jaboticaba peel shows possible health applications, reports that prove its benefits are scarce. Furthermore, there are few studies that have evaluated its effects on the development of obesity and its complications. Thus, the purpose of this study was to evaluate the role of freeze-dried jaboticaba peel on obesity, hormonal and lipid profiles and insulin resistance on experimental animals fed high-fat diet.

2 MATERIALS AND METHODS

2.1 Preparation of freeze-dried jaboticaba peel

Jaboticabas (M*yrciaria jaboticaba (vell.) berg*) were acquired in Campinas Central Supply (CEASA), Brazil. The fruits were selected, washed in tap water and the peels were separated manually. The peels were frozen, freeze-dried and milled [10] to get an homogeneous powder. The product was stored at -80 ° C in dark containers, prior to analysis and preparation of the diet used in biological assay.

2.2 Determination of chemical composition and quantification of anthocyanins in freeze-dried peel jaboticaba

Analyses of humidity, total protein and ash were performed according to methods described by Association of Official Analytical Chemists [11]. The total lipids were determined by Bligh & Dyer [12] and the determination of soluble and insoluble fiber was

performed according to ASP et al. [13]. Carbohydrate content was determined by difference.

The extraction of anthocyanins from freeze-dried jaboticaba peel was performed according to Wu [14], with subsequent determination in liquid chromatography equipped with a diode array detector and C18 column. The identification and quantification of total and individual anthocyanins were performed according Leite et al. [16].

2.3 Animals and diets

Thirty-five Sprague-Dawley, male, recently weaned, weighting 58 ± 18.77g were obtained from the Multidisciplinary Center for Biological Research at UNICAMP (CEMIB). This experiment was approved by the Ethics Committee on Animal Experiments (CEEA / UNICAMP), protocol number 2226-1, and followed all the ethical requirements of the Brazilian College of Animal Experimentation (COBEA). The animals were randomly distributed into five groups (n = 7) and remained at individual cages with food and water under the system of free access, with temperature and humidity controlled, with a range of 22 ± 1 °C and 60-70% respectively, and light / dark cycle of 12 hours, throughout the experimental period.

Two control diets were given during the experiment: a normolipidic control diet, prepared in accordance with the American Institute of Nutrition [17], AIN-93G, with protein concentration of 12% [18] and a high-fat control diet, AIN 93G-modified with 12% protein and 35% (g) of fat, 4% vegetable oil (soybean) and 31% of animal origin (swine fat) [19]. Beyond the control diets, three experimental diets were given: high-fat diet added with freeze-dried jaboticaba peel (FJP) in three different concentrations (1, 2 and 4% w/w).

Adjustments were done in order to get diets with same contents of calories and fibers. Control group received normolipidic control diet and group HF high-fat control diet throughout the experiment; groups HFJ1%, HFJ2% and HFJ4% received high-fat control diet during the first 4 weeks and diets added with FJP until the end of 10 weeks. Diet consumption was monitored every 2 days and weight gain once a week.

Humidity, ash, protein [11] and total lipids [12] were determined on all the experimental diets. The concentration of carbohydrates was obtained by difference and energy value was determined in isoperibol automatic calorimeter (PARR 1261) with oxygen pump (PARR 1108).

After 10 experimental weeks, the animals were sacrificed by decapitation preceded by 12 hour fasting, Blood was collected in tubes with and without anticoagulant EDTA to obtain plasma and serum, respectively. After exsanguination, the organs were removed, cleaned with saline, weighed, and the liver was frozen in liquid nitrogen and stored in a freezer at - 80 ° C for further analysis. The carcasses of the animals was weighed, packaged and frozen at - 20 °C.

2.4 Evaluation of body composition

The assessment of body composition was performed according to Park et al. [20]. The carcasses were frozen, sliced, dried, crushed and stored at -80 ° C until the time of the determinations of humidity, total ash and total nitrogen [11]. The determination of total fat was made by the method of Bligh & Dyer [12].

2.5 Measurement of serum hormone animals

Enzyme-linked immunosorbent assay (ELISA) method was used to determine serum levels of insulin, leptin, ghrelin and adiponectin by commercial kits *Millipore*®.

2.6 Analysis of serum lipid profile and glucose levels of animals

Serum levels of total cholesterol, HDL-cholesterol, triglycerides and glucose were measured, respectively, by enzymatic method, direct enzymatic and totally enzymatic with colorimetric commercial kits *Laborlab*®. The quantification of free fatty acids was performed by enzymatic colorimetric kit HR Series NEFA-HR 2, ACS-ACOD method *Wako*®.

2.7 Determination of insulin resistance

The state of glucose tolerance and insulin sensitivity were determined before the start of supplementation with the freeze-dried peel jaboticaba and the end of the experimental period using the intraperitoneal Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT), respectively. For both tests, the animals were fasted for 12 hours. For GTT, blood glucose was determined at t=0 through a small incision in the caudal vein, followed by injection of glucose solution 25% (1.1 mmol / kg) in the peritoneal cavity. The glucose was again measured at 30, 60, 90 and 120 minutes in order to determine the rate of reduction of glucose level. The results, expressed in mg / dL, were used to calculate the area under the curve (AUC) in glucose versus time graph. As for the ITT, blood glucose was measured through caudal vein, at baseline and at 5, 10, 15, 20, 25 and 30 minutes after intraperitoneal administration of 0.75 MU / kg of human rapid insulin [19, 6]. The results

(expressed in mg/dL) were used to calculate the decay constant of the glucose curve ($_K$ITT). The measurement of blood glucose was performed using blood glucose test strips Optium mini blood glucose monitoring system, *Abbott*®, for the GTT and ITT. Homeostatic Model Assessment (HOMA-IR) was calculated according with Prior et al. [7], using the data from serum glucose and insulin concentrations. HOMA-IR, which estimates the presence or absence of insulin resistance, was calculated by the multiplication of the concentration of glucose (mmol / L) by insulin (mU / L), dividing the result by the factor 22.5.

2.8 Statistical analysis

For data analysis we used the statistical program Statistical Analysis System (SAS) 9.1.3. [21]. The results were submitted to analysis of variance (ANOVA) and means treatment were compared by Tukey test with an alpha of 0.05.

3 RESULTS

The peel jaboticaba is composed mainly of carbohydrates, including soluble and insoluble fiber, and water (Table 1). Possibly, the high content of carbohydrate found reflects the vast amount of simple and structures sugars which, in general, are the major constituents of fruit peel [22].

Table 1. Proximate composition of the freeze-dried jaboticaba peel.

Components (g%)	Mean ± SD [1]
Moisture	15.33 ± 0.19
Protein[2]	4.89 ± 0.10
Lipids	1.72 ± 0.02
Ashes	3.52 ± 0.02
Insoluble fiber	20.00 ± 2.00
Soluble fiber	5.00 ± 0.50
Carbohydrates	49.46
Energy value[3]	2.32

[1]Analyses of the moisture, protein, lipids and ashes were performed in triplicate, and the results were expressed in mean ± SD. [2]Conversion factor used to calculate protein: N = 6,25. [3]Value expressed in kcal/g of the peel.

The composition of experimental diets, well as its anthocyanins concentration is shown in Table 2. The predominant anthocyanins in the FJP, cyanidin-3-glucoside and delphinidin-3-glucoside, were previously determined by Leite et al. [16]. Terci [23] and Reynertson et al. [24] had described the presence of these same anthocyanins in fresh and freeze-dried jaboticaba, respectively, in similar concentrations, however, they also described the presence of peonidina-3-glycoside in the fruit.

Table 2. Composition of modified AIN-93G diets fed to rats.

INGREDIENTS	**Control**	**HF**[1]	**HFJ1%**[1,2]	**HFJ2%**[1,2]	**HFJ4%**[1,2]
	(g/Kg)	(g/Kg)	(g/Kg)	(g/Kg)	(g/Kg)
Casein (78% prot.)	153,85	153,85	153,85	153,85	153,85
Corn starch	426,63	249,82	249,82	249,82	249,82
Maltodextrin	141,68	82,91	82,91	82,91	82,91
Sucrose	107,33	62,92	62,92	62,92	62,92
Soybean oil	70,00	40,00	40,00	40,00	40,00
Cellulose	50,00	50,00	47,50	45,00	40,00
Mineral mix	35,00	35,00	35,00	35,00	35,00
Vitamin mix	10,00	10,00	10,00	10,00	10,00
L-cystine	3,00	3,00	3,00	3,00	3,00
Choline bitartarate	2,50	2,50	2,50	2,50	2,50
Tert-Butyl Hydroquinone	0,014	0,014	0,014	0,014	0,014
Lard	-	310,00	310,00	310,00	310,00
Freeze-dried peel jaboticaba	-	-	10,00	20,00	40,00
Total	***1000***	***1000***	***1007,54***	***1015,01***	***1030,01***
Anthocyanins (mg/kg diet)					
Cyanidin-3-O-glucoside	-	-	196,4	392,8	785,6
Delphinidin-3-O-glucoside	-	-	63,5	127	254
Total	***-***	***-***	***259,9***	***519,8***	***1039,6***
Energy value (Kcal/g) [3]	**4,252**	**5,834**	**5,809**	**5,772**	**5,784**

[1]HF, HFJ1%, HFJ2% and HFJ4% diets were added 31% of lard and, consequently, starch, sucrose and maltodextrin contents were reduced; [2]HFJ1%, HFJ2% and HFJ4% diets were added to 1, 2 and 4% of freeze-dried jaboticaba peel. [3]Caloric value expressed in Kcal/g diet, determined by calorimetry.

The cumulative consumption of diet did not differ significantly among the groups, although group HFJ4% showed a numerically higher diet intake compared to other groups (Figure 1A). As expected, HF, HFJ1%, HFJ2%, and HFJ4% groups showed much higher energy intakes than the control (Figure 1B).

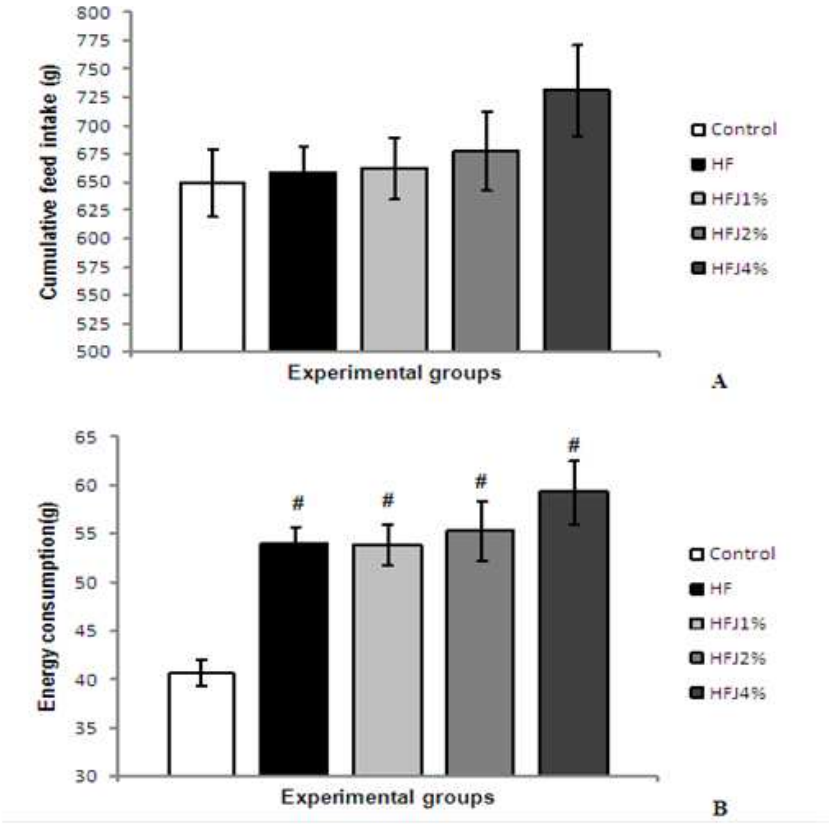

Figure 1. Cumulative feed intake (A) and energy consumption after 10 experimental weeks (B). Control: group fed with control diet; HF: group fed with high-fat diet; HFJ1%: group fed with HF diet added to 1% FJP; HFJ2%: group fed with HF diet added to 2% FJP; HFJ4%: group fed with HF diet added to 4% FJP. Bars represent mean ± SE. Data statistically analyzed using ANOVA and Tukey's test ($p<0.05$). # indicates statistical difference from control group.

Weight gain and cumulative weight gain (Figure 2) clearly reflected the results of feed intake, with no statistical difference between HF and HF groups added with FJP. However, groups HF, HFJ1%, HFJ2% and HFJ4% showed higher cumulative weigh gain

compared to control, demonstrating the efficiency of high-fat diet on weight accumulation. Thus, supplementation of high-fat diet with FJP did not prevent weight gain caused by high-fat diet in experimental animals.

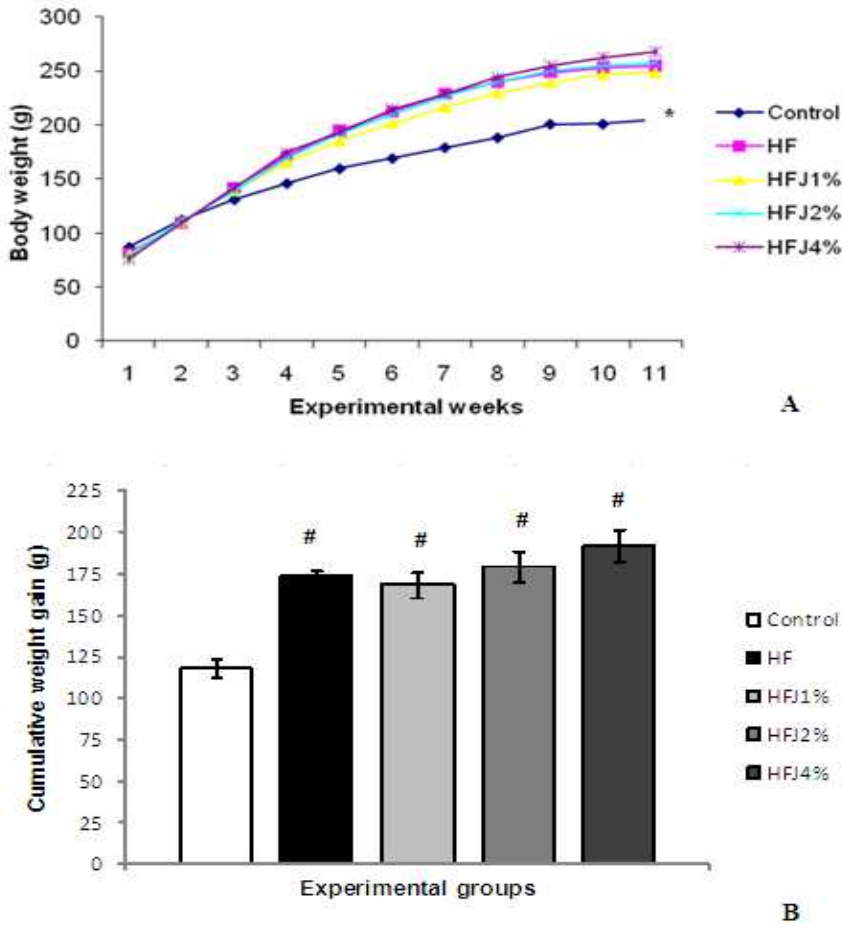

Figure 2. Body weight gain along 10 experimental weeks (A) and cumulative weight gain after 10 experimental weeks (B). Control: group fed with control diet; HF: group fed with high-fat diet; HFJ1%: group fed with HF diet added to 1% FJP; HFJ2%: group fed with HF diet added to 2% FJP; HFJ4%: group fed with HF diet added to 4% FJP. Bars represent mean ± SE. Data statistically analyzed using ANOVA and Tukey's test ($p<0.05$). * indicates statistical difference from HF group and # indicates statistical difference from control group.

The values obtained in the proximate analysis of carcasses (table 3) at the end of the experimental period corroborate the results of weekly and total weight gain. There was no

statistical change in body composition of animals, regardless of treatment received. Significant difference was detected for ashes: the animals that received high-fat diets (with and without FJP) showed lower values of ash contents on the carcass.

Table 3 – Body composition of the rats, after 10 experimental weeks[1].

Composition (%)	Control[3]	HF[3]	HFJ1%[3]	HFJ2%[3]	HFJ4%[3]
Protein[2]	60.4 ± 4.0[a]	58.9 ± 3.3[a]	60.0 ± 3.6[a]	57.5 ± 2.3[a]	60.2 ± 3.6[a]
Lipids	25.4 ± 4.5[a]	28.7 ± 4.0[a]	26.9 ± 5.4[a]	29.2 ± 2.7[a]	28.0 ± 3.1 [a]
Moisture	5.4 ± 1.3[a]	4.6 ± 0.4[a]	5.3 ± 1.0[a]	4.9 ± 0.3[a]	4.6 ± 0.3[a]
Ashes	12.8 ± 1.3[a]	10.8 ± 1.2[b]	11.0 ± 0.5[b]	11.2 ± 1.7[b]	10.7 ± 0.5[b]

[1]Body composition after carcasses freeze-drying. [2]Conversion factor used to calculate protein: N = 6,25. [3]Control: group fed with control diet; HF: group fed with high-fat diet; HFJ1%: group fed with HF diet added to 1% FJP; HFJ2%: group fed with HF diet added to 2% FJP; HFJ4%: group fed with HF diet added to 4% FJP. Data presented as mean ± SD. Data statistically analyzed using ANOVA. Statistical differences (Tukey's test ($p<0.05$)) are represented by different letters.

Table 4 shows the organs weight after the experimental period. The weight of liver of group HFJ4% was statistically ($p<0.05$) higher than the control group. The weight of liver of the other experimental groups varied between the ones from control and HFJ4% groups. Identical results were obtained for heart and pancreas. As for the kidneys, there was no significant difference among the experimental groups. The weight of epididymal, mesenteric and retroperitoneal adipose tissues showed similar results, with a difference ($p<0.05$) between control and HF groups. The percentage of fat in the liver of animals was higher ($p<0.05$) in groups HF and HFJ1% and lower in the control group. Jaboticaba peel did not prevent hepatic accumulation on the amounts offered to the animals.

Table 4. Liver, kidneys, heart, pancreas, epididymal adipose tissue (EAT), mesenteric adipose tissue (MAT), retroperitoneal adipose tissue (RAT) weight and percentage of hepatic fat[1]

	Control[2]	**HF[2]**	**HFJ1%[2]**	**HFJ2%[2]**	**HFJ4%[2]**
Liver (g/100g)	4,03 ± 0,23 [b]	4,89 ± 0,24 [ab]	4,56 ± 0,22 [ab]	4,65 ± 0,35 [ab]	4,87 ± 0,19 [a]
Kidneys (g/100g)	1,42 ± 0,08 [a]	1,24 ± 0,07 [a]	1,14 ± 0,06 [a]	1,17 ± 0,07 [a]	1,22 ± 0,02 [a]
Heart (g/100g)	0,50 ± 0,02 [b]	0,57 ± 0,03 [ab]	0,55 ± 0,02 [ab]	0,59 ± 0,02 [ab]	0,60 ± 0,01 [a]
Pancreas (g/100g)	0,45 ± 0,02 [a]	0,45 ± 0,03 [a]	0,46 ± 0,02 [a]	0,45 ± 0,02 [a]	0,46 ± 0,03 [a]
EAT (g/100g)	1,69 ± 0,10 [b]	2,41 ± 0,27 [a]	1,92 ± 0,15 [ab]	2,25 ± 0,28 [ab]	2,33 ± 0,13 [ab]
MAT (g/100g)	0,80 ± 0,10 [b]	1,17 ± 0,15 [ab]	1,42 ± 0,13 [a]	1,34 ± 0,18 [ab]	1,49 ± 0,18 [a]
RAT (g/100g)	1,07 ± 0,26 [b]	1,89 ± 0,25 [a]	1,37 ± 0,18 [ab]	1,78 ± 0,33 [a]	1,79 ± 0,20 [a]
% Hepatic fat[3]	18,13 ± 1,96[b]	21,46 ± 1,94[a]	22,05 ± 2,54[a]	20,80 ± 3,15 [a,b]	21,08 ± 1,35 [a,b]

[1]Weight of tissues and organs of the rats after 10 experimental weeks. [2]Control: group fed with control diet; HF: group fed with high-fat diet; HFJ1%: group fed with HF diet added to 1% FJP; HFJ2%: group fed with HF diet added to 2% FJP; HFJ4%: group fed with HF diet added to 4% FJP. [3]Values expressed in percentage, obtained by extraction and quantification of lipids by Bligh & Dyer. Data presented as mean ± SE. Data statistically analyzed using ANOVA. Statistical differences (Tukey's test ($p<0.05$)) are represented by different letters.

There were no statistical differences on the plasmatic levels of triglycerides, total cholesterol and free fatty acid among the experimental groups (Table 5), showing that HF diet and the addition these specific amounts of freeze-dried peel jaboticaba during the period of the study did not influenced on these parameters. As for serum glucose, there was a significant difference ($p<0.05$) between control and the other groups (Table 5), indicating that the HF diet caused an increase in blood glucose levels of experimental animals and that consumption of these specific amounts of FJP were not able to revert this effect.

For leptin levels too there were no significant differences among experimental groups. The concentrations of adiponectin were higher in control group ($p<0.05$) when compared to the groups that received high-fat diet, added with jaboticaba or not. The serum ghrelin concentration was lower ($p<0.05$) in the HF, HFJ1% and HFJ4% groups compared to the control. The HFJ2% showed increased ghrelin concentrations with similar values compared to the control group (Table 5).

Table 5. Triacylglycerols, total cholesterol, glucose and hormonal profile in serum and free fatty acids (FFA) in plasm of the rats[1]

	Control[2]	**HF[2]**	**HFJ1%[2]**	**HFJ2%[2]**	**HFJ4%[2]**
Triacylglycerols (mg/dL)	45,53 ± 5,20 [a]	45,96 ± 5,25 [a]	39,67 ± 5,96 [a]	42,58 ± 6,97 [a]	49,49 ± 3,75 [a]
Total cholesterol (mg/dL)	80,36 ± 4,32 [a]	80,38 ± 5,66 [a]	72,48 ± 6,04 [a]	79,39 ± 4,76 [a]	69,24 ± 2,19 [a]
FFA (mMol/L)	0,72 ± 0,036 [a]	0,66 ± 0,029 [a]	0,67 ± 0,088[a]	0,73 ± 0,050 [a]	0,60 ± 0,055 [a]
Glucose (mg/dL)	86,90 ± 4,18[b]	107,31 ± 2,51 [a]	114,74 ± 6,34 [a]	115,45 ± 3,32 [a]	115,43 ± 5,22[a]
Adiponectin (ng/mL)	49238,30 ± 10764,53[a]	18246,54 ± 2622,05 [b]	16242,64 ± 669,31 [b]	14301,07 ± 589,97 [b]	14916,50 ± 587,25 [b]
Ghrelin (ng/mL)	1,26 ± 0,16 [a]	0,61 ± 0,12 [b]	0,56 ± 0,14 [b]	0,80 ± 0,12 [a,b]	0,72 ± 0,05 [b]
Leptin (ng/mL)	2,29 ± 0,49 [a]	3,98 ± 0,48 [a]	3,48 ± 0,45 [a]	3,46 ± 0,49 [a]	3,86 ± 0,49[a]

[1]Triacylglycerols, total cholesterol, glucose and hormonal profile in serum and free fatty acids (FFA) in plasm of the rats after 10 experimental weeks. [2]Control: group fed with control diet; HF: group fed with high-fat diet; HFJ1%: group fed with HF diet added to 1% FJP; HFJ2%: group fed with HF diet added to 2% FJP; HFJ4%: group fed with HF diet added to 4% FJP. Data presented as mean ± SE. Data statistically analyzed using ANOVA. Statistical differences (Tukey's test ($p<0.05$)) are represented by different letters.

The consumption of FJP showed a role on HDL-plasma cholesterol (Figure 3). HF and HFJ1% groups showed statistically lower HDL-cholesterol ($p<0.05$) than the other

groups. The addition of 2% of FJPs on the diet was able to revert the HDL-cholesterol decrease caused by high-fat diet. The addition of 4% of FJPs showed a partial reversion on the decrease of HDL-cholesterol by high-fat diet. This result indicates that the HF diet added to 2% freeze-dried peel jaboticaba was able to protect the animals of decrease this cardiovascular risk marker. As there was no change in total cholesterol concentration of experimental animals, it is speculated that the increase in HDL-cholesterol is a reflection of lowering LDL-cholesterol.

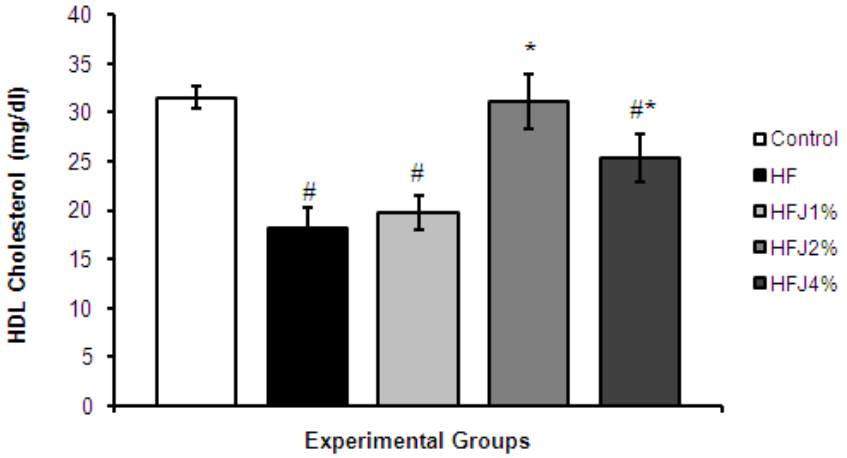

Figure 3. HDL plasma concentrations after 10 weeks of experiment. Control: group fed with control diet; HF: group fed with high-fat diet; HFJ1%: group fed with HF diet added to 1% FJP; HFJ2%: group fed with HF diet added to 2% FJP; HFJ4%: group fed with HF diet added to 4% FJP. Data presented as mean ± SE. Data statistically analyzed using ANOVA. Statistical differences (Tukey's test ($p<0.05$)) are represented by # or *. # indicates statistical difference from the control group and * indicates statistical difference from the HF group.

Insulin resistance (IR) can be expressed using intraperitoneal glucose tolerance test (GTT), insulin tolerance test (ITT) (Figures 4 and 5), serum insulin levels (Figure 6) and HOMA-IR (Figure 7). The results of GTT and ITT (Figures 4 and 5) showed no statistical difference among the groups. However, there is a trend to improvement of insulin

resistance in the groups fed with FJP, especially for the group HFJ1%, when we compared the ITT before and after consumption of FJP.

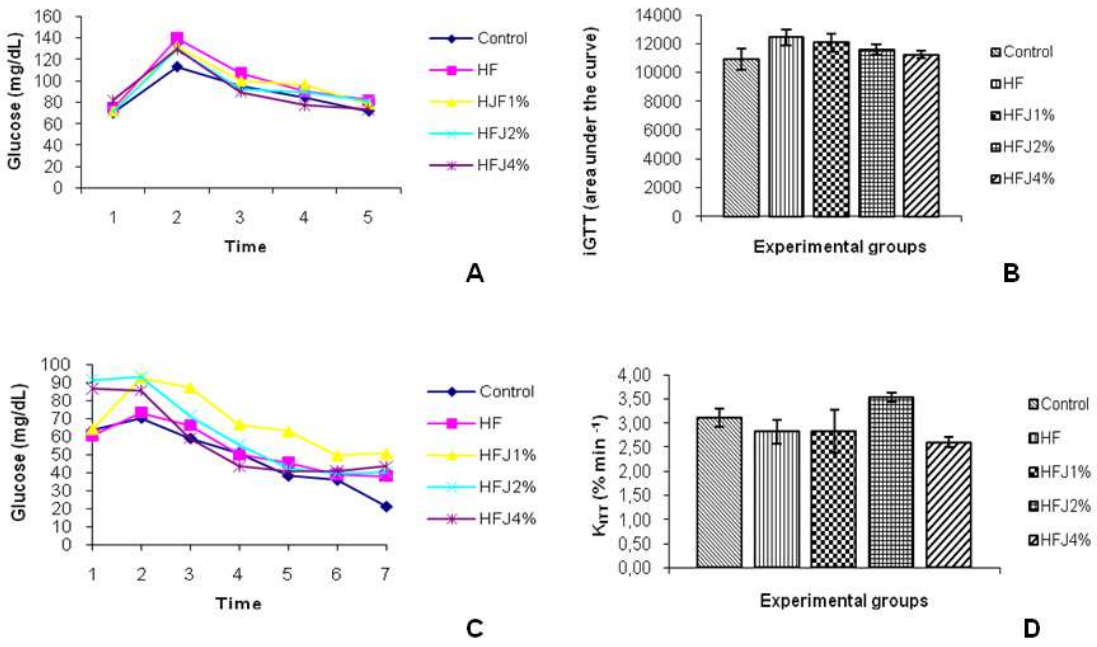

Figure 4. Results of intraperitoneal Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT) before supplementation with freeze-dried jaboticaba peel. A) Mean of blood glucose levels 0, 30, 60, 90 e 120 minutes after intraperitoneal infusion of glucose solution. B) Mean area under the curve and standard error. C) Mean blood glucose 0, 5, 10, 15, 20, 25 e 30 min after intraperitoneal infusion of insulin. D) Mean values do kITT and standard error (curve slope in % min-1). Control: group fed with control diet; HF: group fed with high-fat diet; HFJ1%: group fed with HF diet added to 1% FJP; HFJ2%: group fed with HF diet added to 2% FJP; HFJ4%: group fed with HF diet added to 4% FJP. Data presented as mean ± SE. Data statistically analyzed using ANOVA. Statistical differences (Tukey's test ($p<0.05$)) are represented by # or *. # indicates statistical difference from the control group and * indicates statistical difference from the HF group.

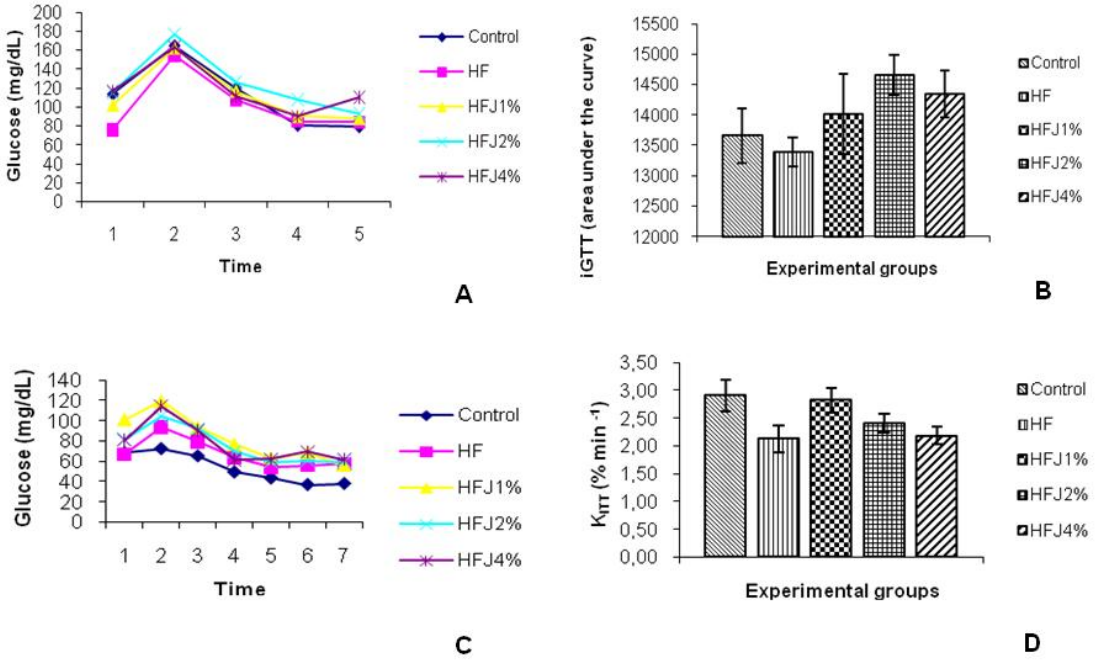

Figure 5. Results of intraperitoneal Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT) after supplementation with freeze-dried jaboticaba peel. A) Mean of blood glucose levels 0, 30, 60, 90 e 120 minutes after intraperitoneal infusion of glucose solution. B) Mean area under the curve and standard error. C) Mean blood glucose 0, 5, 10, 15, 20, 25 e 30 min after intraperitoneal infusion of insulin. D) Mean values do kITT and standard error (curve slope in % min-1). Control: group fed with control diet; HF: group fed with high-fat diet; HFJ1%: group fed with HF diet added to 1% FJP; HFJ2%: group fed with HF diet added to 2% FJP; HFJ4%: group fed with HF diet added to 4% FJP. Data presented as mean ± SE. Data statistically analyzed using ANOVA. Statistical differences (Tukey's test ($p<0.05$)) are represented by # or *. # indicates statistical difference from the control group and * indicates statistical difference from the HF group.

Serum levels of insulin were significantly lower in groups that received FJP, when compared to HF (Figure 6), showing that the FJP was efficient to reduce hyperinsulinemia in these animals. Corroborating the results of insulin, HOMA-IR was significantly lower in groups fed with FJP, when compared to HF, indicating an improvement in insulin resistance.

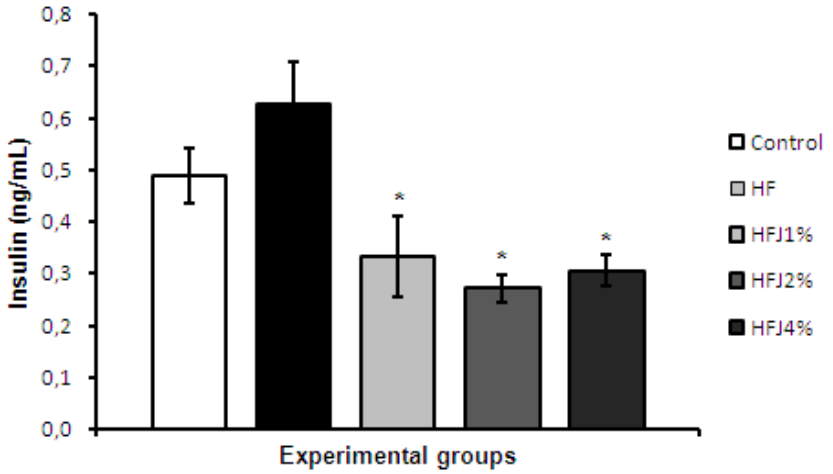

Figure 6. Insulin serum concentrations after 10 weeks of experiment. Control: group fed with control diet; HF: group fed with high-fat diet; HFJ1%: group fed with HF diet added to 1% FJP; HFJ2%: group fed with HF diet added to 2% FJP; HFJ4%: group fed with HF diet added to 4% FJP. Data presented as mean ± SE. Data statistically analyzed using ANOVA. Statistical differences (Tukey's test ($p<0.05$)) are represented by # or *. # indicates statistical difference from the control group and * indicates statistical difference from the HF group.

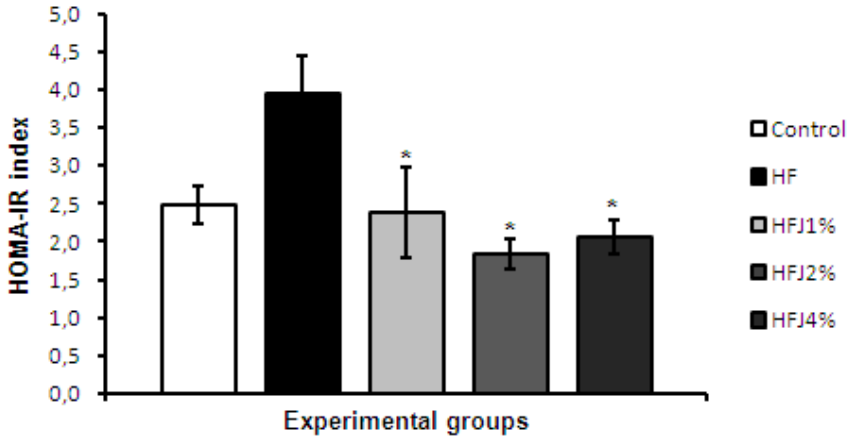

Figure 7. HOMA-IR index after 10 weeks of experiment. Control: group fed with control diet; HF: group fed with high-fat diet; HFJ1%: group fed with HF diet added to 1% FJP; HFJ2%: group fed with HF diet added to 2% FJP; HFJ4%: group fed with HF diet added to 4% FJP. Data presented as mean ± SE. Data statistically analyzed using ANOVA. Statistical differences (Tukey's test ($p<0.05$)) are represented by # or *. # indicates statistical difference from the control group and * indicates statistical difference from the HF group.

4 DISCUSSION

Several studies have evaluated the effects of supplementation of high-fat diet with purified anthocyanins and fruits rich with these compounds on the development of obesity. Studies demonstrated that the supplementation of high-fat diets with cyanidin-3-glucoside (C3G) extracted from different sources promoted reduction in weight gain in mices [5, 26, 27]. However, the consumption of freeze-dried foods as a source of anthocyanins added to high-fat diet did not seem to have that effect to reducer in weight gain and body fat percentage in experimental animals [6, 28].

In our study the supplementation of the diets with FJP did not promote significant changes in weight gain, organ weight and body composition. These results, well as those obtained in previous studies, are not conclusive to disclose all the possible effects of anthocyanins on weight gain and body composition of animal models. It is suggested that the purified anthocyanins have positive effects on reduction of weight gain and metabolic changes results from obesity. However, the data published so far reveal that dietary supplementation with fruits rich in anthocyanins has less effect in body weight reduction compared to the isolated form. In an attempt to explain the possible inefficiency of dried fruit in reducing obesity, it is assumed that there may have some compound in the fresh fruit that slows or impede the absorption of anthocyanins [7].

Although the bone mass of the animals has not been evaluated, it is believed that the small ash content in groups HF may indicate a change in bone mineralization. One possible explanation for this finding was described by Xiao et al. [25]. According to the authors, the adipocytes and osteoblasts originate from mesenchymal stem cells (MSCs) and the

increased formation of adipocytes caused by supplementation with lard may have a reduction in osteoblast cell differentiation, leading to reduced bone formation.

Metabolic complications results from the development of obesity are of great importance due to its health consequences. The lipid profile is a major metabolic variable altered in obesity. Elevated levels of total cholesterol, LDL-cholesterol, triglycerides and low levels of HDL-cholesterol are associated with higher incidence of cardiovascular diseases, as atherosclerosis, and diabetes [31, 32].

Although there is no consensus among researchers, atherosclerosis could be strongly related to hyperlipidemia [33]. There is evidence that oxidative damage plays a role on atherosclerotic development, which has motivated searches for antioxidants, like anthocyanins, that could minimize this process [34].

The HDL-cholesterol is strongly related to the prevention of cardiovascular disease. Kwon et al. [35] reported an increase of 28% in HDL-cholesterol and a reduction of 45 and 26% of triglycerides and cholesterol, respectively, in rats fed with high-fat diet added with anthocyanins (C3G) extracted from black soya, compared with the group control. In the group receiving the high-fat diet plus freeze-dried black soya, reductions of 23 and 20% in triglycerides and cholesterol, respectively, and an increase of 37% in HDL-cholesterol were observed. Similar results were reported by Rodrigues et al. [36] using rutin added to normolipidic rat's diet. The animals that received the supplementation did not show changes in blood glucose, triglycerides and total cholesterol; however, a significant increase in HDL-cholesterol was observed. The authors attribute the increase in HDL-cholesterol to the antioxidant effect of rutin, which increased levels of superoxide

dismutase and reduced lipid peroxidation and, possibly, the oxidation of LDL-cholesterol. It is known that excessive consumption of saturated fat is not able to decrease serum levels of HDL-cholesterol, but significantly increases the levels of LDL-cholesterol. The LDL-cholesterol is key in the atherogenic process because its ability to transport the cholesterol from liver for peripheral tissues, favoring the accumulation of lipid in the tissues and arteries, increasing the risk of atherogenic plaque formation. The protective effect of HDL-cholesterol occurs precisely because its action opposite to LDL, carrying blood cholesterol for liver, where it is metabolized and excreted [37].

Our results show an increase of 41.65% in HDL-cholesterol in animals fed with diet added to 2% freeze-dried peel jaboticaba in relation to the HF group. As there was no reduction in circulating levels of total cholesterol, it is assumed that this increase in HDL-cholesterol is a reflection of decreased levels of LDL-cholesterol. Thus, it is suggested that the consumption of the diet added with FJP showed a protective effect against cardiovascular disease by means of elevation levels of HDL-cholesterol and possible decrease of LDL-cholesterol.

Notable emphasis has been given to the role of hormones produced in adipose tissue in obesity and insulin resistance. The ghrelin, an orexigenic hormone, is another key adipokine in the prevention and treatment of obesity. Studies in animal models suggest that this hormone has an important role on hypothalamic signaling of food intake and energy balance; moreover, they show that some nutrients that could modulate postprandial plasma levels of ghrelin [38, 39]. Our results suggest that the high concentration of fat in the diet was responsible for the decline on serum ghrelin in experimental animals.

Circulating levels of adiponectin correlate negatively with body mass index (BMI), which is high in obese individuals. The metabolic effects of this adipokine include increased insulin sensitivity, increased glucose uptake and fatty acid oxidation in skeletal muscle, and anti-inflammatory and anti-atherogenic effects [40, 41]. As expected, the animals that received high-fat diet showed reduced levels of this hormone compared to control animals, although the analysis of chemical composition did not show fat accumulation on the carcasses of the animals receiving high-fat diet. In addition, low levels of this adipokine can to suggest higher tendency to insulin resistance [40].

Several animal studies have shown that the supplementation of high-fat diet with purified anthocyanins, especially C3G, can promote a decrease in circulating levels of insulin, an anabolic hormone crucial in the genesis of diabetes, and leptin, a hormone important in appetite regulation and signaling [5, 26, 7, 42]. In our study, those effects were detected for insulin, but not for leptin. The consumption of 1, 2 and 4% of FJP reduced insulin serum of experimental animals. In addition, these animals showed lower values of HOMA-IR, indicating improvement in insulin resistance induced by HF diet. These results indicated great potential for FJP as a functional ingredient, source of anthocyanins, able to improve insulin resistance and prevent the development of type 2 diabetes mellitus.

Other researchers showed that animals fed with HF diet supplemented with purified cyanidin-3-glucoside showed decreased blood glucose and serum insulin, confirming that the anthocyanins were able to revert glucose intolerance and insulin resistance promoted by the HF diet [26, 29]. The addition of 0.2% of purified cyanidin-3-glucoside (96%) from purple corn on the diet of diabetic mice resulted on suppressed blood glucose, although no statistical difference in levels of plasma insulin were detected [32]. Takikawa et al [29]

suggests that a decrease in serum glucose and increased insulin sensitivity occurs through activation of AMP-activated protein kinase in white adipose tissue, skeletal muscle and liver of animals. According to the authors, the activation of AMP kinase was accompanied by a decrease in glucose transporter in white adipose tissue and skeletal muscle, suppression of hepatic glucose production and lower lipid deposition in this organ.

In another way, other studies have evaluated the effects of high-fat diet supplemented with freeze-dried foods as a source of anthocyanins on the development of IR and diabetes using different methods (insulin and glucose homeostasis, glucose tolerance test (GTT), and HOMA-IR) and showed no positive effects on these parameters [27, 28, 43].

Our results showed that the addition of FJP did not promote significant differences on serum glucose, glucose tolerance test (GTT) and insulin tolerance test (ITT). However, observing the results of GTT (Figures 4B and 5B) before consumption FJP and after the experiment period, it can be noted that all groups had decreased glucose tolerance evidenced by high values of area under the curve in second test. This was an expected result, due to physiological changes that occur in aging of the animals. Although, observing the ITT (Figures 4D and 5D) it seems that the control group and HFJ1% group showed the same trend on the decay rate of the glucose curve, while the other groups (HF, HFJ2% and HFJ4%) showed slower decay of the glucose curve. Therefore, it is suggested that the supplementation with 1% of FJP could improve the levels of postprandial glucose by insulinemic homeostasis regulation.

We conclude that the consumption of 1, 2 and 4% freeze-dried peel of jaboticaba was able to reverse the hyperinsulinemia in animals fed with HF diet, thus reversing the development of IR. In addition, the concentration of 2% of FJP elevated serum levels of HDL-cholesterol, showing important cardioprotective effect. However, there were no positive effects on weight gain and body composition in the animals. Further studies are needed to elucidate the mechanisms of action of anthocyanins from the peel of jaboticaba on the parameters evaluated.

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6 ABBREVIATIONS USED

FJP – Freeze-dried Jaboticaba Peel

GTT – Glucose Tolerance Test

ITT – Insulin Tolerance Test

HOMA-IR – Homeostatic Model Assessment of Insulin Resistance

HF – High-fat diet

HFJ1% - High-fat diet added to 1% freeze-dried jaboticaba peel

HFJ2% - High-fat diet added to 2% freeze-dried jaboticaba peel

HFJ4% - High-fat diet added to 4% freeze-dried jaboticaba peel

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CAPÍTULO 2

FREEZE-DRIED JABOTICABA PEEL ADDED TO HIGH-FAT DIET INCREASES PLASMA LEVELS OF ANTIOXIDANT ENZYMES, BUT NOT AMEND LIPID PEROXIDATION IN SPRAGUE-DAWLEY RATS

(Artigo em fase de preparação para envio a revista Journal of Agricultural and Food Chemistry)

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ABSTRACT

The objective of this study was to evaluate the effect of freeze-dried jaboticaba peel (FJP) added to high-fat diet on lipid peroxidation and enzymes with antioxidant activity. For this purpose, Sprague-Dawley rats were fed a control diet, high-fat control diet and high-fat diets supplemented with 1, 2 and 4% of FJP. The levels of TBARS and isoprostane, well as the concentrations of total glutathione, glutathione reductase, glutathione peroxidase, catalase and superoxide dismutase were measured in plasma of animals. Levels of TBARS and hydroperoxides were measured in liver. The results were statistically analyzed using ANOVA and Tukey's test, $p < 0.05$. The FJP added to the diet did not reverse the lipid peroxidation in plasma and liver of animals. However, the levels of enzymes glutathione reductase, catalase and superoxide dismutase were increased in plasma of animals. Thus, the consumption of FJP can modulate positively the activity these enzymes, promoting an improvement in antioxidant potential.

Keywords: *Myrciaria Jaboticaba* (Vell.) Berg., oxidative stress, antioxidant enzymes, lipid peroxidation.

1 INTRODUCTION

Anthocyanins are polyphenolic compounds present in many fruits, leaves and flowers. They are responsible for the red, blue and violet colors. The consumption of fruits and vegetables is positively associated with health benefits; some researchers believe that the presence of phenolic compounds can strongly contribute to those effects. Polyphenolic compounds are known to have antioxidant, antiinflammatory and anticancer activities, well as to prevent cardiovascular and neurodegenerative diseases [1,2].

For some decades oxidative stress was faced as the key mechanism of aging and development of a variety of age-related diseases, such as atherosclerosis, several types of cancer, diabetes and Alzheimer's disease [3]. Oxidative damage can be induced by different environmental factors such as smoking, alcohol and diet. The high consumption of fats, especially saturated fat, induces an overproduction of reactive oxygen species (ROS) and the excessive oxidative damage is an important initiator of the pathogenesis of several diseases. Furthermore, in humans, the consumption of high-fat diet and fruits and vegetables are inversely associated. Thus, the organism is exposed to an imbalanced amount of ROS and antioxidants [4,5].

In an attempt to combat oxidative damage, researchers have investigated the role of phytochemicals on this process. Due to their antioxidant capacity, anthocyanins are among the most studied compounds related to this topic. Several *in vitro* studies confirm the antioxidant activity of anthocyanins, but *in vivo* studies there is still great controversy over his action, especially in relation its bioavailability [6]. Moreover, the use of purified anthocyanins is common in studies that evaluate the antioxidant potential and few are the studies that use raw or processed foods.

Jaboticaba is a typical Brazilian fruit. It is generally consumed 'in natura', but its peel is always neglected. The freeze-dried jaboticaba peel (FJP) was used recently in a study by Leite et al [7]. They described that cyanidin-3-glucoside and delphinidin-3-glucoside are the major anthocyanins found in jaboticaba peels. The authors reported that the consumption of jaboticaba peel can increase the antioxidant capacity of rat's plasma.

Thus, the purpose of this study was to evaluate the antioxidant potential of FJP added to high-fat diet through the quantification of lipid peroxidation products in plasma and liver, well as the quantification of plasma concentrations of glutathione and antioxidant enzyme complex (glutathione reductase, glutathione peroxidase, catalase and superoxide dismutase).

2 MATERIALS AND METHODS

2.1 Preparation of freeze-dried jaboticaba peel

Jaboticabas (M*yrciaria jaboticaba (vell.) berg*) were acquired in Campinas Central Supply (CEASA), Brazil. The fruits were selected, washed in tap water and the peels were separated manually. The peels were frozen, freeze-dried and milled [10] to get a homogeneous powder. The product was stored at -80 ° C in dark containers, prior to analysis and preparation of the diet used in biological assay.

2.2 Determination of chemical composition and quantification of anthocyanins in freeze-dried peel jaboticaba

Analyses of moisture, total protein and ash were performed according to methods described by Association of Official Analytical Chemists [8]. The total lipids were

determined by Bligh & Dyer [9] and the determination of soluble and insoluble fiber was performed according to ASP et al. [10]. Carbohydrate content was determined by difference.

The extraction of anthocyanins from freeze-dried jaboticaba peel was performed according to Wu [11], with subsequent determination in liquid chromatography equipped with a diode array detector and C18 column. The identification and quantification of total and individual anthocyanins were performed according Leite et al. [7].

2.3 Animals and diets

Thirty-five Sprague-Dawley, male, recently weaned, weighting 58 ± 18.77g were obtained from the Multidisciplinary Center for Biological Research at Unicamp (CEMIB). This experiment was approved by the Ethics Committee on Animal Experiments (CEEA / UNICAMP), protocol number 2226-1, and followed all the ethical requirements of the Brazilian College of Animal Experimentation (COBEA). The animals were randomly distributed into five groups (n = 7) and remained at individual cages with food and water under the system of free access, with temperature and humidity controlled, with a range of 22 ± 1 °C and 60-70% respectively, and light / dark cycle of 12 hours, throughout the experimental period.

Two control diets were given during the experiment: a normolipidic control diet, prepared in accordance with the American Institute of Nutrition [12], AIN-93G, with protein concentration of 12% [13] and a high-fat control diet, AIN 93G-modified with 12% protein and 35% (g) of fat, 4% vegetable oil (soybean) and 31% of animal origin (swine fat) [14]. Beyond the control diets, three experimental diets were given: high-fat diet added

with freeze-dried jaboticaba peel (FJP) in three different concentrations (1, 2 and 4% w/w). Adjustments were done in order to get diets with same contents of calories and fibers. Control group received normolipidic control diet and group HF high-fat control diet throughout the experiment; groups HFJ1%, HFJ2% and HFJ4% received high-fat control diet during the first 4 weeks and diets added with FJP until the end of 10 weeks. Diet consumption was monitored every 2 days and weight gain once a week.

Humidity, ash, protein [8] and total lipids [9] were determined on all the experimental diets. The concentration of carbohydrates was obtained by difference and energy value was determined in isoperibol automatic calorimeter (PARR 1261) with oxygen pump (PARR 1108).

After 10 experimental weeks, the animals were sacrificed by decapitation preceded by 12 hour fasting, blood was collected in tubes with and without anticoagulant EDTA to obtain plasma. After exsanguination, the liver was removed, cleaned with saline, weighed, and frozen in liquid nitrogen and stored in a freezer at - 80 ° C for further analysis.

2.4 Measurement of plasma levels of antioxidant enzymes of animals

Enzyme-linked immunosorbent assay (ELISA) method was used to determine plasma levels of total glutathione, glutathione peroxidase, glutathione redutase, catalase and superoxide dismutase by commercial kits *Cayman chemical*®.

2.5 Analysis of lipid peroxidation in plasma of animals

The quantification of plasma levels of 8-iso-PGF2α-isoprostane was performed by Enzyme Immunoassay (EIA) and Thiobarbituric Acid Reactive Substances (TBARS) was performed by ELISA, both by commercial kits *Cayman chemical*®.

2.6 Analysis of lipid peroxidation in liver of animals

For determination of hydroperoxides and malondialdehyde in the liver of animals, the organs were freeze-dried and macerated to obtain a powder. This powder was stored in a freezer at - 80 ° C before the analysis.

The determination of hydroperoxides was preceded by the liver lipid extraction using the method of Bligh & Dyer [9] with some modifications. From the filtrate obtained in the final stage of the extraction, 3 ml were withdrawn for hydroperoxides analysis. The peroxide index (PI) was measured according with the official method of AOAC [8], which is based on the oxidation of iode in the presence of potassium iodide relative with the hydroperoxide concentration in the sample. The concentration of hydroperoxide of each sample in mEq/kg was calculated considering the quantity of lipids determined by Bligh & Dyer [9].

The quantification of hepatic levels of malondialdehyde (TBA value) was determined by the method of Sinnhuber; Yu (1958). The results were expressed as mg of malondialdehyde/kg sample [15].

2.7 Statistical analysis

For data analysis we used the statistical program Statistical Analysis System (SAS) 9.1.3. [16]. The results were submitted to analysis of variance (ANOVA) and means treatment were compared by Tukey test with an alpha of 0.05.

3 RESULTS

The jaboticaba peel is composed mainly of carbohydrates, including soluble and insoluble fiber, and water (Table 1). Possibly, the high content of carbohydrate found reflects the vast amount of simple and structures sugars which, in general, are the major constituents of fruit peel [17].

Table 1. Proximate composition of the freeze-dried jaboticaba peel.

Components (g%)	**Mean ± SD** [1]
Moisture	15.33 ± 0.19
Protein [2]	4.89 ± 0.10
Lipids	1.72 ± 0.02
Ashes	3.52 ± 0.02
Insoluble fiber	20.00 ± 2.00
Soluble fiber	5.00 ± 0.50
Carbohydrates	49.46
Energy value [3]	2.32

[1]Analyses of the moisture, protein, lipids and ashes were performed in triplicate, and the results were expressed in mean ± SD. [2]Conversion factor used to calculate protein: N = 6,25. [3]Value expressed in kcal/g of the peel.

The composition of experimental diets, as well as its anthocyanins concentration is shown in Table 2. The predominant anthocyanins in the FJP, cyanidin-3-glucoside and delphinidin-3-glucoside, were previously determined by Leite et al. [7]. Terci [18] and Reynertson et al. [19] had described the presence of these same anthocyanins in fresh and freeze-dried jaboticaba, respectively, in similar concentrations, however, they also described the presence of peonidina-3-glycoside in the fruit.

Table 2. Composition of modified AIN-93G diets fed to rats.

INGREDIENTS	**Control**	**HF**[1]	**HFJ1%**[1,2]	**HFJ2%**[1,2]	**HFJ4%**[1,2]
	(g/Kg)	(g/Kg)	(g/Kg)	(g/Kg)	(g/Kg)
Casein (78% prot.)	153,85	153,85	153,85	153,85	153,85
Corn starch	426,63	249,82	249,82	249,82	249,82
Maltodextrin	141,68	82,91	82,91	82,91	82,91
Sucrose	107,33	62,92	62,92	62,92	62,92
Soybean oil	70,00	40,00	40,00	40,00	40,00
Cellulose	50,00	50,00	47,50	45,00	40,00
Mineral mix	35,00	35,00	35,00	35,00	35,00
Vitamin mix	10,00	10,00	10,00	10,00	10,00
L-cystine	3,00	3,00	3,00	3,00	3,00
Choline bitartarate	2,50	2,50	2,50	2,50	2,50
Tert-Butyl Hydroquinone	0,014	0,014	0,014	0,014	0,014
Lard	-	310,00	310,00	310,00	310,00
Freeze-dried peel jaboticaba	-	-	10,00	20,00	40,00
Total	***1000***	***1000***	***1007,54***	***1015,01***	***1030,01***
Anthocyanins (mg/kg diet)					
Cyanidin-3-O-glucoside	-	-	196,4	392,8	785,6
Delphinidin-3-O-glucoside	-	-	63,5	127	254
Total	***-***	***-***	***259,9***	***519,8***	***1039,6***
Energy value (Kcal/g) [3]	**4,252**	**5,834**	**5,809**	**5,772**	**5,784**

[1]HF, HFJ1%, HFJ2% and HFJ4% diets were added 31% of lard and, consequently, starch, sucrose and maltodextrin contents were reduced; [2]HFJ1%, HFJ2% and HFJ4% diets were added to 1, 2 and 4% of freeze-dried jaboticaba peel. [3]Caloric value expressed in Kcal/g diet, determined by calorimetry.

Uneexpectedly, the markers of lipid peroxidation measured in the plasma of animals were not positively changed by the consumption of FJP. The concentration of TBARS, secondary products of lipid peroxidation did not differ significantly among the groups. For isoprostanes, the levels found were significantly ($p<0.05$) higher in animals fed the FJP (Table 3).

In the liver tissue, the concentration of hydroperoxides, primary products of lipid peroxidation, were significantly lower ($p<0.05$) in groups HFJ2% and HFJ4%, when compared to the control group, but similar to other groups. As the concentration of malondialdehyde (MDA), a secondary product of lipid peroxidation, only the group HFJ2% differed significantly ($p<0.05$) from other experimental groups, with higher TBA value (Table 3).

Table 3. TBARS and 8-iso-PGF2α-isoprostane plasmatic and Peroxide Index (PI) and TBA value in liver of the rats[1]

	Control	**HF**	**HFJ1%**	**HFJ2%**	**HFJ4%**
Plasmatic TBARS (μM/mL)	37,99 ± 11,65 [a]	17,36 ± 2,78 [a]	23,96 ± 3,22 [a]	18,06 ± 3,76 [a]	22,22 ± 1,39 [a]
8-iso-PGF2α-isoprostane plasmatic (pg/mL)	42,63 ± 3,92[c]	58,87 ± 5,24[c]	112,47 ± 11,87[b,c]	213,47 ± 39,95[a]	167,50 ± 16,38[a,b]
Hepatic PI (Meq/Kg)	45,08 ± 4,62 [a]	38,23 ± 3,86 [a,b]	34,38 ± 2,19 [a,b]	28,64 ± 3,28 [b]	31,36 ± 4,16 [b]
Hepatic TBA value (mg/Kg)	6,26 ± 0,90 [b]	5,70 ± 0,60 [b]	6,39 ± 0,64 [b]	8,32 ± 0,53 [a]	6,16 ± 0,18 [b]

[1]TBARS and 8-iso-PGF2α-isoprostane plasmatic and Peroxide Index (PI) and TBA value in liver of the rats after 10 experimental weeks. Control: group fed with control diet; HF: group fed with high-fat diet; HFJ1%: group fed with high-fat diet added to 1% FJP; HFJ2%: group fed with high-fat diet added to 2% FJP; HFJ4%: group fed with high-fat diet added to 4% FJP. Data presented as mean ± SE. Data statistically analyzed using ANOVA. Statistical differences (Tukey's test ($p<0.05$)) are represented by different letters.

Total glutathione results did not show differences among the groups, however the levels of control group were smaller (Figure 1A). Plasma levels of glutathione peroxidase (Figure 1B) were not different between groups. As for the enzyme glutathione reductase (Figure 1C), the HFJ4% group concentration were significantly higher ($p<0.05$) than the HF and control groups and similar groups HFJ1% and HFJ2%. Therefore the consumption of 4% FJP elevated significantly the plasma concentration of glutathione reductase and the diets with 1 and 2% FJP showed a tendency to promote the same effect.

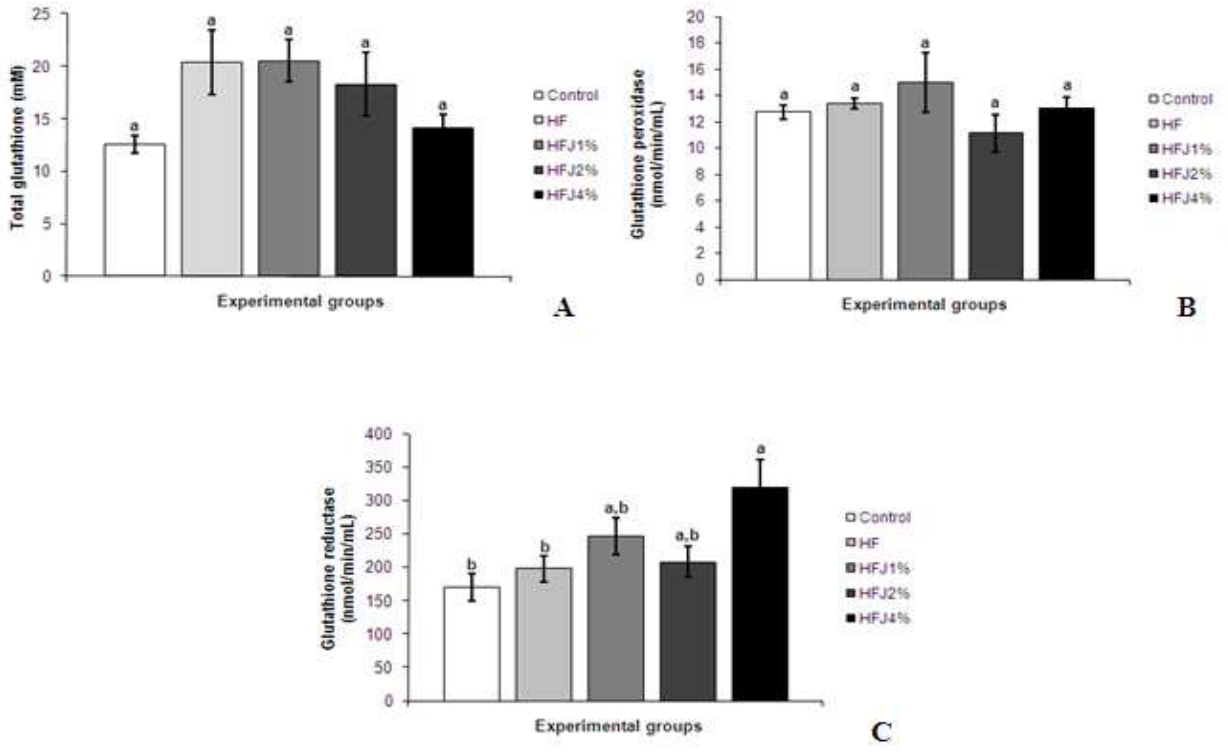

Figure 1. Total glutathione (A), glutathione peroxidase (B) and glutathione reductase in plasma of the animals after 10 weeks of experiment. Control: group fed with control diet; HF: group fed with high-fat diet; HFJ1%: group fed with high-fat diet added to 1% FJP; HFJ2%: group fed with high-fat diet added to 2% FJP; HFJ4%: group fed with high-fat diet added to 4% FJP. Bars represent mean ± SE. Data statistically analyzed using ANOVA. Statistical differences (Tukey's test ($p<0.05$)) are represented by different letters.

The Figure 2 shows the concentrations of catalase in animals plasma. The HFJ2% group showed concentrations significantly ($p<0.05$) greater of catalase when compared with other groups. The HFJ4% group was similar at HFJ2%, but did not differ significantly from HFJ1% and HF groups. The control, HF and HFJ1% groups did not differ significantly as the concentration of catalase. Thus, the consumption of 2% FJP increased the plasma concentration of the enzyme catalase and the consumption of 4% FJP showed a strong tendency to promote the same effect.

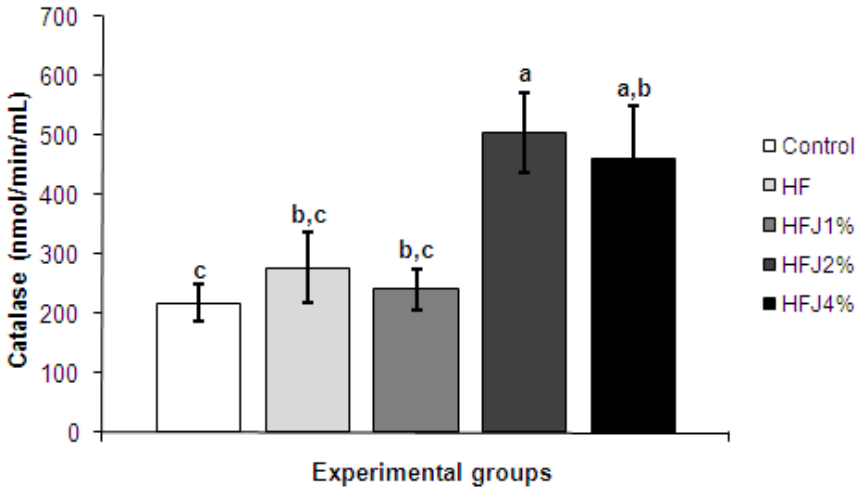

Figure 2. Catalase in plasma of the animals after 10 weeks of experiment. Control: group fed with control diet; HF: group fed with high-fat diet; HFJ1%: group fed with high-fat diet added to 1% FJP; HFJ2%: group fed with high-fat diet added to 2% FJP; HFJ4%: group fed with high-fat diet added to 4% FJP.. Bars represent mean ± SE. Data statistically analyzed using ANOVA. Statistical differences (Tukey's test ($p<0.05$)) are represented by different letters.

Similar result to found for catalase was showed for the enzyme superoxide dismutase (SOD) in Figure 3. The HFJ4% group presented highest concentration for this enzyme, differing significantly ($p<0.05$) from control, HF and HFJ1% groups, and similar to the HFJ2% group. The lowest concentration of this enzyme was showed the HF group. Therefore, the consumption of 4% FJP promoted a significant increase in plasma

concentration of the enzyme SOD and the consumption of 2% of FJP showed a strong tendency to increase the concentration of this enzyme.

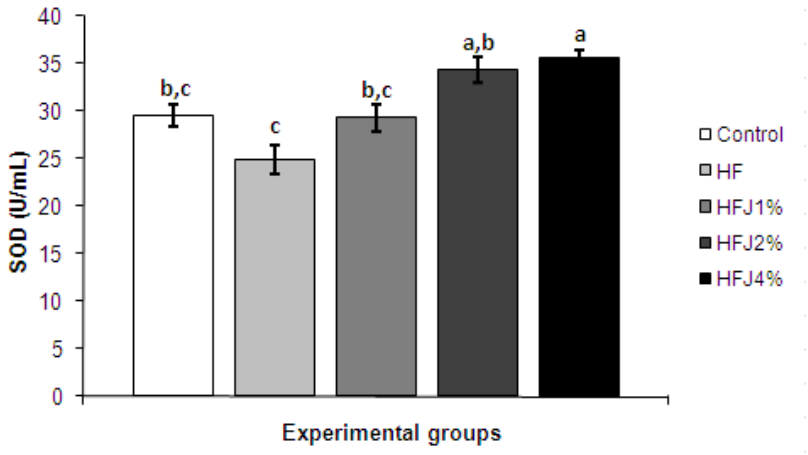

Figure 3. Superoxide Dismutase (SOD) in plasma of the animals after 10 weeks of experiment. Control: group fed with control diet; HF: group fed with high-fat diet; HFJ1%: group fed with high-fat diet added to 1% FJP; HFJ2%: group fed with high-fat diet added to 2% FJP; HFJ4%: group fed with high-fat diet added to 4% FJP.. Bars represent mean ± SE. Data statistically analyzed using ANOVA. Statistical differences (Tukey's test ($p<0.05$)) are represented by different letters.

4 DISCUSSION

Several studies have searched compounds that can act as antioxidants on biological systems, given that the development of important diseases such as cancers, cardiovascular and neurodegenerative diseases is attributed to oxidative damage [20]. Polyphenolic compounds, among which stand out the anthocyanins, have attracted special interest due to its antioxidant capacity and of to modulator of various cellular processes [21].

In two studies, Tsuda et al. [22,23] used different *in vitro* systems to evaluate the antioxidant potential of anthocyanins extracted from common bean (*Phaseulos vulgaris*)

and red bean, primarily cyanidin-3-glucoside (C3G) and its aglycone cyanidin, comparing them with the activity of α-tocopherol. In both studies, all compounds showed effective antioxidant activity in different systems evaluated, and the cyanidin was the most potent, followed by C3G, with a significant decrease in the production of malondialdehyde (MDA), a product of lipid peroxidation. These results suggest that C3G, well as its aglycone, cyanidin, have potent inhibitor effect in lipid peroxidation *in vitro.*

Han et al. [24] evaluated the effects of red potato flakes (RPF), rich in anthocyanins on lipid peroxidation and mRNA levels of hepatic enzyme superoxide dismutase (SOD) in rats fed 25% of RPF for 4 weeks. The results showed a decrease in serum TBARS levels and increased hepatic expression of the enzyme SOD. Thus, a diet rich in RPF had a positive effect on serum lipid peroxidation and antioxidant enzyme system, suggesting that the anthocyanins in the diet can increase the antioxidant potential *in vivo.*

In study that evaluated the effect of tart cherry juice, a source of C3G, on oxidative stress in healthy adult men and women who consumed 240ml of juice tested twice daily for 14 days, the antioxidant potential of these substances was again proven. The intervention with the tart cherry juice reduced plasma levels of F2-isoprostane, indicating a reduction of lipid peroxidation. There was also a reduction in basal urinary excretion of oxidized nucleic acids, but not of urinary isoprostanes. Therefore, the consumption of this drink was able to promote greater protection against oxidative damage in plasma of individuals participating [25].

More recently, Leite et al [7] evaluated the antioxidant potential of freeze-dried jaboticaba peel (FJP), rich in C3G, added to the diet of Wistar rats. The results showed that

consumption of 2% FJP increased the plasma antioxidant potential (1.7 times by TEAC method and 1.3 times by ORAC), but the addition of 4% FJP showed not antioxidant effect. Those results suggest that high doses of FJP not ensure greater antioxidant potential and can to act as pro-oxidant in biological systems.

In our study, plasma levels of isoprostanes and TBARS indicate that there was not improvement in lipid peroxidation in animals fed with FJP. The same way, there was not a positive change in markers of lipid peroxidation in liver tissue of experimental animals. The peroxide value showed decreased in groups HFJ2% and HFJ4%, while the TBA value was high in the group HFJ2%. It is known that hydroperoxides are primary indicators of lipid oxidation, produced in the stages of initiation and propagation process; in the sequence this substances are cleaved and give origin to other compounds, secondary indicators of lipid peroxidation, such the malondialdehyde (MDA) [26].

The combat against oxidative damage is endogenously mediated by enzymatic antioxidant system, compound for the enzymes superoxide dismutase (SOD), glutathione reductase, glutathione peroxidase and catalase, which are responsible for removal and inactivation of Reactive Oxygen and Nitrogen Species (RONS) produced during the metabolism [27]. It is known that some compounds can to have a positive influence on the concentrations of these enzymes, improving the endogenous potential antioxidant. Several studies have evaluated the effects of polyphenolic compounds on the antioxidant enzyme systems *in vitro* and *in vivo.*

Han et al [28] evaluated the effects of purple potato flakes (PPF), which is rich in anthocyanins, on the antioxidant status of rats fed high-cholesterol diet. Hepatic glutathione

levels were significantly higher in animals that received supplementation. As for antioxidant hepatic enzymes, there was an increase in the activity of glutathione reductase and glutathione-S-transferase. According the authors, the results indicate that anthocyanins from purple potato flakes can inhibit oxidative damage and modulate the activity of hepatic antioxidant enzymes, which gives these compounds important role in protection against the lipid peroxidation effects.

In another study, to demonstrate the antioxidant activity of anthocyanins of aged red wine, Tedesco et al. [29] used an *in vitro* experimental model of human erythrocyte which catalase activity was inhibited by sodium acid. Some of the erythrocytes were treated with H_2O_2 and were measured the formation of methemoglobin in the presence and absence of H_2O_2, well as catalase activity in different concentrations of the wine source of anthocyanins. With this assay, they demonstrated that the anthocyanins of red wine were able to increase the catalase activity, even in the presence of an inhibitor.

Suwannaphet et al. [30] demonstrated that rats fed a fructose rich diet supplemented with 1% grape seed extract (GSE) showed significantly increased superoxide dismutase and catalase activity in liver, and suppression of lipid peroxidation. However, the rats fed with GSE showed not significant change in hepatic glutathione peroxidase activity. Thus, the ingestion of GSE can to be a viable therapeutic strategy to combat the oxidative stress induced by a fructose rich diet.

Our results showed that consumption of FJP in different proportions tested increased the concentration of the enzyme glutathione reductase, catalase and SOD, but did not alter the concentrations of total glutathione and glutathione peroxidase. The

consumption of 2 and 4% seem to be more effective in the modulation of these enzymes. These results are in agreement with those set out in the literature and suggest that anthocyanins of FJP are able to improve the enzymatic potential antioxidant *in vivo.*

In conclusion, the consumption of freeze-dried jaboticaba peel added to HF diet at concentrations of 1, 2 and 4% were not able to reverse lipid peroxidation in plasma and liver tissue of animals. However, the consumption of 2 and 4% FJP elevated considerably the levels of enzymes with antioxidant activity. It is believed that anthocyanins of FJP modulated positively the enzymatic activity, increasing the antioxidant potential in plasma of animals. Further studies are needed to elucidate the mechanisms of action of anthocyanins of FJP on modulation of these enzymes.

5 ACKNOWLEDGEMENTS

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6 ABBREVIATIONS USED

FJP – Freeze-dried Jaboticaba Peel

HF – High-fat diet

HFJ1% - High-fat diet added to 1% freeze-dried jaboticaba peel

HFJ2% - High-fat diet added to 2% freeze-dried jaboticaba peel

HFJ4% - High-fat diet added to 4% freeze-dried jaboticaba peel

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4 CONCLUSÃO GERAL

Nós concluímos que o consumo de 1, 2 e 4% de casca de jabuticaba liofilizada (CJL) foi capaz de reverter a hiperinsulinemia nos animais alimentados com dieta HF, combatendo o desenvolvimento de resistência à insulina. Além disso, o consumo de 2% de CJL elevou os níveis séricos de HDL-colesterol, mostrando importante efeito de proteção cardiovascular. Contudo, não foram observados efeitos positivos sobre o ganho de peso, composição corporal e níveis séricos das adipocinas dosadas nos animais.

Quanto ao status antioxidante, o consumo de CJL não reverteu a peroxidação lipídica no plasma e fígado dos animais, porém o consumo de 2 e 4% de CJL elevou consideravelmente os níveis plasmáticos das enzimas com atividade antioxidante (catalase, SOD e glutationa redutase). Isso indica que as antocianinas da CJL podem modular positivamente a atividade enzimática, aumentando o potencial antioxidante plasmático dos animais.

Futuros estudos são necessários para elucidar os mecanismos pelos quais as antocianinas da CJL atuam sobre a insulinemia, perfil lipídico e potencial antioxidante. Além disso, ainda é necessário estabelecer uma dose de suplementação na qual esses compostos atuem positivamente sobre os parâmetros estudados.

5 ANEXO

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

C E R T I F I C A D O

Certificamos que o Protocolo nº **2226-1**, sobre **"Avaliação do efeito do pó liofilizado da casca de jabuticaba sobre o estresse oxidativo, o tratamento da obesidade e parâmetros de saúde *in vivo*"**, sob a responsabilidade de **Prof. Dr. Mário Roberto Maróstica Junior / Sabrina Alves da Silva**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética no Uso de Animais – CEUA/Unicamp em **30 de setembro de 2010**.

C E R T I F I C A T E

We certify that the protocol nº **2226-1**, entitled "_____", is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **September 30, 2010**.

Campinas, 30 de setembro de 2010.

Profa. Dra. Ana Maria A. Guaraldo
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