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INVESTIGAÇÃO DE VIAS DE ESTRESSE OXIDATIVO EM RATOS WISTAR HIPERCOLESTEROLÊMICOS SUPLEMENTADOS COM EXTRATO DOS FRUTOS DE *Vaccinium ashei* R.

DISSERTAÇÃO DE MESTRADO

Deise Jaqueline Ströher

Uruguaiana, RS, Brasil.
2013

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

Orientadora: Prof. Drª. Vanusa Manfredini

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Área de concentração: Bioprospecção molecular

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Estarei contando isso com um suspiro,
em algum lugar
daqui a muitas eras.
Duas estradas se bifurcaram numa floresta, e eu...
Eu escolhi a estrada menos percorrida
E isso fez toda a diferença.
Robert Frost

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Gostaria de agradecer a Deus e a Nossa Senhora Aparecida, pela saúde, sabedoria e pela presença constante em minha vida.

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RESUMO

Dissertação de Mestrado

Programa de Pós-Graduação em Bioquímica

Fundação Universidade Federal do Pampa

INVESTIGAÇÃO DE VIAS DE ESTRESSE OXIDATIVO EM RATOS WISTAR HIPERCOLESTEROLÊMICOS SUPLEMENTADOS COM EXTRATO DOS FRUTOS DE *Vaccinium ashei* R.

AUTORA: Deise Jaqueline Ströher

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Data e Local da Defesa: Uruguaiana, 22 de outubro de 2013.

A hipercolesterolemia é caracterizada pelo aumento do colesterol total circulante. Uma dieta rica em colesterol aumenta os níveis da lipoproteína de baixa densidade (LDL), que atua como um fator pró-aterogênico, desencadeando processo inflamatório que leva à formação da placa aterosclerótica. Os efeitos dietéticos de plantas sobre o perfil lipídico tem sido documentados e mostram ser úteis na redução dos níveis de colesterol plasmático, prevenindo a aterosclerose. O mirtilo é uma importante fonte alimentar de polifenóis, antocianinas, flavonoides e possui ação antioxidante, conferido o título de alimento funcional. O objetivo do trabalho foi investigar vias de estresse oxidativo em ratos *Wistar* hipercolesterolêmicos suplementados com extrato liofilizado dos frutos de *Vaccinium ashei* R. Os ratos hipercolesterolêmicos foram divididos em 6 grupos ($n=6$): grupo 1: controle (salina); grupo 2: sinvastatina (10mg/Kg) como controle positivo; grupo 3: extrato de mirtilo (25mg/Kg); grupo 4: extrato de mirtilo (50mg/Kg); grupo 5: extrato de mirtilo (25mg/Kg) e sinvastatina (10mg/Kg) e o grupo 6: extrato de mirtilo (50mg/Kg) e sinvastatina (10mg/Kg). Após 14 dias consecutivos de administração, os animais foram eutanasiados e o sangue total e artéria retirados para análises posteriores. O grupo que recebeu o extrato de mirtilo mostrou redução estatisticamente significativa do perfil lipídico (colesterol total, colesterol LDL e triglicerídeos) e um aumento no colesterol HDL. A presença de polifenóis no extrato dos frutos do mirtilo contribuiu para efeito hipolipêmico que ficou mais evidente quando o extrato foi associado à sinvastatina. A dieta hipercolesterolêmica aumentou os níveis séricos da creatina-quinase e homocisteína, porém a administração do extrato diminuiu significativamente os níveis destes marcadores quando comparado ao grupo que não recebeu o extrato. Além disso, o extrato na dose de 50mg/Kg associado à sinvastatina mostrou proteger contra o espessamento da aorta, reduziu a peroxidação lipídica e o dano oxidativo ao DNA induzido por hipercolesterolemia. Os teores de vitamina C e polifenóis séricos aumentaram após 14 dias de administração dos extratos, bem como a atividade das enzimas antioxidantes, superóxido dismutase, catalase e glutationa peroxidase. Assim, os resultados sugerem que o extrato liofilizado de *Vaccinium ahei* R. apresenta um efeito antiaterogênico além de atividade antioxidante e hipocolesterolêmica.

Palavras chave: Vaccinium ashei Reade, hipercolesterolemia, parâmetros biquímicos, atividade antioxidante, histologia.

ABSTRACT

Dissertation of Master's Degree

Program of Post-Graduation in Biochemistry

Federal University of Pampa

INVESTIGATION OF WAY OF OXIDATIVE STRESS IN RATS WISTAR HYPERLIPIDEMICS SUPPLEMENTED WITH EXTRACT OF FRUIT *Vaccinium ashei* R.

AUTHOR: Deise Jaqueline Ströher

ADVISOR: Vanusa Manfredini

Date and Place of Defense: Uruguaiana, October 22rd, 2013.

The hypercholesterolemy is characterized by the total circulating cholesterol increase. A diet rich in cholesterol increase the levels of lipoprotein of low density (LDL), that acts as a proatherogenic, triggering inflammatory process that leads to the formation of the atherosclerotic plaque. The dietetic effects of the plants on the lipid profile, have been documented and show to be useful in the plasmatic cholesterol levels reduction, preventing the atherosclerosis. The blueberry fruit is an important source of food rich in polyphenols, anthocyanins, flavonoids, and it has antioxidant action, giving to it the title of functional food. The objective of this study was to investigate ways of oxidative stress in hypercholesterolemic *Wistar* rats, supplemented with lyophilized extract from the *Vaccinium ashei* R fruits. The hypercholesterolemic rats were divided into six groups (n=6): group 1: control (saline); group 2: simvastatin (10mg/Kg) as positive control; group 3: blueberry extract (25mg/Kg); group 4: blueberry extract (50mg/Kg); group 5: blueberry extract (25mg/Kg) and simvastatin and the group 6: blueberry extract (50mg/Kg) and simvastatin. After 14 consecutive days of administration, the animals were euthanized and the total blood and arteries were removed to be analyzed later. The group that received the blueberry extract shown statistically significant reduction of the lipid profile (total cholesterol, LDL-cholesterol and triglycerides) and an increase of the HDL-cholesterol. The presence of polyphenols in the blueberry extract contributed to hypolipidemic effect what is more evident when the extract is associated with to the simvastatin. The hypercholesterolemic diet increased the serum levels of creatine kinase and homocysteine, nevertheless, the administration of the extract decrease significantly the levels of these makers when they are compared to the group that did not received the extract. Moreover, the extract at a dose of 50mg/kg associated with simvastatin showed protection against the aorta thickening, it reduced the lipid peroxidation and oxidative damage to DNA, induced by hypercholesterolemia.The contents of vitamin C and serum polyphenols increased after 14 days of the extracts administration, as the activity of the oxidant enzymes, superoxide dismutase, catalase and glutathione peroxidase. In this manner, the results point that the lyophilized extract of *Vaccinium ahei* R. shows an antiatherogenic effect, besides the antioxidant and hypocholesterolemic activities.

Keywords: Vaccinium ashei Reade, hypercholesterolemia, biochemical parameters, antioxidant activity, histology.

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LISTA DE ABREVIATURAS

AF – Alimentos funcionais

ANVISA – Agência Nacional de Vigilância Sanitária

CAT – Catalase

CEP - Comitê de ética em pesquisa

DCV – Doenças Cardiovasculares

DNA – Ácido desoxirribonucleico

DTNB - Ácido 5,5'-ditio-bis(2-nitrobenzóico)

ERN – Espécies reativas de nitrogênio

ERO – Espécies reativas de oxigênio

GPx – Glutationa peroxidase

GSH – Glutationa redutase

H_2O_2 - Peróxido de hidrogênio

HDL- Lipoproteína de alta densidade

HOCl – Ácido hipocloroso

LDL – Lipoproteína de baixa densidade

LDL ox – Lipoproteína de baixa densidade oxidada

MCP-1 – Proteína quimiotática para monócitos 1

M-CSF – Fator estimulante de colônias de monócitos

MM-LDL-OX – Lipoproteína de baixa densidade minimamente oxidada

MS - Ministério da Saúde

N_2O – Óxido nitroso

$NO^{\bullet}$ - Óxido nítrico

O_2 - Oxigênio singleto

$O_2^{\bullet-}$ - Ânion superóxido

$OH^{\bullet}$ - Radical hidroxila

$ONOO^-$ - Peroxinitrito

RL – Radicais livres

$RO^{\bullet}$ - Radical alcoxila

$ROO^{\bullet}$ - Radical peroxila

SOD – Superóxido dismutase

TBARS - Espécies reativas ao ácido tiobarbitúrico

SUMÁRIO

APRESENTAÇÃO

A presente dissertação foi dividida em três partes principais. Na **parte I** encontram-se a **INTRODUÇÃO**, **REFERENCIAL TEÓRICO** e **OBJETIVOS**. Os resultados que fazem parte desta dissertação estão apresentados sob a forma de manuscritos, os quais se encontram no item **MANUSCRITOS**, **parte II** deste trabalho. As seções materiais e métodos, resultados, discussão dos resultados e referências bibliográficas, encontram-se nos próprios manuscritos e representam a íntegra deste estudo. O item **CONCLUSÃO** encontra-se na **parte III** desta dissertação, apresenta interpretações e comentários gerais sobre os resultados apresentados nos manuscritos deste trabalho. O item **REFERÊNCIAS BIBLIOGRÁFICAS** refere-se somente às citações que aparecem nos itens introdução, referencial teórico e conclusão desta dissertação. No item **PERSPECTIVAS**, estão expostos os possíveis estudos para dar continuidade a este trabalho.

PARTE I

1.0 INTRODUÇÃO

As doenças cardiovasculares (DCV) são a maior causa de morbimortalidade, tanto em países desenvolvidos quanto em países em desenvolvimento. Os principais fatores de risco associados ao desenvolvimento das DCV são o tabagismo, obesidade e dislipidemia (SPOSITO *et al*., 2007).

A dislipidemia constitui o maior fator de impacto no desenvolvimento da doença aterosclerótica, em particular a presença de concentrações aumentadas de lipoproteína de baixa densidade (LDL) (SPOSITO *et al*., 2007). Nesse sentido a hipercolesterolemia mostra-se um fator muito importante, pois este evento pode desencadear outras patologias, como a aterosclerose, infarto do miocárdio, acidente vascular cerebral, diabete mellitus e outras doenças renais e hepáticas (SCHIAVO *et al*., 2003).

O estresse oxidativo ocupa um local de destaque nas pesquisas com aterosclerose, visto que a modificação oxidativa da LDL é a hipótese mais referendada em ser responsável para o início e a progressão do processo aterosclerótico (ROSENSON, 2004). Ele ocorre quando há um desequilíbrio no estado redox do organismo, gerando um excesso de radicais livres (RL), que são capazes de danificar biomoléculas como proteínas citosólicas, lipídeos de membrana e o ácido desoxirribonucleico (DNA) (SPEIT *et al*., 1996; HALLIWELL & GUTTERIDGE, 2007).

A fim de contrapor este quadro o organismo humano possui mecanismos de defesa antioxidante, que atuam intracelular e extracelularmente para manter o equilíbrio redox da célula, assegurando que o aumento das espécies reativas de oxigênio (ERO) seja transitório (RIBEIRO *et al*., 2008).

A utilização de plantas medicinais como recurso terapêutico é uma tendência milenar, que se encontra em plena ascensão, contribuindo significativamente para sanar as necessidades primárias de assistência à saúde. Aproximadamente 80% da população mundial utiliza a medicina tradicional, fato que se deve a cultura ou a falta de

alternativas (WHO, 2011). No Brasil, além do uso de plantas medicinais, a utilização de alimentos funcionais (AF) encontra-se em expansão e contribui significativamente para auxiliar a promoção da saúde. Os AF são ingredientes que produzem efeitos metabólicos e/ou fisiológicos e/ou efeitos benéficos à saúde, além de suas funções nutricionais básicas. Este efeito ocorre em sua maioria quando estes são consumidos como parte da dieta (ERLUND *et al*., 2003).

As frutas do gênero *Vaccinium* podem ser consideradas como os primeiros AF descritos. O mirtilo é uma espécie frutífera originária de algumas regiões da Europa e América do Norte, onde é muito apreciado por seu sabor exótico, pelo valor econômico e por seus poderes medicinais, sendo considerada como "fonte de longevidade". Além disso, possui um alto conteúdo de polifenóis, como os flavonóides e antocianidinas, com poder antioxidante contido nos pigmentos de cor azul-púrpura dos frutos (BASU *et al*., 2010; WU *et al*., 2010).

Estudos sugerem que os frutos mirtilo apresentam muitos benefícios à saúde, tais como atividade antioxidante, atividade antidiabética (LEDUC *et al.*, 2006) e capacidade de proteger contra o acidente vascular cerebral e o câncer (WANG *et al.*, 2005). Além disso, a ingestão do extrato de frutos de mirtilo tem mostrado melhorar a memória de curto prazo, o equilíbrio e a coordenação em ratos velhos (JOSEPH, *et al.*, 1999).

Assim, considerando o importante papel dos elevados níveis plasmáticos dos lipídeos no desenvolvimento da resistência a insulina, aterosclerose e DCV (BARBALHO *et al*., 2009), este trabalho teve como objetivo investigar vias de estresse oxidativo em ratos *Wistar* hipercolesterolêmicos suplementados com extrato dos frutos de *Vaccinium ashei* Reade**.**

2.0 REVISÃO BIBLIOGRÁFICA

2.1 Doenças Cardiovasculares e Aterosclerose

Dietas ricas em colesterol, bem como a modificação dos padrões dietéticos que incluem o aumento no consumo de energia, açúcares, sal, gorduras totais, trans e saturadas (MAGALHÃES, CHAGAS e LUZ, 2005) aliadas à inatividade física, possibilitam o aumento na prevalência de dislipidemia, que é considerada um dos principais fatores de risco para DCV. As DCV e suas complicações permanecem a principal causa de morte em países industrializados (LOPEZ *et al.*, 2006).

A DCV é caracterizada pela elevação nos níveis plasmáticos de triacilgliceróis, colesterol total e sua fração LDL, associados à diminuição dos valores da lipoproteína de alta densidade (HDL) (BRUCKNER, 2008). Nos últimos anos, o uso do grupo de fármacos denominado estatinas, foi estabelecido como uma terapia eficaz para reduzir os níveis de LDL e, consequentemente, o risco cardiovascular (BAIGENT *et al*., 2010).

O desenvolvimento das DCV é influenciada por diferentes fatores que incluem hipertensão arterial, hipercolesterolemia, tabagismo, diabetes mellitus, obesidade, herança genética, sedentarismo e o estresse (KAWAMORI et al., 1992; BECKSTROM et al., 2007). Além destes, homocisteinemia aumentada e função plaquetária alterada também são citados como fatores que predispõem ao desenvolvimento da placa de ateroma (PICCINATO; CHERRI; MORIYA, 2001).

Durante a última década, muitos trabalhos tem mostrado informações detalhadas sobre os acontecimentos inflamatórios que ocorrem na aterosclerose. No entanto, a maneira pela qual esses eventos contribuem para a formação, evolução e complicação de lesões ainda não é completamente entendido. Diferentes autores tem estabelecido ligações entre a hipercolesterolemia e o processo inflamatório que ocorre na aterosclerose. Porém, sabe-se que as LDL, quando retidas e oxidadas na camada íntima da artéria, são as principais indutoras da ativação inflamatória da célula endotelial, que leva ao início e a progressão do processo aterosclerótico (STEINBERG *et al.*, 2009; NAVAB *et al*., 2004).

A oxidação da LDL ocorre em pequena proporção ainda na circulação sanguínea e continua após a entrada da LDL na camada íntima das artérias, em ambiente pró-oxidante (KOVANEN e PENTIKAINEN, 2003). As partículas responsáveis por esta oxidação da LDL são os radicais livres (RL) que podem ser as espécies reativas de oxigênio (ERO) e as espécies reativas de nitrogênio (ERN).

As partículas de LDL difundem-se passivamente através das células endoteliais por transporte vesicular, o qual não necessita de receptores, e aderem à parede do vaso por interações entre a apoproteína B, presente na sua estrutura, e os proteoglicanos da matriz subendotelial (Figura 1) (LUSIS, 2000).

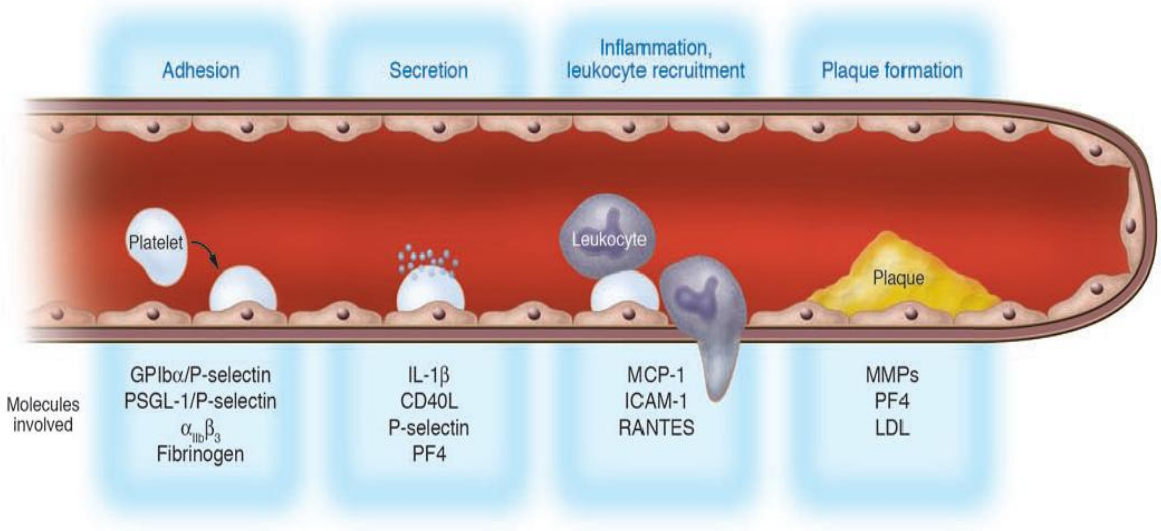


Figura 1 – Etapas do processo de formação da placa aterosclerótica
(Adaptado de GAWAZ *et al.*, 2005).

Retidas na camada íntima da artéria, a LDL sofre modificações oxidativas, que parecem ocorrer em dois estágios. O primeiro, antes que os monócitos sejam ativados, resulta na oxidação dos lipídios da LDL, com pequena alteração na apoproteína B, resultando na LDL minimamente oxidada (MM-LDL-OX). A MMLDL-OX estimula as células endoteliais a produzirem moléculas aterogênicas e pró-inflamatórias, como a proteína quimiotática para monócitos 1 (MCP-1), que promove a quimiotaxia de monócitos e linfócitos T para o espaço subendotelial; moléculas de adesão e o fator

estimulante de colônias de monócitos (M-CSF), que ativa a diferenciação de monócitos em macrófagos (ROSS, 1999).

O segundo estágio de oxidação da LDL, formando a LDL oxidada (LDLox), ocorre quando monócitos são recrutados para a lesão e convertidos em macrófagos, contribuindo com sua enorme capacidade oxidativa. Nesta fase, os lipídios da LDL são adicionalmente oxidados e a parte protéica também é modificada, impedindo o reconhecimento da lipoproteína pelo receptor de LDL, tornando-a reconhecível apenas pelos receptores presentes nos macrófagos (BROWN e GOLDSTEIN, 1990).

Dentro dos macrófagos, a LDLox é degradada, o colesterol livre é esterificado, conferindo às células o aspecto de espuma. O resultado é o grande acúmulo de colesterol e a formação de células espumosas, originando a primeira lesão da aterosclerose: a estria gordurosa (STEINBERG, 1997).

Posteriormente, as células musculares lisas começam a migrar da camada média da parede arterial para a íntima ou espaço subendotelial, se proliferam e secretam colágeno, dando origem à lesão intermediária. Nesta fase, o espessamento da íntima provoca o remodelamento, ou seja, uma dilatação da artéria compensatória ao estreitamento do lúmen. Mais uma vez, sob o estímulo da LDLox, entre outros, as células do sistema imune local liberam enzimas, citocinas e fatores de crescimento que podem induzir necrose. Ciclos repetidos de acúmulo e ativação de células mononucleares, migração e proliferação das células musculares lisas com produção de colágeno, levam ao aumento progressivo da lesão, até que se estruture uma capa fibrosa ao redor de um núcleo lipídico e de tecido necrótico, a chamada lesão avançada (ROSS, 1999).

As lesões avançadas estáveis são mais resistentes à ruptura e se caracterizam por células musculares lisas envoltas de densa matriz de colágeno, com baixo conteúdo de células inflamatórias e de lipídios no centro necrótico. Por outro lado, as regiões onde as lesões apresentam volumoso centro necrótico e um grande infiltrado de células espumosas, capa fibrosa frágil e fina, com pouca quantidade de colágeno, são mais susceptíveis à ruptura, caracterizando lesões instáveis (LEE e LIBBY, 1997).

A ruptura da capa fibrosa expõe material lipídico altamente trombogênico, levando à formação de um trombo sobrejacente. Este processo, também conhecido por

aterotrombose, é um dos principais determinantes das manifestações clínicas da aterosclerose (SPOSITO *et al*., 2007).

A LDLox participa de todas as etapas do processo de aterosclerose, desde a disfunção endotelial até a formação das placas ateroscleróticas (SIQUEIRA *et al*., 2006). Produtos derivados da LDLox são citotóxicos, podendo promover a apoptose celular. Em adição, a LDLox também contribui para o processo inflamatório na aterosclerose por inibir a produção de óxido nítrico (NO), que é um vasodilatador, e por estimular a produção de citocinas, como a interleucina-1, e aumentar a agregação plaquetária (SINGH e JIALAL, 2006).

2.2 Radicais livres, Estresse Oxidativo e Defesas Antioxidantes

O termo radical livre (RL) é frequentemente usado para designar qualquer átomo ou molécula contendo um ou mais elétrons desemparelhados nos orbitais mais externos, o que torna essas moléculas altamente reativas e capazes de reagir com qualquer composto que esteja próximo, passando a assumir uma ação oxidante (HALLIWELL e GUTTERIDGE, 1999).

Entretanto, o termo não é ideal para designar todos os agentes reativos, pois, alguns destes não possuem elétrons desemparelhados, como é o caso do peróxido de hidrogênio (H_2O_2). Apesar de o H_2O_2 não ser um RL, ele pode ser bastante danoso às células, principalmente devido à reação entre ele e o ânion superóxido, formando o radical hidroxila (OH•), altamente reativo (HALLIWELL, 1991).

Os RL cujo elétron encontra-se centrado nos átomos de oxigênio ou nitrogênio são denominados, respectivamente, de ERO e ERN (ABRAHÃO, 2007). As principais ERO distribuem-se em dois grupos, as radicalares: ânion superóxido ($O_2^{\bullet-}$), radical hidroxila ($OH^{\bullet}$), peroxila ($ROO^{\bullet}$) e alcoxila ($RO^{\bullet}$); e as não radicalares: oxigênio singleto (O_2), peróxido de hidrogênio (H_2O_2) e ácido hipocloroso (HOCl). Dentre as ERN incluem-se óxido nítrico ($NO^{\bullet}$), óxido nitroso (N_2O) e peroxinitrito ($ONOO^-$), dentre outros (GILLHAM *et al*., 1997).

Em condições fisiológicas normais, as ERO possuem um papel importante em seres vivos, como a regulação da resposta imunológica, participando do processo fagocítico de defesa contra infecções e atuando como fatores de transcrição na sinalização intracelular, induzindo apoptose (HALLIWELL, 1994; BIESALSKI, 2002). No entanto, em determinadas condições, pode ocorrer um aumento na produção de ERO e/ou a redução na sua eliminação pelas defesas antioxidantes, causando um desequilíbrio fisiológico, que resulta no chamado estresse oxidativo (FINKEL e HOLBROOK, 2000; GUTTERIDGE e HALLIWELL, 2000).

O desequilíbrio entre a produção e a remoção das ERO, podem ocasionar vários eventos nocivos, como a apoptose de células saudáveis, o envelhecimento precoce, a alteração da função celular e o aparecimento de doenças degenerativas como aterosclerose, câncer, doença de Alzheimer ou a doença de Parkinson (FINAUD, *et al*., 2006). Além disso, o estresse oxidativo está envolvido na oxidação das LDL e tem

demonstrado ser um importante fator no desenvolvimento de doenças cardiovasculares (MAYNE, 2003).

Um antioxidante pode ser definido como uma substância que, em baixa concentração em relação a um determinado substrato, retarda ou previne a oxidação do substrato oxidável (HALLIWEL *et al*., 1995). Quando o mecanismo de ação for através de sua reação com os RL, o novo radical formado deve ser estável e incapaz de propagar a reação (SHAHIDI *et al*., 1992).

Para combater os danos deletérios causados pelo estresse oxidativo, o organismo humano possui mecanismos de defesa antioxidante, os quais atuam intracelular e extracelularmente e mantém o equilíbrio redox da célula, assegurando que o aumento das ERO seja transitório. Existem dois mecanismos antioxidantes: o enzimático e o não enzimático, os quais agem cooperativamente para manter o equilíbrio dos RL no organismo, e, em consequência disso, diminuir o dano às estruturas biológicas (RIBEIRO *et al*., 2008; BELLÓ, 2002).

O mecanismo de defesa enzimático é a primeira linha de defesa do organismo contra os danos oxidativos. O sistema é constituído por um conjunto de enzimas, tais como a superóxido dismutase (SOD), glutationa peroxidase (GPx), glutationa redutase (GSH), catalase (CAT), tiorredoxinas, peroxirredoxinas e inúmeras outras redutases (RIBEIRO *et al*., 2008). Já o mecanismo não-enzimático é constituído por um grande número de compostos de baixo peso molecular, ingeridos pela dieta (nutrientes e não-nutrientes) como as vitaminas A, C, E e os flavonoides, ou sintetizados no organismo, como a glutationa (RIBEIRO *et al*., 2008; MANACH *et al*., 2004).

Quando a produção de RL e/ou espécies reativas supera a capacidade de ação dos antioxidantes, ocorre a oxidação de biomoléculas, gerando metabólitos específicos que são os marcadores do estresse oxidativo. Tais marcadores são derivados, sobretudo, da oxidação de lipídeos, proteínas e DNA (HALLIWEL e WHITEMAN, 2004; VINCENT, *et al*., 2007; MAYNE, 2003).

Nos últimos anos, tem aumentado a busca por compostos naturais eficazes, não tóxicos, com atividade antioxidante e que possam ser utilizados na prevenção e tratamento de doenças. Tentativas de utilização de antioxidantes sintéticos para bloquear ou atenuar os efeitos prejudiciais de ERO têm produzido resultados negativos (COZMA, 2004; CHEN, *et al*., 2005; PAPAHARALAMBUS *et al*., 2007), e cada vez

mais atenção tem sido dada aos produtos naturais (BANDYOPADHYAY, *et al*., 2004). Nesse contexto, inserem-se os AF, considerados promotores de saúde por estarem associados à diminuição dos riscos de doenças crônicas.

2.3 Alimentos Funcionais

Ainda não existe um consenso mundial a respeitos do que são os AF, no entanto, a definição mais comum indica que um alimento pode ser considerado funcional se for demonstrado que pode influenciar positivamente uma ou mais funções alvo no corpo, além de possuir efeitos nutricionais adequados, de maneira a ser tanto relevante para o bem-estar e a saúde, quanto para a redução do risco de doença (ROBERFROID, 2002).

Os AF representam um conceito, mais do que um grupo definido de alimentos, porém a área é precisamente a de alimentos. Eles possuem um valor nutricional, aspecto, propriedades sensoriais e demais atributos de todo alimento, porém, não são um veículo de fármacos com ação farmacológica. Representam uma conjunção com princípios ativos, que quando consumidos em uma quantidade razoável exercem ações benéficas à nível fisiológico (NOONAN e NOONAN, 2004).

No Brasil, o Ministério da Saúde (MS), através da Agência Nacional de Vigilância Sanitária (ANVISA), regulamentou os AF através das seguintes resoluções: ANVISA/MS 16/99; ANVISA/MS 17/99; ANVISA/MS 19/99. Segundo a ANVISA, AF são aqueles que produzem efeitos metabólicos ou fisiológicos através da atuação de um nutriente ou não-nutriente no crescimento, desenvolvimento, manutenção e em outras funções normais do organismo humano.

Para Lajolo (2001), “alimento funcional é o alimento semelhante em aparência ao alimento convencional, consumido como parte da dieta usual, capaz de produzir demonstrados efeitos metabólicos e fisiológicos úteis na manutenção de uma boa saúde física e mental, podendo auxiliar na redução do risco de doenças crônico-degenerativas, além das suas funções nutricionais básicas”.

De acordo com Pimentel e colaboradores (2005) os AF são classificados baseados na sua natureza química e molecular. Para eles os alimentos funcionais são classificados em sete grupos, sendo eles: isoprenóides, compostos fenólicos, proteínas, carboidratos e derivados, ácidos graxos e lipídeos, minerais e os microbióticos.

Em muitas partes do mundo os AF e os nutracêuticos possuem conceituações semelhantes, porém há uma diferença fundamental entre eles, que faz com que os AF se relacionem à venda e consumo dos mesmos como alimentos, ao passo que os

nutracêuticos são ingredientes funcionais isolados e podem ser consumidos sob diferentes formas, dadas pela indústria farmacêutica.

O Brasil é um país rico em produtos naturais e AF ainda não explorados (CUPPARI, 2002). Aliado a isso, o aumento da expectativa de vida da população e o crescimento exponencial de doenças crônicas tais como a obesidade, a aterosclerose, a hipertensão, o diabetes e o câncer têm ocasionado um crescente interesse na busca por novos AF capazes de prevenir ou reduzir o risco de ocorrência dessas e outras patologias.

2.4 Mirtilo

2.4.1 Características gerais e produção

O mirtilo é a fruta do mirtileiro, planta frutífera de clima temperado que é membro da família *Ericaceae* e pertence ao gênero *Vaccinium* (RASEIRA E ANTUNES, 2004), originária de algumas regiões da Europa e América do Norte, onde é conhecida popularmente como "blueberry". O mirtilo é um fruto tipo baga, possui uma coloração azul-escura e um sabor agridoce (KLUGE *et al*., 1994) e, é muito apreciado por seu sabor exótico, pelo valor econômico e por seus poderes medicinais, sendo considerado como fruto "fonte da longevidade" (ANTUNES; MADAIL, 2005).

A produção de mirtilo está concentrada principalmente nos Estados Unidos e no Canadá, onde, o primeiro, é responsável por 66% e o segundo por 33% da produção mundial (STRIK, 2005). Os trabalhos com o mirtilo no Brasil iniciaram em 1983, na Embrapa Clima Temperado (Pelotas-RS), com a introdução da coleção de cultivares de baixa exigência em frio do grupo *"rabbiteye"* (SILVA *et al*., 2008), espécie considerada pelos produtores como a que oferece as maiores possibilidades para adaptação (ECK *et al*., 1990).

O crescente interesse pelas frutas tem mobilizado o mercado mundial a aumentar a oferta do fruto, expandindo seu cultivo em países da América do Sul, como Chile, Argentina e Uruguai (BAÑADOS, 2006; RASEIRA e ATUNES, 2004). O Brasil é um produtor ainda recente de mirtilo (FACHINELLO, 2008) e o potencial cultivo do fruto, aponta o *Vaccinium ashei* R como a espécie mais adaptável às condições de clima frio do Sul do Brasil (Figura 2).

Figura 2 - *Vaccinium ashei* Reade

Fonte: Embrapa

Atualmente, a produção se concentra nas regiões sul e sudeste do país, nos municípios Vacaria e Caxias do Sul (Rio Grande do Sul, RS), Barbacena (Minas Gerais, MG), e Campos do Jordão (São Paulo, SP) (SANTOS, 2004), porém, o estado do Rio Grande do Sul ainda é o que mais se destaca na produção de mirtilo (KLUGE *et al*., 1994).

Segundo Raseira e Antunes (2004), existem muitas espécies de mirtilo, sendo que as principais espécies com expressão comercial são divididas em três grupos, de acordo com o genótipo, hábito de crescimento, tipo de fruto produzido e outras características. Estes grupos são:

a) "highbush": sua produção, dentre os demais grupos, é a de melhor qualidade, tanto em tamanho quanto em sabor dos frutos. A principal espécie deste grupo é *Vaccinium corymbosum* L.;

b) "rabbiteye": compreende a espécie *Vaccinium ashei* Reade. Em relação ao grupo anterior, produz frutos de menor tamanho e de menor qualidade. Apresenta maior produção por planta, e seus frutos têm maior conservação em pós-colheita. Apresenta maior importância comercial em regiões com menor disponibilidade de frio, por causa da sua tolerância a temperaturas mais elevadas e à deficiência hídrica;

c) "lowbush": tem hábito de crescimento rasteiro e produz frutos de pequeno tamanho, cujo destino é a indústria processadora.

2.4.2 Compostos fenólicos

Os vegetais produzem uma grande variedade de substâncias que não possuem ação direta na fotossíntese, respiração, síntese de proteínas, de carboidratos e de lipídeos, sendo considerados compostos produzidos pelo metabolismo secundário (TAIZ & ZEIGER, 2004). Os compostos fenólicos ou polifenóis fazem parte do metabolismo secundário vegetal e possuem diversas funções nos vegetais, tais como proteção contra raios ultravioleta, proteção contra insetos e bactérias, controle da ação de hormônios vegetais, além de atrair animais com finalidade de polinização (ZUANAZZI e MONTANHA, 2004).

Quimicamente, os compostos fenólicos podem ser definidos como substâncias que possuem um anel aromático contendo um ou mais subtituintes hidroxila (LEE *et al*., 2005) e a atividade antioxidante destes compostos depende da sua estrutura, particularmente do número e posição dos grupos hidroxila e da natureza das substituições nos anéis aromáticos (BALASUNDRAM, SUNDRAM e SAMMAN, 2006).

O mirtilo (*Vaccinium* sp.) apresenta em sua composição alta concentração de compostos fenólicos (WU *et al*., 2004), sendo superior a maioria das frutas (WOLFE *et al*., 2008). No entanto, estudos demonstram que há uma grande variação qualitativa e quantitativa na composição fenólica do mirtilo e que, esta variação é dependente de fatores intrínsecos (gênero, espécie e cultivar) e extrínsecos (condições ambientais, cultivo, manejo e condições de armazenamento) (WANG *et al*., 2008; GIOVANELLI e BURATTI, 2009).

2.4.3 Flavonóides

Os flavonóides constituem o maior grupo dos compostos fenólicos, sendo descritos mais de 8.000 compostos (BEECHER, 2003). Estes são pigmentos responsáveis pelas cores amarelas, laranjas e vermelhas das flores, sendo importantes para o desenvolvimento e para defesa das plantas (RICE-EVANS, 2003). Estes compostos possuem uma estrutura comum de difenilpropanos (C6 – C3 – C6), constituídos de dois anéis aromáticos e um heterociclo oxigenado ligados através de três

carbonos (Figura 3), os quais se subdividem em seis subclasses como isoflavona, antocianina, flavanona, catequina, flavona e flavonol (ROSS & KASUM, 2002) representadas na Figura 4.

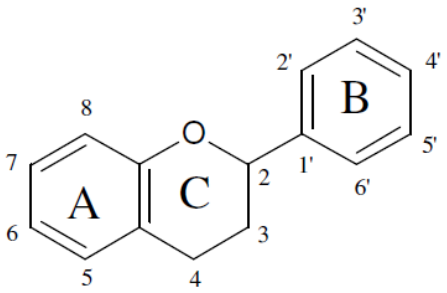


Figura 3 - Estrutura geral dos flavónoides
(Adaptado de: BALASUNDRAM, SUNDRAM e SAMMAN, 2006).

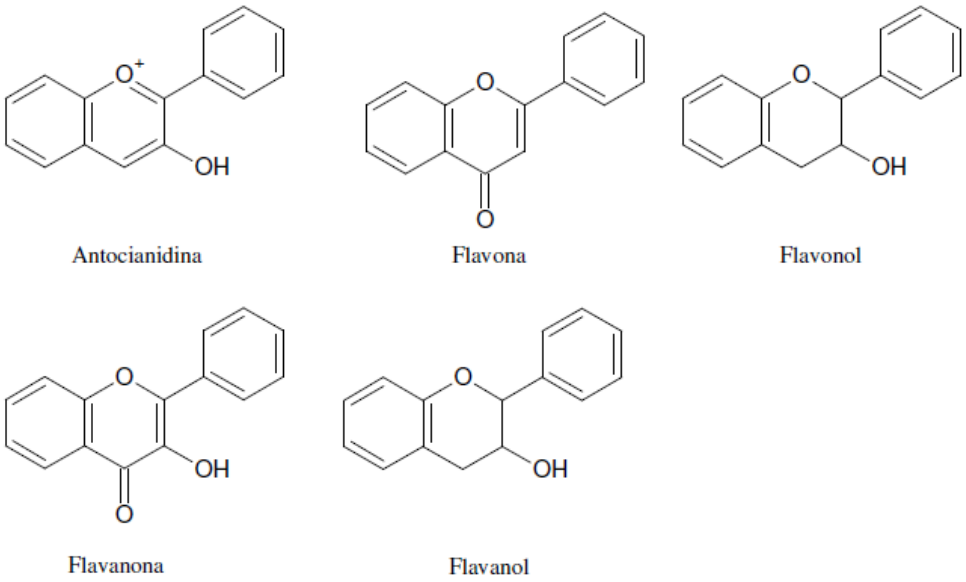


Figura 4 - Estrutura geral das diferentes subclasses de flavonóides
(Adaptado de: BALASUNDRAM, SUNDRAM e SAMMAN, 2006).

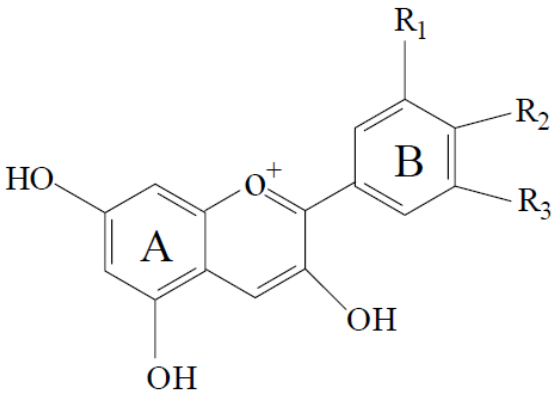
2.4.4 Antocianinas

As antocianinas (do grego: *anthos*, flor e *kyanos*, azul) pertencem a uma das subclasses dos flavonóides, sendo responsáveis pela coloração atraente de diversas

flores e frutas. Seu espectro de cor pode variar de salmão, rosa, vermelho, magenta, violeta, roxo e azul (MANHITA, *et al*., 2006; CISSE *et al*., 2009).

A estrutura das antocianinas é constituída por dois anéis benzênicos e um anel heterocíclico central, contendo oxigênio (KONCZAK, 2004). Essa estrutura é completada por uma ou mais moléculas de açúcar ligadas em diferentes posições hidroxiladas da estrutura básica (DELGADO-VARGAS & PAREDES-LÓPEZ, 2003) (Figura 5).

As antocianinas diferem entre si pelo número de grupos hidroxila, número e natureza dos açúcares unidos à molécula, posição desse açúcar e pelo número e natureza dos ácidos alifáticos ou aromáticos unidos aos açúcares da molécula (KONG *et al*., 2003) e, devido à sua polaridade, são mais solúveis em solventes polares que em apolares (DELGADO-VARGAS & PAREDES-LÓPEZ, 2003). A Figura 5 ilustra a estrutura de uma antocianina.



Antocianinas	R_1	R_2	R_3
Cianidina	OH	OH	-
Peonidina	OCH_3	OH	-
Delfinidina	OH	OH	OH
Malvidina	OCH_3	OH	OCH_3
Petunidina	OCH_3	OH	OH

Figura 5 - Estruturas das antocianinas mais comumente encontradas na natureza. (Adaptado de: JACKSON, 1994).

As antocianinas são glicosídeos que apresentam em sua estrutura química um resíduo de açúcar na posição 3, facilmente hidrolizado, como produtos desta hidrólise

obtém-se o componente glicídico e a aglicona, denominadas antocianidina (DEWICK, 2002).

Aproximadamente 22 antocianidinas são conhecidas (FRANCIS, 2000), dentre elas, apenas seis estão presentes em alimentos: pelargonidina, cianidina, delfinidina, peonidina, petunidina e malvidina, que se distinguem entre si pelo número de hidroxilas e pelo grau de metoxilação no anel B, conforme apresentado na Figura 4 (SCALBERT; WILLIAMSON, 2000; LIMA; GUERRA, 2003).

Dentre as frutas vermelhas, como o morango e amora, o mirtilo apresenta maior capacidade antioxidante, a qual é diretamente ligada ao seu alto teor de antocianinas (KALT *et al*., 1999). Lohachoompol e colaboradores (2008) identificaram e quantificaram antocianinas em várias cultivares de mirtilo produzidas na Austrália. As cultivares estudadas foram: Crunchie, Star e Sharpe ("*highbush*", *Vaccinium corymbosum*); Clímax, Powderblue e Brightwell ("*rabbiteye*", *Vaccinium ashei*). Foram identificadas as antocianinas: delfinidina-3-glicosídeo, delfinidina-3-galactosídeo, cianidina-3-galactosídeo, delfinidina-3-arabinosídeo, cianidina-3-glicosídeo, petunidina-3-galactosídeo, cianidina-3-arabinosídeo, petunidina-3-glicosídeo, peonidina-3-galactosídeo, petunidina-3-arabinosídeo, peonidina-3-glicosídeo, malvidina-3-galactosídeo, peonidina-3-arabinosídeo, malvidina-3-glicosídeo e malvidina-3-arabinosídeo. As antocianidinas majoritárias encontradas foram a delfinidina, petunidina e malvidina.

2.4.5 Atividades biológicas

Estudos epidemiológicos indicam que o consumo de alimentos ricos em componentes bioativos está associado à redução de DCV, acidente vascular cerebral e câncer (BAGCHI *et al*., 2004; PAPANDREOU *et al*.,2009). O mirtilo é uma importante fonte alimentar de antocianinas, polifenóis e flavonóides e parecem ter a maior capacidade antioxidante entre frutas e legumes (PRIOR *et al*., 2000), conferindo ao mirtilo o título de AF (CHO *et al*., 2004; HUANG *et al*., 2012).

Muitos estudos demonstram que os frutos mirtilo têm uma ampla gama de benefícios à saúde, tais como atividade antioxidante, tanto *in vitro* (LI *et al*., 2013; CASTREJÓN, *et al*., 2008) quanto *in vivo* (DULEBOHN *et al*., 2008; MOLAN, 2008),

que pode ser associado ao seu alto teor de antocianinas (RASEIRA & ANTUNES, 2004). Atividades anti-hipertensiva (SHAUGHNESSY *et al*., 2009; KALEA *et al*., 2009), antiobesidade (PRIOR *et al*., 2009), antidiabética (DEFURIA *et al*., 2009; STULL *et al*., 2010) e atividade antitumoral (YI, *et al*., 2005; NETO, 2007) também foram descritas. DeFuria *et al*., (2009) mostraram em seu estudo que a expressão de genes inflamatórios foram reduzidos em ratos após o consumo de mirtilo, sugerindo uma resposta anti-inflamatória.

As folhas de mirtilo também tem atraído a atenção de pesquisadores uma vez que tem sido relatado que os polifenóis (especialmente proantocianidinas) podem suprimir a expressão do vírus da hepatite C (TAKESHITA, *et al*., 2009). As folhas de mirtilo tem demonstrado exercer também atividade antimicrobiana e antioxidante, pois possuem uma grande quantidade de compostos bioativos, como os flavonóides (LI *et al*., 2013).

Inoue e colaboradores, em 2011, analisaram o efeito da infusão das folhas de mirtilo sobre o perfil lipídico e o acúmulo de triglicerídeos hepáticos em ratos com sobrepeso. O extrato das folhas, obtidas a partir de uma infusão, demonstraram um efeito hipolipemiante e os autores atribuem o efeito as proantocianidinas e flavonóides presentes nas folhas.

3.0 OBJETIVOS

3.1 Objetivo geral:

Investigar vias de estresse oxidativo em ratos Wistar hipercolesterolêmicos suplementados com o extrato dos frutos de *Vaccinium ashei* Reade.

3.2 Objetivos específicos:

- Obter o extrato liofilizado dos frutos de *Vaccinium ashei* Reade;
- Avaliar o potencial antioxidante do extrato de mirtilo *in vitro*, através da determinação da atividade scavenger do radical DPPH e do radical ABTS;
- Quantificar o conteúdo de polifenóis totais do extrato liofilizado;
- Traçar o perfil lipídico (colesterol total e frações e triglicerídeos), glicêmico, hematológico e marcadores cardíacos, antes e após 14 dias de suplementação;
- Determinar a atividade das enzimas antioxidantes em eritrócitos: catalase (CAT), superóxido dismutase (SOD) e glutationa peroxidase (GPx);
- Obter os níveis de vitamina C em plasma;
- Determinar o conteúdo de polifenóis em plasma;
- Determinar o dano oxidativo em proteínas plasmáticas;
- Avaliar o nível de peroxidação lipídica através da medida das espécies reativas ao ácido tiobarbitúrico (TBARS) em plasma;
- Determinar o dano oxidativo no DNA de leucócitos do sangue periférico, através do ensaio cometa e micronúcleo;
- Análise de cortes histológicos de aortas.

PARTE II

MANUSCRITO I

Em fase de preparação para submissão para The Journal of Nutrition

Hypolipidemic Effect of *Vaccinium ashei* Reade (blueberry) in Experimentally Induced Hypercholesterolemic Wistar Rats

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Hypolipidemic Effect of *Vaccinium ashei* Reade (blueberry) in Experimentally Induced Hypercholesterolemic Wistar Rats

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RUNNING TITLE: Hypolipidemic Effect of *Vaccinium ashei* Reade in Hypercholesterolemic Rats

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ABSTRACT

Blueberry (*Vaccinium ashei* Reade) is a fruit rich in bioactive compounds such as polyphenols, especially anthocyanins, and have different pharmacological properties. In the present study, we found that supplementation for 2 weeks with lyophilized extract of blueberry (BE), decreased the level of total cholesterol, LDL cholesterol and triglycerides, as well as increased plasma levels of HDL cholesterol in hypercholesterolemic rats. When the extract was associated to the simvastatin, the hypocholesterolemic effect demonstrated to be better, especially on the group receiving blueberry extract at a dose of 50 mg/Kg associated with simvastatin. Moreover, we observed that treatment with BE, protected against weight gain. However, no significant change was observed in the glycemic and hematology profile. The BE also showed an antiatherogenic effect, which may be promising for the development of new drugs.

Keywords: blueberry, hypercholesterolemia, lipid profile, atherosclerosis.

1.0 INTRODUCTION

Hypercholesterolemia has been implicated in atherosclerosis, which is the leading cause of death among world populations. Atherosclerosis is considered as a chronic and progressive disease, arising from the inflammatory processes and oxidative stress within vessel wall.[1,2] High cholesterol diet increases serum low-density lipoprotein (LDL) levels results in increased oxidized LDL levels.[3] Oxidation of LDL acting as a strong pro-atherogenic factor by triggering a complex inflammatory process[4] that triggers accumulation of macrophage white blood cells in the artery wall. Rupture of the plaque deposits oxidized cholesterol into the artery wall leading to atherosclerotic plaque formation.[5,6]

The 3‑hydroxymethylglutaryl coenzyme A reductase (HMG CoA reductase) inhibitors, or statins, are a widely used group of hypocholesterolemic drugs, which are effective in reducing atherosclerotic cardiovascular events, largely by reducing plasma LDL concentrations.[7] The Simvastatin is one of the most commonly prescribed statins worldwide.[8]

Dietary flavonoids have emerged as potential candidates to protect against cardiovascular disease (CVD.[9] Epidemiological studies associate regular consumption of flavonoid-rich foods and beverages with a decreased risk of CVD mortality, which is mainly due to the potential of these bioactive components in to increasing serum antioxidant capacity and thereby protect against LDL oxidation and prevent CVD.[10]

There has been a growing interest in natural products as an alternative to pharmaceutical medications and their contribution to maintenance or improvement of health. The cholesterol-lowering effects of dietary plants has been well studied and various plants were shown to be helpful in lowering plasma cholesterol levels[11,12] and are considered to be useful means to prevent disorders such as atherosclerosis.[13]

Blueberries are an important dietary source of anthocyanins, polyphenols and flavonoids and appear to have the highest antioxidant capacity among fruits and vegetables,[14] conferring on blueberries the title of a functional food[15,16] and creating an opportunity for their use in the nutraceutical industry.[17]

The blueberry (*Vaccinium* spp) is a fruit species native to parts of Europe and North America where the fruit is considered as "source of longevity".[18] Several studies provide evidence of antioxidative,[19] antiinflammatory,[20,21] antihypertensive,[22,23] antiobesity,[24] and, antidiabetic[25,26] effects of blueberries.

Considering the important role of elevated plasma levels of lipids in the development of insulin resistance, atherosclerosis and cardiovascular disease[27] and there are however no reports on the effect of *Vaccinium ashei* Reade on hypercholesterolemia, this work was therefore aimed to investigate the effect of extract of fruits of *Vaccinium ashei* R on biochemical, hematological and histopathological profile in hypercholesterolemic rats.

2.0 MATERIALS AND METHODS

2.1 Sample plant

Mature fruits of rabbiteye blueberry (*Vaccinium ashei* Reade) cultivars Bluegem were collected in november of the year 2012 and transported fresh to the city of Uruguaiana, RS where they were immediately processed. The fruits were generously provided by EMBRAPA – Clima Temperado, Pelotas, Brazil, RS, established through collaboration with Maria do Carmo Bassols Raseira.

2.2 Preparation of the extract

For the preparation of the extract were used 100g of blueberry macerated using a porcelain grail and pistil, in the dark, to preserve the antioxidant properties of its constituents

and then mixed with 100 mL of methanol: ethanol: acetone (45:45:10 v/v) according to the method described by Vizzotto and Pereira (2009)[28], with adaptations. After 24 hours the extract was filtered and subjected to reduced pressure in a rotary evaporator to remove the solvent. The extract was transferred to Falcon tubes, frozen and subsequently lyophilized. The lyophilized extract was stored at -70°C until treatment.

2.3 Animal Experimentation

For this study we used male Wistar rats (60-65 g), 30 days old, were obtained from the Central Animal Laboratory of the Federal University of Santa Maria, Rio Grande do Sul, Brazil. During treatment, rats were housed at a constant room temperature, humidity, and light cycle (12:12h light-dark), free access to tap water and fed with standard chow *ad libitum*. All experiments were conducted in compliance with the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology and approved by the Ethics Committee on Animal Use Experimentation of the Federal University of Pampa, CEUA, Uruguaiana, Rio Grande do Sul, Brazil (Protocol n ° 035/2012).

2.4 Preparation of feed and Induction of hypercholesterolemia

Commercial feed was supplemented with 7,5% of pig grease and with 2,5 % corn oil for each 100 g of feed according to the described methodology of Fietz and Salgado (1999)[29], with adaptations. The hyperlipidemic feed was prepared daily for all experimental groups.

After the induction of the hypercholesterolemy, the groups kept receiving the same diet until the end of the experiment. 44 rats that consumed the hypercholesterolemic diet were selected. From them, 2 rats were euthanized after the first week of hypercholesterolemia induction to be used as comparison parameter, and were dosed the blood levels of total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides. This procedure was

developed weekly until the hypercholesterolemia confirmation, totalizing 30 days of induction.

2.5 Experimental Desing

The 36 rats were divided into 6 groups of 6 rats each. The group 1 (control) received saline solution in a daily dose of 1 mL, group 2: oral suspension of simvastatin 10 mg/Kg (SIM), group 3: blueberry extract 25 mg/Kg (BE25), group 4: blueberry extract 50 mg/Kg (BE50), group 5: blueberry extract 25 mg/Kg associated to simvastatin 10 mg/Kg (BE25+SIM), group 6: blueberry extract 50 mg/Kg associated to simvastatin 10 mg/Kg (BE50+SIM). All treatments were administered by gavage. The register of the body weight of the animals was performed during all experimental period to accompany the development of the animals and to determine the extracts and administrated drugs volume.

2.6 Blueberry extract prepare and administration

The blueberry-based solution was obtained by the dissolution of the extract previously lyophilized, in water. The solution was daily prepared, in the moment immediately before the administration. The extracts were administered by gavage for 14 consecutive days. Animals were euthanized 24 h after the last treatment, in fasting, to obtain the whole blood and aorta.

2.7 Biochemical, hematological and cardiac markers analysis

The hemograms (complete blood count) were performed in an automatic counter Cell-Dyn 3200 Hematology Analyzer (Abbott Diagnostic, St Clara, CA, USA) and total cholesterol, HDL-cholesterol, triglycerides total, glucose and creatine-kinase (CK) levels using automatic analyzers A-25 Biosystems (Biosystems SA, Barcelona, Spain) for *in vitro* diagnostics. Creatine kinase-MB (CK-MB) using Architect Abbott for *in vitro* diagnostics. Determination of plasma total homocysteine using HPLC coupled to mass spectrometry (LC-MS/MS), according to the technique described by Nelson *et al.*, (2003)[30]. LDL cholesterol

values were computed according to the Friedewald formula. All biochemical assays were carried out in triplicate.

2.8 Collection of Aorta and Histopathological analysis

After 14 days of treatment, the animals were euthanized and the thoracic abdominal cavity was opened. The heart together with the aorta (2–3 cm length) was excised from each animal. The aorta was cut at the origin and removed from the heart. The entire aortas were rapidly dissected out and tissue sections (5 mm) fixed by immersion at room temperature in 10% formalin solution. For the histological examinations, paraffin embedded tissue sections of aorta were stained with hematoxylin-eosin (H&E). The tissue samples were for examined and photographed under a light microscope for observation of structural abnormality. The aortic diameter measurements were made with the program Image Pro-Plus.

2.9 Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Comparisons between groups were performed using a two-way analysis of variance (ANOVA), followed by post hoc of Bonferroni for multiple comparison tests. Results were considered statistically significant when $p<0.05$. The statistical analysis was performed using the software GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

3.0 RESULTS

3.1 Effects of BE on body weight gain

During the period of hypercholesterolemia induction (week 1–4) there has not been significant difference on the body weight gain among rats. After 4 weeks, the hypercholesterolemic rats received the treatments along with the hypercholesterolemic diet. At the end of 2 weeks, the treatment of the group which received simvastatin (SIM) showed a significant decrease ($p<0.05$) of the body weight when compared to the control group. The

groups that received the blueberry extract (BE25 and BE50) demonstrated a significant decrease o the body weight ($p<0.05$) when compared to the control groups and the SIM. The groups which received the BE associated to the simvastatin (BE25+SIM e BE50+SIM) showed a significant decrease ($p<0.05$) of the body weight when compared to the group which received SIM **(Table 1)**.

3.2 Effect of the BE treatment on Biochemical Profile

The effect of BE administration, for 2 weeks on lipid and glicemic profile in rats with induced hypercholesterolemia is illustrated in **Figure 1**. The BE25, BE50 and BE25+SIM administration along with the hypercholesterolemic diet was able to significantly decrease ($p<0.05$) serum total cholesterol levels compared with that of control and SIM groups. The BE50+SIM treated group also showed a significantly decrease ($p<0.05$) in serum total cholesterol levels with that of control group, but the treated group BE50+SIM was comparable to the SIM in terms of cholesterol lowering effect **(Figure 1A)**.

The HDL-cholesterol levels significantly increased ($p< 0.05$) in the group treated with BE dose of 50 mg/Kg (BE50) and the BE25+SIM group when compared to the control group, but the group treated with BE50+SIM showed an increase in HDL-cholesterol less when compared BE50 and BE25+SIM groups **(Figure 1B)**.

The BE50+SIM administration showed significantly decrease ($p<0.05$) LDL-cholesterol levels with compared that of control, SIM and BE25, BE50 groups, showing a better effect in lowering LDL-cholesterol than the SIM alone **(Figure 1C)**.

The triglycerides levels significantly decreased ($p<0.05$) in all treated groups compared with the control group, but the groups treated with the BE or SIM showed no significant difference between them **(Figure 1D)**.

Treatment with BE showed no significant differences among the groups in glicemic profile **(Figure 1E)**.

3.3 Effect of the BE treatment on Hematological Profile

The plasma platelets counts were higher in hypercholesterolemic rats. However, the results showed significant decrease in plasma levels of platelets and leukocytes in the group treated with BE25+SIM. The neutrophils also decreased in the group treated with BE50+SIM. The other parameters evaluated showed no significant results (**Table 2**).

3.4 Effect of the BE treatment on Markers of cardiac damage

The creatine-kinase (CK) and its isoform CK-MB are used as cardiac disease marker. The hypercholesteromic diet induced an increase on the CK-total levels on the control group, although at the end of 14 days of treatment, the group which received SIM showed a decrease ($p<0.05$) on the CK-total levels when compared to the control group. The groups that received BE25 and BE50 also showed a decrease ($p<0.05$) on the CK-total levels when compared to the control group and SIM. The CK-total levels also demonstrated a statistically significant decrease ($p<0.05$) on the groups which received the BE25+SIM and BE50+SIM demonstrating a better result than the groups that received only the extract. The group that received SIM has not showed significant differences on the CK-MB levels when compared to the control group. The groups that received BE with doses of 25 mg/Kg and 50 mg/Kg and the groups which received the same doses associated to the simvastatin showed a statistically significant decrease ($p<0.05$) on the CK-MB levels when compared to the control groups and SIM. The hypercholesterolemic diet also induced to a significant increase ($p<0.05$) on the serum levels of homocysteine on the control group when comparing to the groups treated with the BE or SIM. The groups that received SIM, BE25 and BE25+SIM showed a statistically significant decrease ($p<0.05$) on the levels of homocysteine, compared to the group which

only received a hypercholesterolemic diet (control group). The groups which received the isolated extract of 50 mg/Kg (BE50) and the group that homocysteine received the extract of 50 mg/Kg associated to (BE50+SIM) demonstrated a statistically significant decrease similar to the serum levels of homocysteine, when compared to the other groups. The results are showed on **Table 3**.

3.5 Effect of the BE treatment on Histopathological Profile

Histopathological analysis of the aorta of hypercholesterolemic rats showed spaces of fat droplets within the tunica intima and media. These findings indicate that hypercholesterolemia disturbed the prooxidant-antioxidant balance in favor of peroxidation in the aorta tissues together with atherosclerotic changes in the aorta of rats. However, histopathological observation showed significant decrease of aortic lesions in the BE25, BE50, BE25+SIM and BE50+SIM groups when compared to control and SIM groups, and the improvement is more evident when the extract (50mg/Kg) is associated with simvastatin. (**Figure 2**)

4.0 DISCUSSION

Hypercholesterolmia is a great concern in the occidental countries as the main etiology for the atherosclerosis and cardiovascular diseases (CVD). The consumption of a rich diet on cholesterol increases the level of lipid peroxidation, which is also one of the initial processes of atherosclerosis. Studies have demonstrated that some antioxidants are associated to an anti-hypercholesterolemic effect and can prevent the atherosclerosis, protecting the LDL from oxidation.[31,32]

The statins are a category of drugs widely used to decrease cholesterol and it has been proved to be efficient on the reduction of morbidity and mortality of these two conditions related to high levels of cholesterol. Additional effects of the statins have been demonstrated

and showed to be important on the stabilization of the atherosclerotic plaque. These effects (also called pleiotropic), refer to the endothelial protection, to the reduction of the lipid peroxidation and to the control of the inflammatory and hemostais.[33,34,35,36] However, other studies have demonstrated the security profile of the statins and some concerns came up about the collateral effects that this category of drugs may provoke in a long term.[37]

Our results showed that the groups which received treatment with blueberry extract in doses of 25 mg/Kg and 50 mg/kg (BE25 and BE50) for 2 weeks showed a significantly decrease in body weight. This result corroborates the findings of Song *et al*., 2013[38], we used a HFD-induced obesity rat model to investigate the anti-obesity effects of blueberry extract. The groups receiving blueberry extract at the dosage of 60 mg/kg and 150 mg/kg orally for 5 weeks and the result showed that the body weight of rats were significantly lower.

Studies show that the natural antioxidants, as the polyphenols, can be used for the effective correction of high levels of total cholesterol and triglycerides in the blood.[39] In our study the groups treated with blueberry extract were able to significantly reduce levels of total cholesterol and triglycerides, when compared to the control group. Again, these data support the study of Song *et al*., 2013[38] indicating that the blueberry extract effectively regulates the metabolism of cholesterol and triglycerides in obese rats induced by hypercholesterolemic diet.

Coffy, in 2008[40], carried out a study with hypercholesterolemics rats treated with blueberry extract, showed serum reduction on LDL-cholesterol levels on the percentage of 46% and the increase of the HDL-cholesterol in about 10%. On this study, the blueberry extract on the doses of 25 mg/Kg e 50 mg/Kg (BE25 e BE50), it was capable of reducing the total cholesterol and the LDL-cholesterol in a significant way. When the extract was

associated to the simvastatin, the hypercholesterolemic effect demonstrated to be better, specially on the group BE50+SIM.

In our study, the blueberry extract (BE) was also capable of diminishing the level of serum triglycerides of the hypercholesterolemic rats and proved to have an activity similar to the isolated simvastatin. Inoue and collaborators, in 2011[41], analized the effect of the infusion of the blueberry leaves on the lipid profile and the accumulation of hepatic triglycerides on overweight rats. The leaves extract, obtained from an infusion, showed an hypolipemic effect and the authors attach this effect to the presence of the proanthocyanidins and flavonoids present on the leaves.

The HDL-cholesterol performs an important role on the defense against oxidative damage of the membranes.[42,43] The main role of the HDL on the lipid metabolism is the absorption and cholesterol transportation from the peripheral tissues to the liver through a reverse transportation of cholesterol. Diminished levels of HDL-cholesterol are strongly associated to a high risk of CDV.[44,45] In our study, the HDL-cholesterol levels increased in the groups that received the treatment with blueberry extract and it was significantly ($p< 0.05$) higher in the group that received the extract on the dose of 50 mg/Kg. These results prove the favorable performance of the BE on the lipid profile of *Wistar* rats.

However, in our study, the BE has not showed effect on the fasting glycaemia, datum also found by Basu and collaborators in 2010.[46]

The supplementation with fruits, vegetables and seeds that contain flavonoids, contribute to the hypolipidemic effect. The blueberry is an important feeding source of antocianins, polyphenols and flavonoids[47], and these bioactive compounds are probably the responsible for the reduction of the hypercholesterolemia.

The CK has been used as a cardiac disease marker. The increase of the induced cholesterol by the diet has increased the levels of the CK-total and CK-MB on the control group, although in the groups that received treatment with BE, there have been a significant decrease of the enzyme levels. However, the group that received the BE50+SIM showed a meaningful improvement when compared to the isolated extract. The high levels of homocysteine in the blood are also associated to an increased risk of CVD.[48] In this case, there have been significant differences of the groups that received the BE from the ones which have not received. Based on the results achieved, we can state that the blueberry had an important role in the prevention of cardiac damage of the rats submitted to the hypercholesterolemic diet.

Histopathological analysis of the aorta of hypercholesterolemic rats showed significant decrease of aortic lesions in groups treated with BE. These results suggest a protective effectiveness of blueberry extract against atherosclerosis in hypercholesterolemic rats. The potential mechanisms may involve reduction in oxidative stress by both inhibition of lipid peroxidation an as enhancement of antioxidant defense. Wu *et al.*, 2010[49] have reported that dietary supplementation with 1% freeze-dried blueberries (BB) for 20 weeks decreased aortic lesions. The authors accordingly have suggested that the atheroprotective effect of BB may have been related to its antioxidative effect.

In conclusion, the BB supplementation in hypercholesterolemic rats showed protect against weight gain, improve the lipid profile, reversing the high levels of total cholesterol, LDL and triglycerides, and showed an antiatherogenic effect. These results show that blueberry have positive effects, and therefore, they may have potential for use in the development of functional food or nutraceuticals.

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FIGURE LEGENDS

Figure 1: Effect of blueberry extract in hypercholesterolemic rats after treatment. In A: serum total cholesterol levels; B: HDL-cholesterol levels; C: LDL-cholesterol levels; D: triglycerides levels; E: glucose levels. Data are expressed as means±S.D. Different letters are significantly ($p<0.05$) different by two-way ANOVA followed by Bonferroni's comparison pos hoc test.

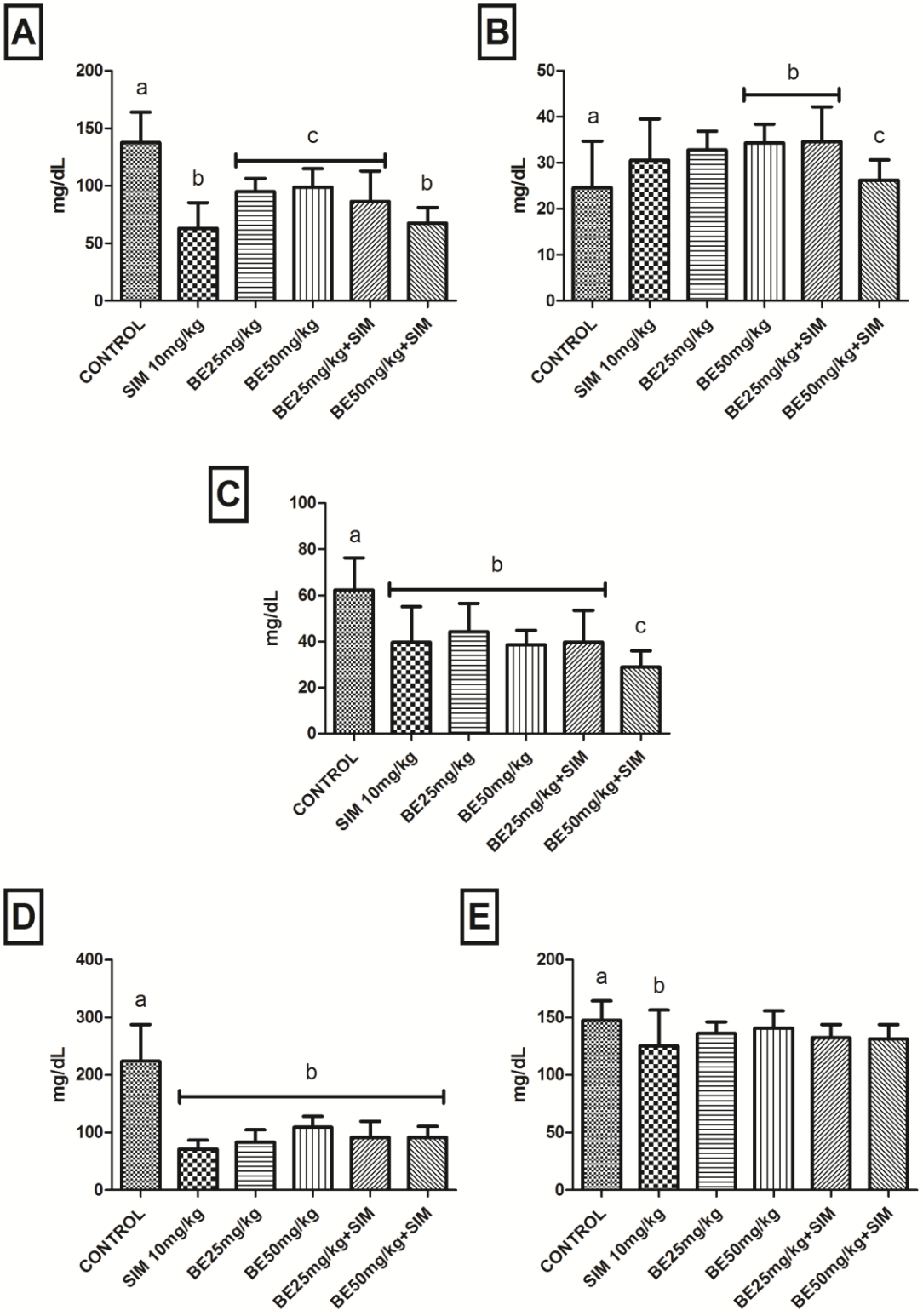
Figure 2: Histological section and thickening measure of the aorta of hypercholesterolemic rats after treatment. In 1: control group (100x); 1B: simvastatin (200x); 1C: blueberry extract 25 mg/Kg (100x); 1D: blueberry extract 50 mg/Kg (40x); 1E: blueberry extract 25 mg/Kg + simvastatina 10 mg/Kg (40x); 1F: blueberry extract 50 mg/Kg + simvastatina 10 mg/Kg (40x). (H&E). In 2: Measure aortic thickening (μm) in groups subjected to different treatments. Different letters are significantly different by two-way ANOVA followed by Bonferroni's comparison pos hoc test. Different letters are significantly ($p<0.05$) different by two-way ANOVA followed by Bonferroni's comparison pos hoc test.

Table 1 - Body weight of rats during the induction of hypercholesterolemia (weeks 1-4) and during treatment (weeks 5-6).

	BODY WEIGHT					
GROUPS	**INDUCTION OF HYPERCHOLESTEROLEMIA**				**TREATMENT**	
	Week 1	**Week 2**	**Week 3**	**Week 4**	**Week 5**	**Week 6**
CONTROL	131.0 ±3.79	159.9 ± 9.00	180.1 ± 11.71	210.2 ± 13.19	238.3 ± 12.72	260.3 ± 12.80[a]
SIM	127.9 ± 4.41	148.7 ± 24.98	159.0 ± 24.02	191.1 ± 28.04	183.1 ± 55.47	189.3 ± 70.34[b]
BE 25	130.5 ± 3.07	158.2 ± 6.12	174.4 ± 10.38	208.2 ± 10.56	235.7 ± 15.95	253.6 ± 20.17[b,c]
BE 50	131.5 ± 4.08	160.1 ± 10.43	179.0 ± 13.45	213.4 ± 18.19	242.2 ± 16.43	257.3 ± 16.17[b,c]
BE 25+SIM	128.8 ± 3.79	158.2 ± 9.08	169.0 ± 11.43	204.9 ± 6.25	224.8 ± 10.02	242.6 ± 11.06[c]
BE 50+SIM	129.5 ± 5.49	159.0 ± 14.59	168.6 ± 13.10	200,8 ± 14.59	217.9 ± 12.33	233.9 ± 18.92[c]

Values are expressed as Mean ± SD of each group (n=6). Different letters are significantly different by two-way ANOVA followed by Bonferroni's comparison pos hoc test.

Figure 1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mg/dL
200
150
100
50
0
a
b
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b
CONTROL
SIM 10mg/kg
BE25mg/kg
BE50mg/kg
BE25mg/kg+SIM
BE50mg/kg+SIM
B
mg/dL
50
40
30
20
10
0
a
b
c
CONTROL
SIM 10mg/kg
BE25mg/kg
BE50mg/kg
BE25mg/kg+SIM
BE50mg/kg+SIM
C
mg/dL
100
80
60
40
20
0
a
b
c
CONTROL
SIM 10mg/kg
BE25mg/kg
BE50mg/kg
BE25mg/kg+SIM
BE50mg/kg+SIM
D
mg/dL
400
300
200
100
0
a
b
CONTROL
SIM 10mg/kg
BE25mg/kg
BE50mg/kg
BE25mg/kg+SIM
BE50mg/kg+SIM
E
mg/dL
200
150
100
50
0
a
b
CONTROL
SIM 10mg/kg
BE25mg/kg
BE50mg/kg
BE25mg/kg+SIM
BE50mg/kg+SIM

Table 2 – Hematological parameters of the hypercholesterolemic rats exposed to different treatments.

PARAMETERS	GROUPS					
	CONTROL	**SIM**	**BE 25**	**BE 50**	**BE 25+SIM**	**BE 50+SIM**
Hemoglobin (g/dL)	13.58 ± 2.71	13.82±1.54	13.90±0.53	12.90±3.31	12.96±2.98	15.15±0.90
Hematocrit (%)	39.83±9.17	40.56±4.27	40.60±1.33	41.10±1.82	42.83±2.64	43.35±2.19
MCV (fL)	55.17±1.72	54.40±0.82	55.67±1.13	57.33±1.53	56.20±1.65	54.75±1.12
MHC (pg)	19.48±1.09	18.56±0.42	19.05±0.51	20.12±0.66	19.24±0.87	19.20±0.50
MCHC (%)	35.28±1.91	34.10±0.40	34.18±0.33	35.23±0.96	19.24±0.87	19.20±0.50
Erythrocytes (10^6/mm³)	6.49±2.08	7.45±0.81	7.29±0.23	7.17±0.39	6.80±1.74	7.76±0.27
Leukocytes (10^3/mm³)	5.76±1.23	2.60±0.95[a]	6,58±2,25	6,98±1,93	3,84±1,87[b]	6,30±0,53
Neutrophils (%)	22,17±3,43	25,00±7,12	23,17±7,11	21,40±6,05	24,60±7,98	14,50±3,68[a]
Lymphocytes (%)	72,83±2,17	69,75±7,17	73,50±7,43	69,17±11,93	70,40±6,56	79,25±4,15
Eosinophils (%)	2,33±0,76	2,20±0,41	1,66±0,76	1,66±0,76	2,20±0,77	2,50±0,89
Monocytes (%)	1,83±0,70	2,60±0,50	1,66±0,76[a]	3,50±0,78	3,40±1,40	2,75±0,44
Basophil (%)	0.00±0.00	0.00±0.00	0.16±0.38	0.16±0.38	0.00±0.00	0.00±0.00
Platelets (10^3/mm³)	730,2±246,8	741,5±211,9	814,4±114,5	713,7±132,5	379,7±332,3[a]	786,0±92,97

Data are expressed as means±SD. Different letters are significantly ($p<0.05$) different by two-way ANOVA followed by Bonferroni's comparison pos hoc test.

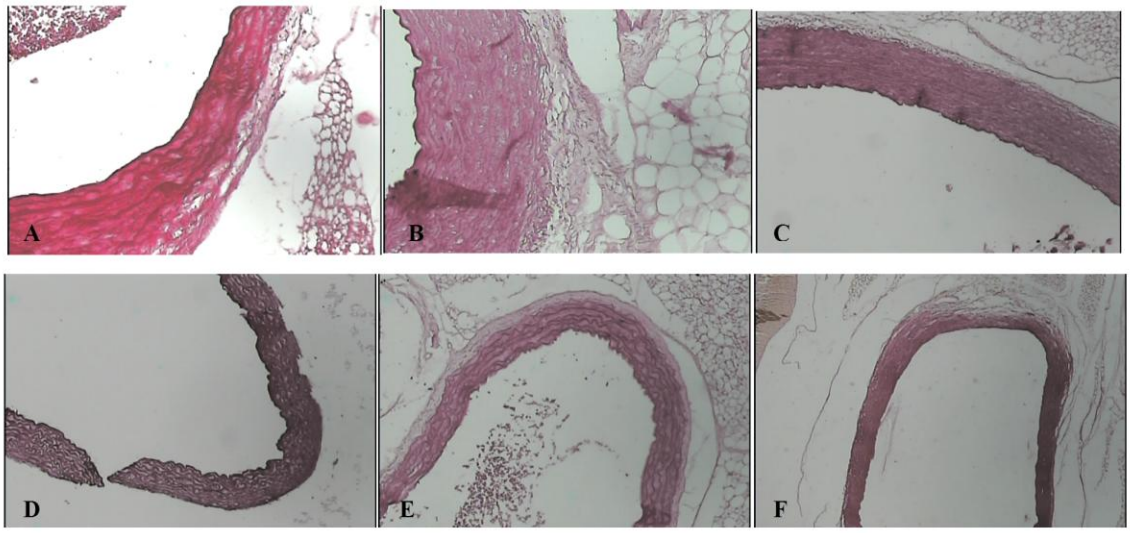
Table 3 – Markers of cardiac damage in the hypercholesterolemic rats exposed to different treatments.

GROUPS	CK-TOTAL (U/L)	CK-MB (U/L)	HOMOCYSTEINE (µmol/L)
CONTROL	132.5±39,67[a]	4.72±0.80[a]	17.52±0.48[a]
SIM	83.60±7.30[b]	4.36±0.35[a]	11.02±0.22[b]
BE 25	66.33±2.11[c]	3.16±0.09[b]	10.77±0.11[b]
BE 50	49.67±5.84[d]	2.35±0.18[c]	8.98±0.06[c]
BE 25 + SIM	27.40±1.54[e]	1.58±0.18[d]	10.80±0.06[b]
BE 50 + SIM	21.67±5.46[e]	0.90±0.21[e]	8.49±0.04[c]

Means with different letters at a time differ significantly, $p< 0.05$. The values sharing common letters are not significantly different at $p< 0.05$. CK-total: serum total creatine kinase. CK-MB: CK isoform. Different letters are significantly different by two-way ANOVA followed by Bonferroni's comparison pos hoc test.

Figure 2

1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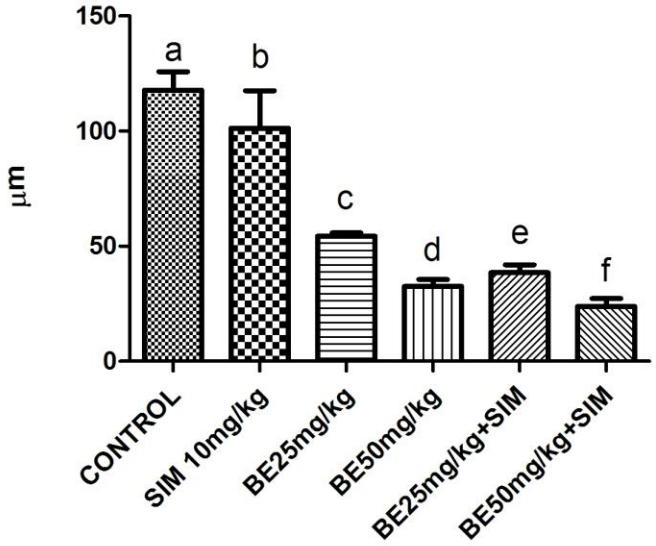
B
C
D
E
F

2


150
100
50
0
µm
a
b
c
d
e
f
CONTROL
SIM 10mg/kg
BE25mg/kg
BE50mg/kg
BE25mg/kg+SIM
BE50mg/kg+SIM

MANUSCRITO II

Em fase de preparação para submissão para Journal of Agricultural and Food Chemistry

Antioxidant effect of *Vaccinium ashei* Reade (blueberry) extract in hypercholesterolemic rats

Deise Jaqueline Ströher, Ritiele Pinto Coelho, Angélica Aparecida da Costa Güllich, Bruna Cocco Pilar, Jamila Benvegnú Bruno, Jacqueline da Costa Escobar Piccoli, Vanusa Manfredini.

Antioxidant effect of *Vaccinium ashei* Reade (blueberry) extract in hypercholesterolemic rats

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ABSTRACT

Blueberries are an important dietary source of polyphenols, anthocyanins, flavonoids and has greater antioxidant capacity than most other fruits and vegetables, being considered a functional food. *Vaccinium ashei* lyophilized extract was administered in diet-induced hypercholesterolemic rats during 2 weeks and parameters of oxidative stress, antioxidant enzymes, polyphenols and vitamin C were mensured. The current study demonstrated that blueberry extract (BE) has antioxidant potential *in vitro* as verified by DPPH radical and ABTS scavenging activities as well as by the high total polyphenols content. In addition, treatment with the BE showed a decrease of lipid peroxidation and protein and an increase in activities of antioxidant enzymes like catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx). A significant increase in the levels of vitamin C and polyphenols in plasma also were found. Dietary consumption of polyphenols rich foods may contribute to overall antioxidant status, particularly in reducing oxidative stress associated with hypercholesterolemia.

Keywords: Vaccinium ashei Reade, hypercholesterolemia, polyphenols, oxidative stress.

1.0 INTRODUCTION

Oxidative stress has been demonstrated to play a causal role in different vascular diseases, such as hypertension, diabetic, hypercholesterolemia and atherosclerosis[1]. Reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radicals, and free radical-meditated reactions, can cause oxidative damage to cellular structures and functional molecules (e.g., DNA, proteins, and lipids), contributing to the development of cardiovascular diseases[2]. Dietary compounds such as polyphenols, found in fruits and vegetables, can play an important role in the improvement of antioxidant status because they are able to neutralize ROS[3,4].

With the current upsurge of interest in the efficacy and use of naturally derived antioxidants, functional foods have received much attention in recent years[5]. Blueberries are known as "super fruits" for their potential in the nutraceutical markets[6,7] because contain anthocyanins, polyphenols and flavonoids beyond a high level of vitamin C (ascorbic acid), folic acid and resveratrol[8] and appear to have the highest antioxidant capacity among fruits and vegetables, conferring on blueberries the title of a functional food[9,5].

Many reports have suggested that blueberry fruits have a wide range of health benefits such as antioxidant activity *in vivo*[10] and *in vitro*[11], antidiabetic activity[12,13], and the ability to protect against cancer and stroke[14].

With the current upsurge of interest in the efficacy and use of naturally derived antioxidants, functional foods and nutraceuticals in recent years, and, considering that Brazil has recently become a blueberry producer with a small production[15] the objectives of this study were to evaluate the role of blueberry (*Vaccinium ashei* R) on oxidative stress parameters and antioxidant defenses in hypercholesterolemic rats after treatment of blueberry extract.

2.0 MATERIALS AND METHODS

2.1 Chemicals

All the chemicals were from Sigma Chemical Co. (St. Louis, MO, USA) and of analytical grade.

2.2 Plant Material

Mature fruits of rabbiteye blueberry (*Vaccinium ashei* Reade) cultivars Bluegem were collected in november of the year 2012 and transported fresh to the city of Uruguaiana, RS where they were immediately processed. The fruits were generously provided by EMBRAPA – Clima Temperado, Pelotas, Brazil, RS, established through collaboration with Maria do Carmo Bassols Raseira.

2.3 Preparation of the extract

For the preparation of the extract were used 100 g of blueberry macerated using a porcelain grail and pistil, in the dark, to preserve the antioxidant properties of its constituents and then mixed with 100 mL of methanol: ethanol: acetone (45:45:10 v/v) according to the method described by Vizzotto and Pereira (2009)[16], with adaptations. After 24 hours the extract was filtered and subjected to reduced pressure in a rotary evaporator to remove the solvent. The extract was transferred to Falcon tubes, frozen and subsequently lyophilized. The lyophilized extract was stored at -70°C until treatment.

2.4 Evaluation of antioxidant potential from blueberry extract in vitro

In order to determine the antioxidant potential of this obtained blueberry extract we evaluated the scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, 2,2'-azino-bis-3-ethylbenzthiazoline-6- sulphonic acid (ABTS) as well as we quantified the total polyphenols content.

2.4.1. DPPH radical scavenging activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging activity of blueberry extract was determined as described by Sharma and Bhat[17]. The DPPH radical solution (50µL) was dissolved in methanol and added to a medium containing blueberry extract at different concentrations (0.5-10,1 mg/mL). The medium was incubated at room temperature for 30 min. The decrease in absorbance was measured at 517 nm, which depicted the scavenging activity of blueberry extract against DPPH. The DPPH scavenging capacity of the compound was calculated as

DPPH radical scavenging activity = $100 - [(ABS_{SAMPLE} - ABS_{BLANK}) / ABSC_{ONTROL}) \text{ X } 100]$

Where, ABS_{SAMPLE} is the absorbance of the test compound, ABS_{BLANK} is the absorbance of the blank and $ABS_{CONTROL}$ is the absorbance of the control reaction. IC_{50} value (concentration of sample where absorbance of ABTS decreases 50% with respect to absorbance of blank) of the sample was determined. Ascorbic acid was used as positive control.

2.4.2 ABTS radical scavenging assay:

The ABTS method (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) is based on the deactivation of the antioxidant radical cation ABTS•+, which is measured by the decrease in absorbance at 734 nm. The ABTS method was performed as described by Re *et al.*[18]. Absorbance was read at 734 nm, 7 minutes after the extract addition. The ABTS scavenging capacity of the compound was calculated as

ABTS radical scavenging activity = $100 - [(ABS_{SAMPLE} - ABS_{BLANK}) / ABSC_{ONTROL}) \text{ X } 100]$

Where, ABS_{SAMPLE} is the absorbance of the test compound, ABS_{BLANK} is the absorbance of the blank and $ABS_{CONTROL}$ is the absorbance of the control reaction. IC_{50} value (concentration of sample where absorbance of ABTS decreases 50% with respect to absorbance of blank) of the sample was determined. Ascorbic acid was used as positive control.

2.4.3 Determination of total polyphenols content

Total polyphenols content of the blueberry extract was measured by spectrophotometry using the Folin-Ciocalteu method[19], with modifications. Briefly, 1 mL of 1 N Folin-Ciocalteu reagent was added to a 1 mL of sample, and this mixture was allowed to stand for 2-5 min before the addition of 2 mL of 20% Na_2CO_3. The solution was then allowed to stand for 10 minutes before reading at 750 nm in Spectrophotometer (UV-1800 Shimadzu, Japan) using 1 cm quartz cells. The total polyphenol content was expressed as milligram of gallic acid equivalent per milliliter (mg GAE mL^{-1}).

2.5 Evaluation of blueberry extract in vivo

2.5.1 Animals Experimentation

For this study we used male Wistar rats (60-65 g), 30 days old, were obtained from the Central Animal Laboratory of the Federal University of Santa Maria, Rio Grande do Sul, Brazil. During treatment, rats were housed at a constant room temperature, humidity, and light cycle (12:12h light-dark), free access to tap water and fed with standard chow *ad libitum*. All experiments were conducted in compliance with the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology and approved by the Ethics Committee on Animal Use Experimentation of the Federal University of Pampa, CEUA, Uruguaiana, Rio Grande do Sul, Brazil (Protocol n° 035/2012).

2.5.2 Preparation of feed and Induction of hypercholesterolemia

Commercial feed was supplemented with 7,5% of pig grease and with 2,5 % corn oil for each 100g of feed according to the described methodology of Fietz and Salgado (1999), with adaptations. The hyperlipidemic feed was prepared daily for all experimental groups.

After the induction of the hypercholesterolemy, the groups kept receiving the same diet until the end of the experiment. 44 rats that consumed the hypercholesterolemic diet were

selected. From them, 2 rats were euthanized after the first week of hypercholesterolemia induction to be used as comparison parameter, and were dosed the blood levels of total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides. This procedure was developed weekly until the hypercholesterolemia confirmation, totalizing 30 days of induction.

2.5.3 Experimental Desing

The 36 rats were divided into 6 groups of 6 rats each. The group 1 (control) received saline solution in a daily dose of 1 mL, group 2: oral suspension of simvastatin 10 mg/Kg (SIM), group 3: blueberry extract 25 mg/Kg (BE25), group 4: blueberry extract 50 mg/Kg (BE50), group 5: blueberry extract 25 mg/Kg associated to simvastatin 10 mg/Kg (BE25+SIM), group 6: blueberry extract 50 mg/Kg associated to simvastatin 10 mg/Kg (BE50+SIM). All treatments were administered by gavage. The register of the body weight of the animals was performed during all experimental period to accompany the development of the animals and to determine the extracts and administrated drugs volume.

2.5.4 Blueberry extract prepare and administration

The blueberry-based solution was obtained by the dissolution of the extract previously lyophilized, in water. The solution was daily prepared, in the moment immediately before the administration. The extracts were administered by gavage for 14 consecutive days. Animals were euthanized 24 h after the last treatment, in fasting, to obtain the whole blood.

2.6 Evaluation of oxidative stress parameters

2.6.1 Oxidative damage

Oxidative damage markers, lipid peroxidation[21], protein carbonyls[22] in plasma were measured by the spectrophotometric methods. The assessment of DNA damage was made by comet assay[23] and frequency of micronucleus[24] in leukocytes.

2.6.2 Antioxidant defenses

The activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in erythrocyte were achieved using commercial kits (Randox Brazil LTDA).

2.7 Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Comparisons between groups were performed using a two-way analysis of variance (ANOVA), followed by post hoc of Bonferroni for multiple comparison tests. Results were considered statistically significant when $p<0.05$. The statistical analysis was performed using the software GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

3.0 RESULTS/DISCUSSION

3.1 Radical scavenging capacity of lyophilized blueberry extract

Antioxidant activity of the tested lyophilized blueberry extract (*Vaccinium ashei* R) was determined by both DPPH and ABTS methods and showed good antioxidant capacity. The results are shown in **Figure 1**.

We verified that the lyophilized blueberry extract (0.5 and 10 mg/mL) demonstrated a significant DPPH scavenging activity (2.5 and 52.43 % of inhibition, respectively) (**Figure 1A**) and significant ABTS scavenging activity (4.8 and 73.78 % of inhibition, respectively) (**Figure 1B**). The obtained data illustrated that, ABTS method showed higher antioxidant activity than DPPH method, because the ABTS method, which is more sensitive than the DPPH method.

The lyophilized blueberry extract analyzed in this study showed highest antioxidant activity: DPPH, IC_{50} 9.57 mg/mL and ABTS, IC_{50} 6.85 mg/mL. These results confirm the blueberry extract as a source of phenol compounds with high antioxidant activity.

3.2 The total polyphenols content

We detected that the polyphenols content in the lyophilized blueberry extract was 190.620 mg GAE/mL.

4.1 Evaluation of oxidative stress parameters

4.1.2 Oxidative damage

The incidence of atherosclerosis increases with hypercholesterolemia.[25] Oxidative modifications in proteins, lipids, and DNA are considered to be among the molecular mechanisms leading to endothelial dysfunction and atherosclerosis.[26]

The **Figure 2** shows the results of biomarkers for oxidative damage. The malondialdehyde (MDA) has the potential not only to assess the extent of oxidative damage, but also to predict potential efficacy of therapeutic strategies aimed at reducing oxidative stress.[27]

Hypercholesterolemia is associated with elevated levels of MDA and is also known to increase the production of ROS. In this study plasma MDA level was used to investigate the effect of the blueberry extract on hypercholesterolemic rats. MDA level was measured as TBARS (thiobarbituric acid reactive substances) method. This method has been criticized for its lack of specificity, but it is one of the easiest and the most frequently used marker of lipid peroxidation.[28]

Several investigators found that high cholesterol diet had an increasing effect on lipid peroxidation in plasma and tissues.[29,30] The results of this study show significant increases (p<0.05) in plasma MDA levels in control group when compared with other groups. However, the groups treated with BE25 and BE50+SIM show significant decreases (p<0.05) MDA levels in plasma when compared with control group (**Figure 2A**). This result suggests that this BE may have an antioxidant effect on hypercholesterolemic rats because the polyphenols,

present in BE, are incorporated into membrane lipid and act as hydrogen donors, trapping free radicals and inhibiting the formation of lipid radicals.[31] The antioxidant effects of blueberries have also been reported by studies using cellular and animal models of oxidative stress.[32,33]

Protein carbonylation is a type of protein oxidation that can be promoted by ROS. It usually refers to a process that forms reactive ketones or aldehydes that can be reacted by 2,4-dinitrophenylhydrazine (DNPH) to form hydrazones. Direct oxidation of side chains of lysine, arginine, proline, and threonine residues, among other amino acids, in the "primary protein carbonylation" reaction produces DNPH detectable protein products.[34] To assess the effects of BE supplementation on hypercholesterolemic rats, the levels of carbonyl proteins measured and the results shown in **Figure 2B**. Protein carbonyls are normally used as a biomarker for protein damage caused by oxidized amino acid residues in stress conditions.[35]

We observed a significant effect of the dietary intervention with BE in protect the proteins against the reactive species. This effect is enhanced when the extract is associated with simvastatin (BE50+SIM). This result corroborated those showed by Aydin *et al*., 2009[36], comparison of cholesterol-fed rabbits for 8 weeks with rabbits fed cholesterol fed (8 weeks) plus atorvastatin administration (4 weeks) revealed that a significant decreases in protein carbonyl and MDA in atorvastatin-treated rabbits. Atorvastatin therapy caused significant decreases in both protein carbonyl levels.

In **Figure 2C** is showed the result of micronucleus frequency. Our data show no alteration in these parameter.

The ROS can damage DNA, lipids, and proteins, it is necessary to measure damage of more than just one of these cellular components. DNA damage is the most severe form of oxidative damage because it can cause permanent mutations that are passed on to progeny cells. The comet assay was performed to determine damage index DNA and the results were

shown in **Figure 2D**. We observed a potential effect of the BE in comet assay, a genotoxicity test which has been widely used in recent years to analyze protective effect on DNA damage. In our study the groups treated with BE showed a decrease DNA damage in leukocytes of rats hypercholesterolemics and this effect is enhanced when the extract is associated with simvastatin (BE50+SIM). This result corroborated those showed by Barros *et al.*[37], showing DNA damage levels were significantly reduced in the hippocampal regions of mice that had been supplemented with a much lower level of 2.6-3.2 mg/kg of body weight of blueberry anthocyanins in drinking water for 30 days. Possible mechanisms for these genoprotective effects include protection of DNA from alkylation or formation of anthocyanin-DNA complexes, which stabilize the molecule against oxidative attack.[38,39]

4.1.3 Antioxidant defenses

In **Figure 3**, we show the results of antioxidant defenses biomarkers in plasma. Polyphenols are plant secondary metabolites, widely present in commonly consumed foods of plant origin, and they are accruing a body of evidence as bioactive components in a wide range of biological systems.[40] These compounds are considered to carry many potential beneficial health effects.

We observed a significant effect of the treatment with BE increase the polyphenol concentration in the groups BE 25, BE50 and BE50+SIM, showing that treatment with the extract was able to increase, after 14 days of treatment, the content of polyphenols in plasma of hypercholesterolemic rats (**Figure 3A).**

Ascorbic acid or vitamin C is one of the important water soluble vitamins, being associated to many health benefits. It acts as a cofactor in the enzymatic biosynthesis of hormones, being also a potent antioxidant, as it reduces nitrogen and ROS into stable molecules.[41]

After 14 days of treatment with the BE, there was a significant increase in the levels of ascorbic acid in plasma. This increase was more evident in the group that received the highest dose extract (50mg/kg), and the group that received the combined extract SIM. This result shows that the blueberry is a source of ascorbic acid and is able to increase the antioxidant capacity in hypercholesterolemic rats (**Figure 3B).**

To minimize the oxidative damage caused by ROS, cells possess a wide range of enzymatic systems including, glutathione peroxidase (GPx). The results of the antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) e glutathione peroxidase (GPx) in serum hypercholesterolemic rats is showed in **Figure 3**.

The activity of the antioxidant enzyme CAT is shown in **Figure 3C.** The groups that received SIM, BE25 and BE25+SIM showed increased serum activity of this enzyme. However, the dose of 50mg/kg showed an even better effect on the increase in CAT activity when compared to the control group.

SOD is the major cellular antioxidant defense enzyme against superoxides in vascular cells and the may act synergistically to combat the oxidative stress implicated in atherosclerosis.[42] In our study, after 14 days treatment with blueberry extract, the activity of this enzyme also increased, especially in the groups that received the dose of 50mg/Kg extract. Again, when the extract was associated with SIM, increased SOD activity was more significant (**Figure 3D)**. Wu *et al*,. 2010[43], in their study, also found the activity of SOD increased in female mice that received AIN-93G diet incorporated with 1% freeze-dried blueberry powder.

GPx is a selenoenzyme that plays a key role in protecting the organism from oxidative damage by catalyzing the reduction of harmful hydroperoxides with thiol cofactors.[44] In our study, the activity of GPx enzyme increased when compared to the control group, the groups

treated with SIM, BE25 and BE50+SIM. However, the groups that received a higher dose of extract (50mg/Kg), GPx activity had a greater antioxidant activity (**Figure 3E).**

Another interesting finding of this study was that the activity of antioxidant enzymes increased when the blueberry extract was associated with simvastatin.

The current study demonstrated a decrease of lipid peroxidation and protein as well as an increase of antioxidants enzymes, in hypercholesterolemic rats after 2 weeks of treatment with BE. These results are consistent with the hypothesis that flavonoids and other polyphenols can have effects in decrease oxidative damage and many studies have demonstrated high levels of these compounds in BE. [45,46]

The lyophilized extract of *Vaccinium ashei* R. was shown to have many benefits, which may be related high polyphenols levels, in particularly the anthocyanidins and proanthocyanidins, responsible for their very strong antioxidant activity.

In summary, hypercholesterolemia increases the oxidative stress in plasma. BE is efficient in slowing the progression of hypercholesterolemia induced oxidative stress and improving their functions but BE50+SIM is more effective than BE.

ABBREVIATIONS USED

BE – blueberry extract

BE25 – blueberry extract 25mg/Kg

BE50 – blueberry extract 50mg/Kg

BE25+SIM – blueberry extract 25mg/Kg associated with simvastatin

BE50+SIM - blueberry extract 50mg/Kg associated with simvastatin

DPPH - 2,2-diphenyl-1-picrylhydrazyl

ABTS - 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid

CAT - catalase

SOD – superoxide dismutase

GPx - glutathione peroxidase

ROS – reactive oxygen species

DNA - deoxyribonucleic acid

SIM - simvastatin

MDA - malondialdehyde

TBARS - thiobarbituric acid reactive substances

DNPH - 2,4-dinitrophenylhydrazine

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FIGURE CAPTIONS

Figure 01: Antioxidant activities *in vitro* of the lyophilized blueberry extract of *Vaccinium ashei* R determined at different concentrations: (A) DPPH radical-scavenging activity (B) ABTS radical-scavenging activity. AA – ascorbic acid; BE – blueberry extract.

Figure 02: Oxidative damage markers in hypercholesterolemic rats after treatment of blueberry extract. In A: lipid peroxidation levels; B: carbonyl protein contents; C: frequency of micronucleus; D: DNA damage index Data are expressed as means±S.D. Different letters means statistically different results ($p<0.05$).

Figure 03: Antioxidative defenses markers in in hypercholesterolemic rats after treatment of blueberry extract. In A: polyphenols contents; B: ascorbid acid contents; C: catalase activity; D: superoxide dismutase activity; E: glutathione peroxidase activity. Data are expressed as means±S.D. Different letters means statistically different results ($p<0.05$).

FIGURE GRAPHICS

Figure 1

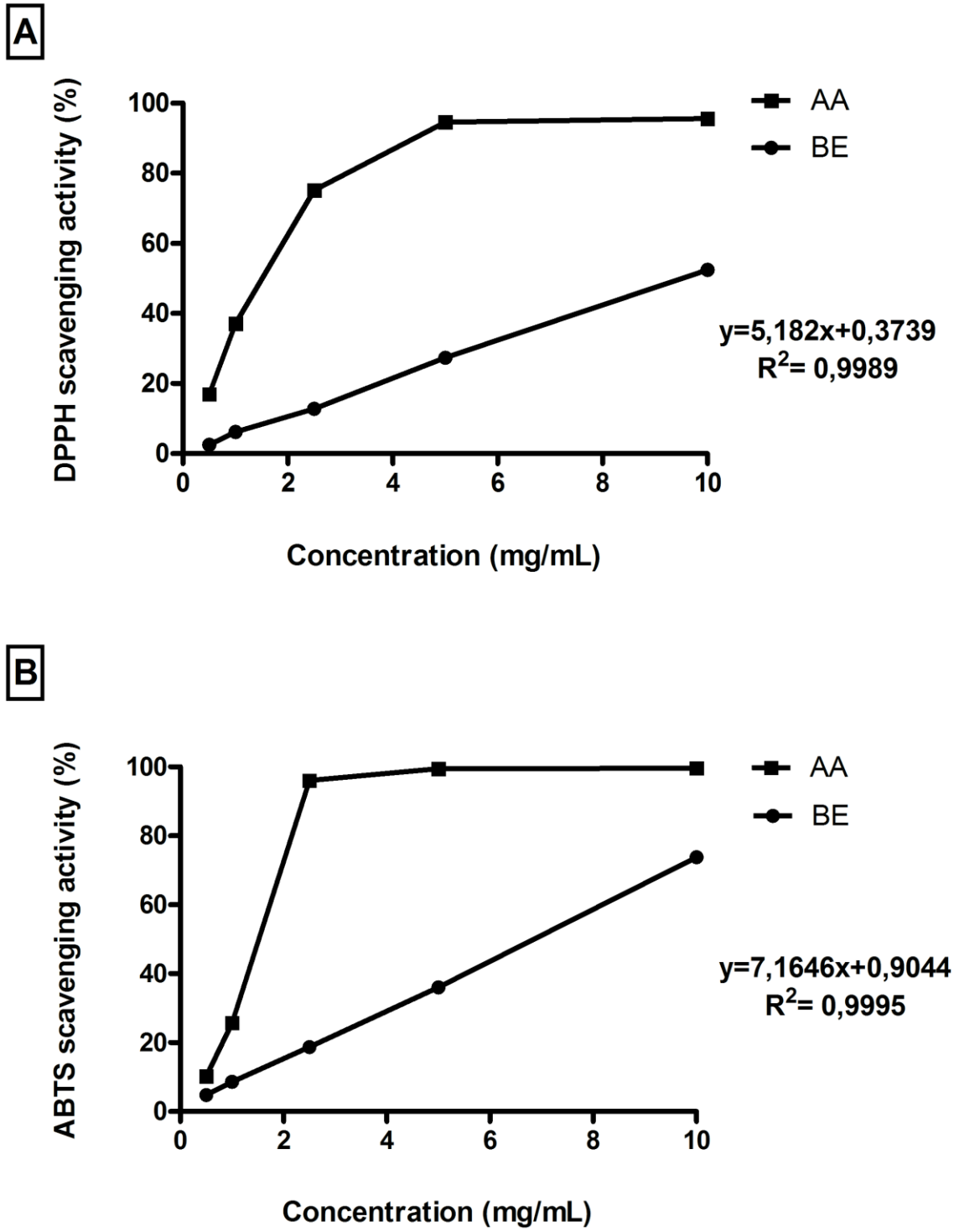


Figure 2

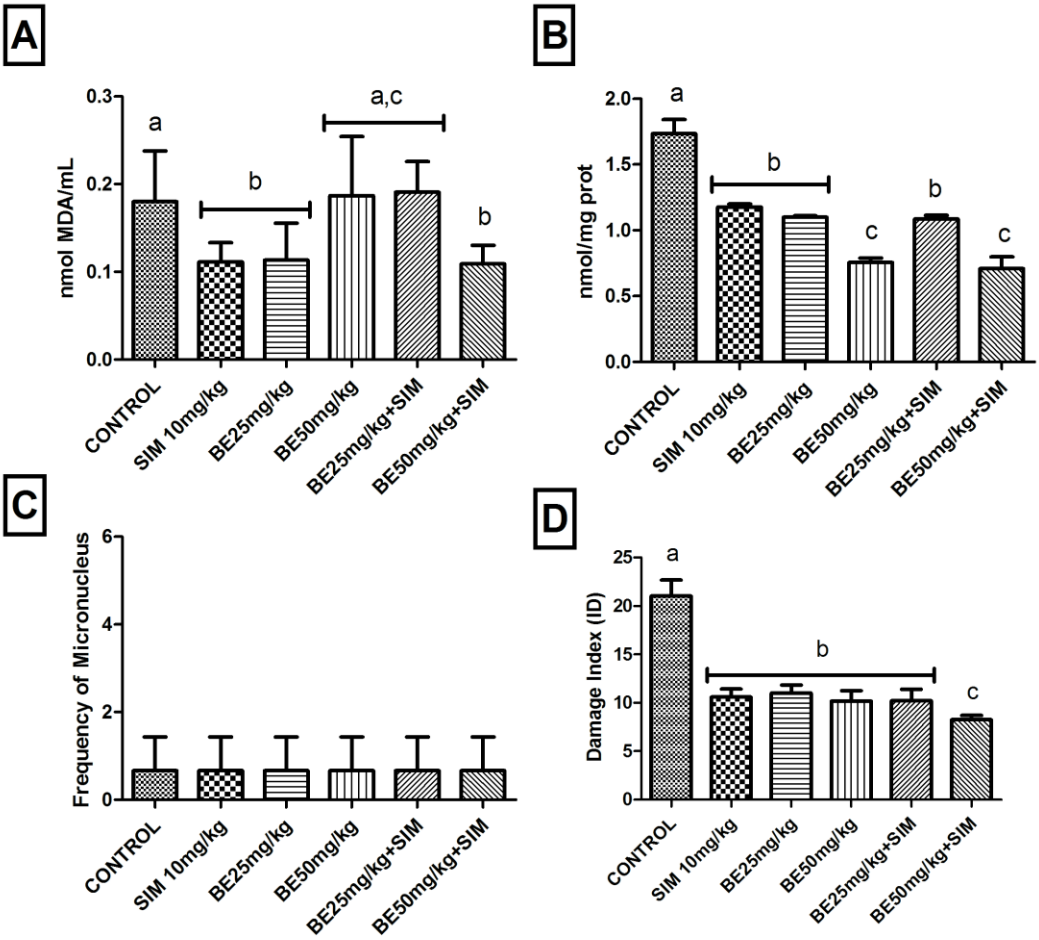
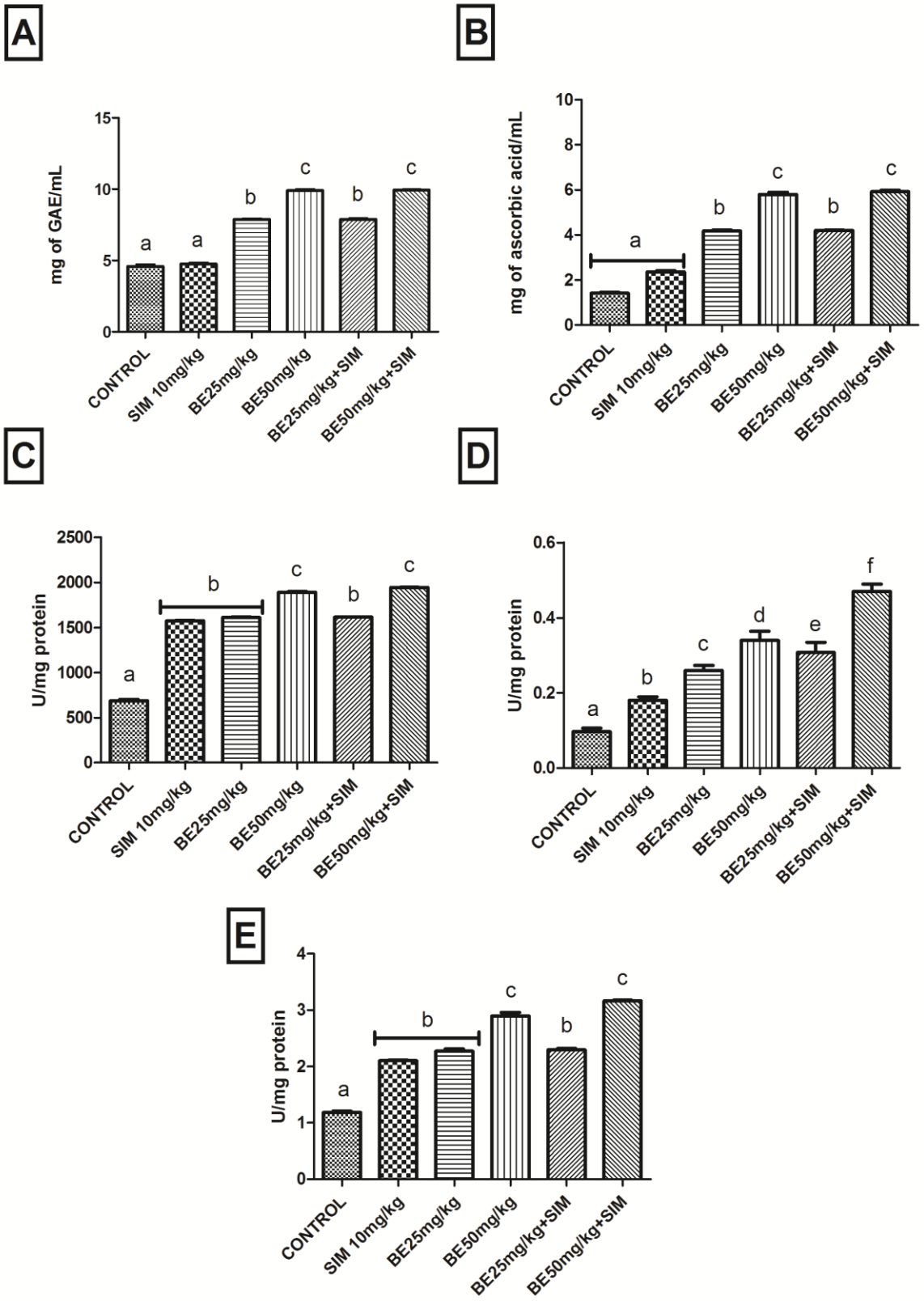

A
nmol MDA/mL
0.3
0.2
0.1
0.0
a
b
a,c
b
CONTROL
SIM 10mg/kg
BE25mg/kg
BE50mg/kg
BE25mg/kg+SIM
BE50mg/kg+SIM
B
nmol/mg prot
2.0
1.5
1.0
0.5
0.0
a
b
c
b
c
CONTROL
SIM 10mg/kg
BE25mg/kg
BE50mg/kg
BE25mg/kg+SIM
BE50mg/kg+SIM
C
Frequency of Micronucleus
6
4
2
0
CONTROL
SIM 10mg/kg
BE25mg/kg
BE50mg/kg
BE25mg/kg+SIM
BE50mg/kg+SIM
D
Damage Index (ID)
25
20
15
10
5
0
a
b
c
CONTROL
SIM 10mg/kg
BE25mg/kg
BE50mg/kg
BE25mg/kg+SIM
BE50mg/kg+SIM

Figure 3


A
mg of GAE/mL
15
10
5
0
a
a
b
c
b
c
CONTROL
SIM 10mg/kg
BE25mg/kg
BE50mg/kg
BE25mg/kg+SIM
BE50mg/kg+SIM
B
mg of ascorbic acid/mL
10
8
6
4
2
0
a
b
c
b
c
CONTROL
SIM 10mg/kg
BE25mg/kg
BE50mg/kg
BE25mg/kg+SIM
BE50mg/kg+SIM
C
U/mg protein
2500
2000
1500
1000
500
0
a
b
c
b
c
CONTROL
SIM 10mg/kg
BE25mg/kg
BE50mg/kg
BE25mg/kg+SIM
BE50mg/kg+SIM
D
U/mg protein
0.6
0.4
0.2
0.0
a
b
c
d
e
f
CONTROL
SIM 10mg/kg
BE25mg/kg
BE50mg/kg
BE25mg/kg+SIM
BE50mg/kg+SIM
E
U/mg protein
4
3
2
1
0
a
b
c
b
c
CONTROL
SIM 10mg/kg
BE25mg/kg
BE50mg/kg
BE25mg/kg+SIM
BE50mg/kg+SIM

PARTE III

4.0 CONCLUSÃO

De acordo com os resultados apresentados nesta dissertação pode-se inferir que o extrato liofilizado de mirtilo:

- apresenta atividade antioxidante *in vitro,* evidenciada pela atividade sequestradora do radical DPPH e ABTS, o que pode ser atribuído ao seu elevado teor de compostos fenólicos totais (190.620 mg GAE/mL);
- evitou o ganho de peso corporal, diminuiu os níveis plasmáticos de colesterol total, colesterol LDL e triglicerídeos, bem como aumentou o colesterol HDL. E, este efeito é ligeiramente melhor quando o extrato na dose de 50 mg/Kg está associado à sinvastatina.
- não mostrou efeito significativo sobre o perfil glicêmico;
- diminuiu o espessamento da aorta, mostrando um efeito protetor contra a aterosclerose;
- reduziu significativamente os marcadores de dano cardíaco (CK-total e CK-MB) e homocisteína;
- reduziu significativamente os marcadores de estresse oxidativo, como a peroxidação lipídica e carbonilação de proteínas;
- aumentou significativamente os níveis de polifenóis e ácido ascórbico no plasma;
- diminuiu o dano no DNA de leucócitos;
- aumentou a atividade das enzimas antioxidantes SOD, CAT e GPx, mostrando um importante papel na atividade antioxidante.

Estes resultados mostram que o mirtilo têm efeitos positivos e, portanto, tem potencial para utilização no desenvolvimento de nutracêuticos.

5.0 PERSPECTIVAS

Este trabalho tem como perspectivas:

- Medir marcadores inflamatórios como PCR, IL-1B, IL-6, IL-10 e TNF-α;
- Determinar a concentração das apolipoproteínas (B e A-I);
- Avaliar as principais antocianinas presentes no extrato liofilizado de *Vaccinium ashei* Reade.

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7.0 ANEXOS

Protocolo de aprovação do projeto pelo CEUA-UNIPAMPA

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA
(Lei nº 11.640, de 11 de janeiro de 2008)

Pró-Reitoria de Pesquisa

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Fone: (55) 3413 4321, E-mail: ceua@unipampa.edu.br

PROTOCOLO Nº 035/2012

Título: INVESTIGAÇÃO DE VIAS DE ESTRESSE OXIDATIVO EM RATOS WISTAR HIPERCOLESTEROLÊMICOS SUPLEMENTADOS COM EXTRATO DOS FRUTOS DE Vaccinium ashei R. (MIRTILO)

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Após a análise detalhada do projeto de pesquisa a relatoria da CEUA-Unipampa emite parecer **FAVORÁVEL** para o cadastro do protocolo e execução do referido projeto.

Luiz E. Henkes
Professor Adjunto
Coordenador do CEUA/Unipampa

Certificado de apresentação do trabalho

40º Congresso Brasileiro de Análises Clínicas

13º Congresso Brasileiro de Citologia Clínica

4ª Jornada Latinoamericana de Genética Forense

16 a 19 de junho de 2013 | Costão do Santinho - Florianópolis - SC

CERTIFICADO

CERTIFICAMOS que o trabalho

PERFIL LIPÍDICO E GLICÊMICO DE RATOS WISTAR HIPERCOLESTEROLÊMICOS TRATADOS COM EXTRATO DOS FRUTOS DE VACCINIUM ASHEI R (MIRTILO)

dos autores **DEISE JAQUELINE STRÖHER, MURIEL PANDO PEREIRA, RITIÉLE PINTO COELHO, ANGÉLICA APARECIDA DA COSTA GÜLLICH, BRUNA COCCO PILAR, JAMILA BENVEGNÚ BRUNO, MARISTELA WITFEL, LEANDRO LEAL GALARÇA, VANUSA MANFREDINI** foi apresentado na sessão de temas livres do Congresso.

Rio de Janeiro, 19 de junho de 2013

Realização: SBAC – Sociedade Brasileira de Análises Clínicas

Entidades Parceiras: SBCC; SLAGF – Sociedade Latinoamericana de GENÉTICA FORENSE

Dr. Irineu Keiserman Grinberg – Presidente da Sociedade Brasileira de Análises Clínicas

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Dra. Maria Elizabeth Menezes – Presidente da Sociedade Latinoamericana de Genética Forense e Presidente da Comissão Científica do 40º CBAC

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