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LORENA NERIS BARBOZA

EFEITOS ARTERIOPROTETORES DE *Cuphea carthagenensis* (JACQ.) J.F. MACBR. EM COELHOS NOVA ZELÂNDIA SUBMETIDOS À DIETA RICA EM COLESTEROL

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Dissertação apresentada ao Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná como requisito parcial para obtenção do título de Mestre em Farmacologia.

Orientador: Profº. Dr. Arquimedes Gasparotto Junior

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MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
Setor CIÊNCIAS BIOLÓGICAS
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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição Dissertação de Mestrado de **LORENA NERIS BARBOZA**, intitulada: " **Efeitos arterioprotetores Cuphea carthagenensis (JACQ.) J.F. MACBR. em coelhos Nova Zelândia submetidos à dieta rica colesterol**", após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer p sua APROVAÇÃO, completando-se assim todos os requisitos previstos nas normas desta Instituição pa obtenção do Grau de **Mestre em FARMACOLOGIA**.

Curitiba, 25 de Fevereiro de 2016.

Prof ARQUIMEDES GASPAROTTO JUNIOR (UFPR)
(Presidente da Banca Examinadora)

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ATA DE SESSÃO PÚBLICA DE DEFESA DE DISSERTAÇÃO PARA A OBTENÇÃO DO GRAU DE MESTRE EM FARMACOLOGIA

No dia vinte e cinco de Fevereiro de dois mil e dezesseis às 08:30 horas, na sala 0, Centro Politécnico, do Setor de CIÊNCIAS BIOLÓGICAS da Universidade Federal do Paraná, foram instalados os trabalhos de arguição da mestranda **LORENA NERIS BARBOZA** para a Defesa Pública de sua Dissertação intitulada: " **Efeitos arterioprotetores de Cuphea carthagenensis (JACQ.) J.F. MACBR. em coelhos Nova Zelândia submetidos à dieta rica em colesterol**". A Banca Examinadora, designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná, foi constituída pelos seguintes Professores Doutores: ARQUIMEDES GASPAROTTO JUNIOR (UFPR), EMERSON LUIZ BOTELHO LOURENÇO (UFPR), JOSÉ EDUARDO DA SILVA SANTOS (UFPR). Dando início à sessão, a presidência passou a palavra a discente, para que a mesma expusesse seu trabalho aos presentes. Em seguida, a presidência passou a palavra a cada um dos Examinadores, para suas respectivas arguições. A aluna respondeu a cada um dos arguidores. A presidência retomou a palavra para suas considerações finais e, depois, solicitou que os presentes e a mestranda deixassem a sala. A Banca Examinadora, então, reuniu-se sigilosamente e, após a discussão de suas avaliações, decidiu-se pela APROVAÇÃO da aluna. A mestranda foi convidada a ingressar novamente na sala, bem como os demais assistentes, após o que a presidência fez a leitura do Parecer da Banca Examinadora, outorgando-lhe o Grau de **Mestre em FARMACOLOGIA**. Nada mais havendo a tratar a presidência deu por encerrada a sessão, da qual eu, ARQUIMEDES GASPAROTTO JUNIOR, lavrei a presente ata, que vai assinada por mim e pelos membros da Comissão Examinadora.

Curitiba, 25 de Fevereiro de 2016.

Prof ARQUIMEDES GASPAROTTO JUNIOR (UFPR)
(Presidente da Banca Examinadora)

Prof EMERSON LUIZ BOTELHO LOURENÇO (UFPR)

Prof JOSÉ EDUARDO DA SILVA SANTOS (UFPR)

NOTA EXPLICATIVA

Esta dissertação é apresentada em formato alternativo – artigo para publicação – de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná, na qual consta uma revisão de literatura, objetivos do trabalho e artigo científico abordando os experimentos realizados, resultados e discussão, bem como conclusão. O artigo foi formatado conforme as normas propostas por periódicos de circulação internacional.

A (Paulo e Maria de Lourdes) meus pais, que são a luz em minha vida, meu eterno amor;

A (Lucas e Leticia) meus irmãos, que são o meu olhar cúmplice, meu coração em outro corpo;

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A todos que, de algum modo, contribuíram para a execução deste trabalho.

Para ser grande, sê inteiro: nada
Teu exagera ou exclui.

Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.

Assim em cada lago a lua toda
Brilha, porque alta vive.

Fernando Pessoa

RESUMO

Apesar da *Cuphea carthagenensis* (Jacq.) J. F. Macbr. ser amplamente utilizada na medicina popular brasileira para o tratamento de aterosclerose e doenças do aparelho circulatório, não há dados que comprovam seus benefícios. O objetivo deste estudo foi avaliar possíveis efeitos hipolipemiantes e antiaterogênicos da fração solúvel em etanol obtida a partir de *Cuphea carthagenensis* (ES-CC), utilizando coelhos Nova Zelândia (NZ) submetidos à dieta rica em colesterol (DRC). A dislipidemia e a aterosclerose foi induzida através da administração de dieta comercial padrão suplementada com 1% de colesterol, durante 8 semanas. O ES-CC foi administrado por via oral em doses de 10, 30 e 100 mg/kg, uma vez ao dia, durante quatro semanas, com início a partir da 4ª semana de DRC. Os níveis séricos de triglicerídeos (TG), colesterol total (CT) e as suas frações (LDL-C, VLDL-C e HDL-C) foram mensurados no tempo zero e no final de cada mês de tratamento. Após eutanásia, os segmentos da artéria aorta (arco-aórtico, torácica, abdominal e ilíaca) foram avaliados macro e microscopicamente e então medidas a camada íntima e média das artérias. Também foram determinadas a atividade antioxidante da ES-CC e a sua influência sobre o funcionamento das enzimas antioxidantes hepáticas. A DRC promoveu importantes mudanças na estrutura da parede arterial, incluindo espessamento da camada íntima dos vasos. Além disso, também observamos um significativo aumento da peroxidação lipídica acompanhada da redução dos níveis hepáticos de glutationa e do nitrito plasmático. O tratamento com a ES-CC foi capaz de impedir o aumento do CT, LDL-C, VLDL-C e triglicérides, além de aumentar os níveis de HDL-C em coelhos Nova Zelândia. Estes efeitos foram acompanhados por uma redução significativa do estresse oxidativo e da modulação da atividade da catalase e superóxido dismutase. Ademais, as camadas íntimas e médias dos segmentos arteriais foram significativamente reduzidas com a administração do ES-CC. Este estudo demonstrou que a ES-CC é capaz de reduzir os lipídeos séricos e o estresse oxidativo, contribuindo para atenuar o desenvolvimento da placa aterosclerótica induzida pela DRC.

Palavras-chave: *Cuphea carthagenensis, hipolipemiante, antiaterogênico, antioxidant*

ABSTRACT

Although *Cuphea carthagenensis* (Jacq.) J.F. Macbr. is used in Brazilian folk medicine in the treatment of atherosclerosis and circulatory disorders, no study has been conducted to evaluate these effects. The aim of this study was to evaluate possible hypolipemiant and antiatherogenic activity of the ethanol soluble fraction obtained from *Cuphea carthagenensis* (ES-CC) in an experimental model of atherosclerosis, using New Zealand (NZ) rabbits undergoing cholesterol-rich diet (CRD). Dyslipidemia and atherogenesis were induced by administration of standard commercial diet increased of 1% of cholesterol (CRD) for 8 weeks. The ES-CC was administered orally at doses of 10, 30 and 100 mg/kg, once a day, for four weeks, starting from the 4thweek of CRD diet. Body weight measurements were carried out weekly from the beginning of the experiments for 8 weeks. The serum levels of triglyceride (TG), total cholesterol (TC) and their fractions (LDL-C, VLDL-C and HDL-C) were measured at beginning of the experiments, and at week four and eight. After the rabbits were euthanized, aorta segments (aortic arc, thoracic, abdominal and iliac segments) were evaluated macroscopically and microscopically and the intima and media layers of the arteries were measured. Additionally, the antioxidant activity of ES-CC and its influence on the functioning of hepatic antioxidant enzymes were also determined. The CRD induced dyslipidemia and major structural changes in the aortic wall. In addition, we observed an increase in lipid peroxidation accompanied by a reduction of hepatic glutathione, and serum nitrite. The treatment with ES-CC was able to prevent the increase of TC, LDL-C, VLDL-C and triglycerides levels, as well as increased the HDL-C levels in NZ rabbits. These effects were accompanied by a significant reduction in oxidative stress and modulation of the function of catalase and superoxide dismutase. Moreover, the intima and media layers of the arterial segments were significantly reduced by ES-CC treatment. This study demonstrated that ES-CC reduces the serum lipids and hepatic oxidative stress when orally administered to NZ rabbits. In addition, it was able to reduce the development of atherosclerosis induced by CRD.

Keywords: *Cuphea carthagenensis; lipid-lowering; anti-atherogenic; antioxida*

LISTA DE FIGURAS

ARTIGO CIENTÍFICO

Figure 3: ES-CC treatment reduces sudan-positive lesions in thoracic aorta from New Zealand rabbits undergoing cholesterol-rich diet (CRD). Macroscopic observations of the atheromatous plaque formation on thoracic aorta segment stained with Sudan IV are showed. (A) Average area of atherosclerotic lesions; (B) Negative control group; (C) Positive control group; (D) ES-CC 30 mg/kg; and (E) ES-CC 100 mg/kg. Images in 4X scale. Values are expressed as mean ± S.E.M. (n=5-6) in comparison to the negative control ($^{a}p < 0.05$) or positive control ($^{b}p< 0.05$) using one-way ANOVA followed by Bonferroni test.

Figure 4: ES-CC treatment reduces arterial thickness in the aortic arch and thoracic aorta segment from New Zealand rabbits undergoing cholesterol-rich diet (CRD). Cross-sections of the aortic arch and thoracic aorta segment stained with hematoxylin-eosin after 8 weeks of CRD and 4 weeks of treatment with ES-CC (C, D, E, H, I and J) are showed. Histomorphometric analysis of arterial tunica intima (A and F) and media (B and G) are also disclosed. Images in 40X scale. Values are expressed as mean ± S.E.M. (n=5-6 in each group) in comparison to the negative control ($^{a}p < 0.05$) or positive control ($^{b}p< 0.05$) using one-way ANOVA followed by Bonferroni test.

Figure 5: ES-CC treatment reduces arterial thickness in the abdominal and iliac aorta segments from New Zealand rabbits undergoing cholesterol-rich diet (CRD). Cross-sections of the abdominal and iliac aorta segments stained with hematoxylin-eosin after 8 weeks of CRD and 4 weeks of treatment with ES-CC (C, D, E, H, I and J) are showed. Histomorphometric analysis of arterial tunica intima (A and F) and media (B and G) are also disclosed. Images in 40X scale. Values are expressed as mean ± S.E.M. (n=5-6 in each group) in comparison to the negative control ($^{a}p < 0.05$) or positive control ($^{b}p< 0.05$) using one-way ANOVA followed by Bonferroni test.

Figure 6: The ES-CC attenuates oxidative stress in New Zealand rabbits. The serum concentration of MDA was measured by monitoring the formation of thiobarbituric acid-reactive substances (A); Serum nitrite was measured as a marker of nitric oxide bioavailability (B); Glutathione [GSH] levels (C), glutathione transferase [GST] (D), catalase [CAT] (E), and superoxide dismutase [SOD] (F) activities were measured in the liver tissues. Values are expressed as mean ± S.E.M. (n=5-6 in each group) in comparison to the negative control ($^{a}p < 0.05$) or positive control ($^{b}p< 0.05$) using one-way ANOVA followed by Bonferroni test.

LISTA DE TABELAS - ARTIGO CIENTÍFICO

LISTA DE ABREVIATURAS

ATP: Trifosfato de adenosina;
BH4: Tetrahidrobiopterina;
CAT: Catalase;
CT: Colesterol total;
DRC: Dieta rica em colesterol;
eNOS: Óxido nítrico sintase endotelial;
ERN: Espécie reativa de nitrogênio;
ERO: Espécie reativa de oxigênio;
ES-CC: Fração solúvel em etanol obtido de *Cuphea carthagenensis;*
GPx: Glutationa peroxidase;
H_2O_2: Peróxido de hidrogênio;
HDL-C: Lipoproteína de alta densidade;
HMG-CoA: 3-hidroxi-3-metilglutaril-coenzima A;
HSV-1: Herpes simplex vírus tipo I;
ICAMs: Moléculas de adesão intercelular;
IFN-γ: Interferon gama;
IL-1: Interleucina-1;
IL-1β: Interleucina-1β;
IL-4: Interleucina-4;
IL-6: Interleucina-6;
IL-8: Interleucina-8;
iNOS: Óxido nítrico sintase indutiva;
LDL-C: Lipoproteína de baixa densidade;
LDLox: Lipoproteína de baixa densidade oxidada;
MCP-1: Proteína-1 quimiotáxica dos monócitos;
M-CSF: Fator estimulante de colônia de macrófagos;
NADP: Fosfato de dinucleotídeo de nicotinamida e adenina;
NO: Óxido nítrico;
NO•: Radical óxido nítrico;
NOS: Óxido nítrico sintase;
$O2^{\bullet -}$: Ânion superóxido;
OH•: Radical hidroxila;
OMS: Organização Mundial da Saúde;
$ONOO^-$: Peroxinitrito;
SOD: Superóxido dismutase;
TG: Triglicerídeos;
TNF-α: Fator de necrose tumoral;
VCAMs: Moléculas de adesão vascular;
VLDL-C: Lipoproteína de muito baixa densidade.

LISTA DE ABREVIATURAS – ARTIGO CIENTÍFICO

AA: Aortic arch;
ANOVA: Analysis of variance;
AS: Abdominal segment;
BW: Body weight;
^{o}C: Degree Celsius;
CAT: Catalase;
CETP: Cholesteryl ester transfer protein;
CID: Collision-induced dissociation;
CG-MS: Chromatography-mass spectrometry;
CRD: Cholesterol-rich diet;
eNOS: Endothelial nitric oxide synthase;
eV: Electron-volts;
ES-CC: Ethanol soluble fraction obtained from *Cuphea carthagenensis;*
ETOH: Ethanol;
FWHM: Full width at half maximum;
GSH: Glutathione;
GST: Glutathione-S-transferase;
HDL-C: High-density lipoprotein cholesterol;
HEUP: Herbarium of the Universidade Paranaense;
HR-MS: High-resolution mass spectrometry;
IS: Iliac segment;
LC-MS: Liquid chromatography-mass spectrometry;
LDL-C: Low-density lipoprotein cholesterol;
NADP: Nicotinamide adenine dinucleotide phosphate;
NO: Nitric oxide;
NOx: Nitrate/nitrite serum;
NZ: New Zealand;
PDA: Photodiode array detector;
ROS: Reactive oxygen species;
SEM: Standard error of the mean;
SIMV: Simvastatin;
SOD: Superoxide dismutase;
TBARS: Thiobarbituric acid reactive substances;
TC: Total cholesterol; TG: Triglycerides;
TLC: Thin layer chromatography;
TIC: Total ion current;
TS: Thoracic segment;
UEM: Universidade Estadual de Maringá;
UNIPAR: Universidade Paranaense;
UPLC: Ultra performance liquid chromatography;
V: Volts;
VLDL-C: Very-low-density lipoprotein cholesterol.

SUMÁRIO

1. REVISÃO DE LITERATURA

1.1. Relação entre doenças cardiovasculares, dislipidemia e aterosclerose

A Organização Mundial de Saúde (OMS) indica que as doenças cardiovasculares são a primeira causa de mortalidade no mundo, e já lideram essa classificação desde 2000. Segundo o levantamento realizado pela OMS em 2011, proximamente 17,3 milhões de pessoas morreram em todo mundo vítimas de doenças cardiovasculares; além do mais, 80% desses óbitos são registrados em países ocidentalizados. Estima-se que em 2030 o total de mortes por doenças cardiovasculares possa chegar a 23,6 milhões ao ano.

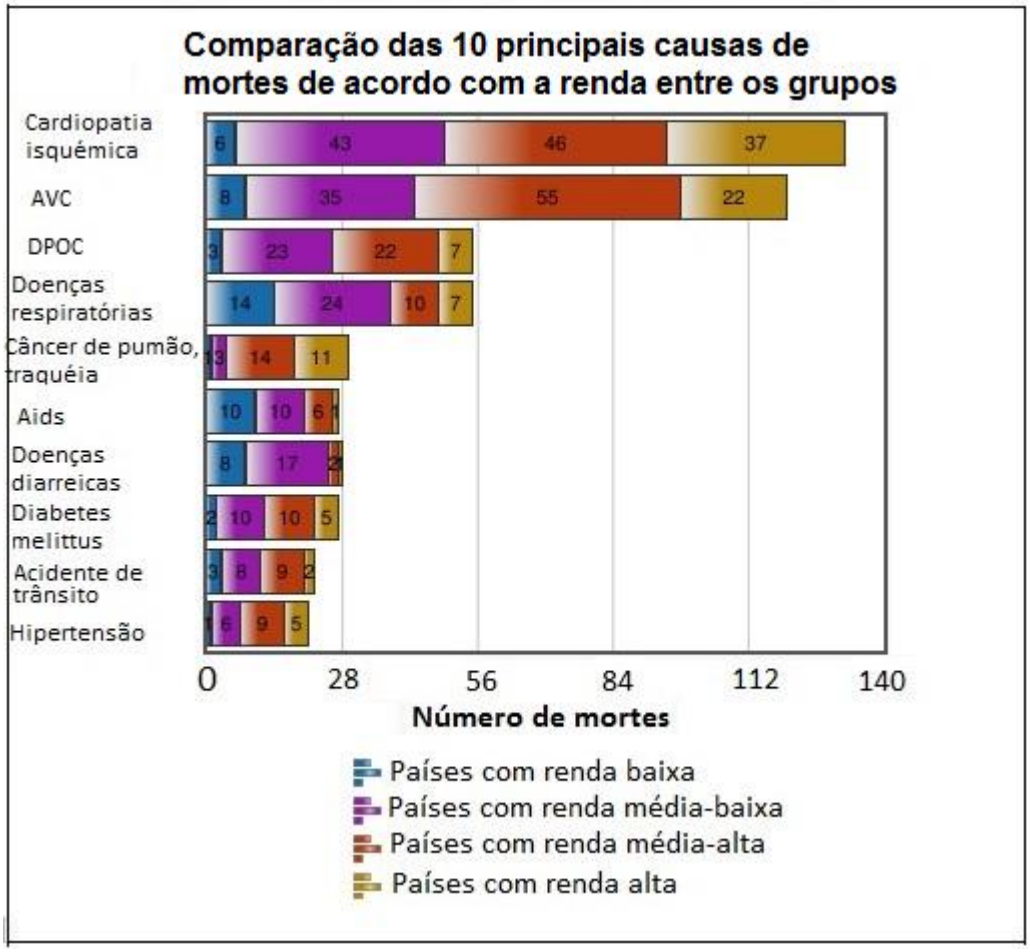

Figura 1: Causas de mortalidade no mundo em 2011 e sua relação entre os diferentes grupos (OMS, 2012).

Nesse sentido a dislipidemia e aterosclerose têm sido bem documentadas, pois estão entre as principais causas subjacentes para o desenvolvimento das doenças cardiovasculares (Libby et al., 2002; McLaren et al., 2011; Buckley et al., 2015). Alterações nos níveis séricos dos lipídios circulantes, principalmente das lipoproteínas de muito baixa densidade (VLDL-C) e lipoproteínas de baixa de densidade (LDL-C), podem influenciar diretamente a deflagração da aterosclerose e conduzir a importantes manifestações clínicas e eventos cardiovasculares agudos (Kruth, 2001; Dashty et al., 2014; Ouweneel et al., 2015). Além disso, quando a dislipidemia está associada a outros fatores de risco como a hipertensão arterial, obesidade, diabetes mellitus, sedentarismo e tabagismo essa co-morbidade se torna ainda mais significativa (Lusis et al, 2000; Buckley et al., 2015; Oliveira et al., 2015).

A dislipidemia e aterosclerose prevalecem como representantes das doenças cardiovasculares ligadas ao envelhecimento, uma vez que se classificam como não modificáveis em indivíduos adultos, cuja incidência aumenta exponencialmente a partir dos 45 anos de idade (Hazzard,1989; Terra et al., 1998; Hui-Hui et al., 2015). Por outro lado, alguns estudos detectaram a prevalência de placas ateroscleróticas em adultos jovens, sugerindo que o processo aterosclerótico possa também ocorrer precocemente (McGill Jr et al., 2000; Satilmis et al., 2015). Além disso, estudos realizados por Napoli et al. (1997) presumiram que em casos de hipercolesterolemia materna, a aterosclerose pode iniciar no estágio fetal e de tal forma progredir lentamente na adolescência até manifestações clínicas na idade adulta.

Atualmente a aterosclerose é considerada uma doença inflamatória crônica, de origem multifatorial que ocorre em resposta à agressão endotelial. É caracterizada pela formação de placas ateroscleróticas no interior dos vasos sanguíneos, acometendo principalmente a camada íntima de artérias de médio e

grande calibre (Ross et al., 1993; Ross et al., 1999; Wang et al., 2012). Embora qualquer artéria possa ser afetada, os principais alvos da doença são as artérias de grande calibre, como a artéria aorta, as coronárias e as artérias cerebrais, sendo responsável por importantes manifestações clínicas tromboembólicas, incluindo as coronariopatias, claudicações intermitentes dos membros inferiores e acidentes vasculares encefálicos (Badimon et al., 1993; Aboyans et al., 2007; Ouweneel et al., 2015).

1.2. Aterogênese

Na patogenia da doença aterosclerótica há um conjunto de marcadores e fatores emergentes que se inter-relacionam, entre eles, o acúmulo de lipídios circulantes, a produção de espécies reativas, a síntese e a proliferação celular, e a ativação de vias pró-inflamatórias (Hulthe et al., 2002; Gottlieb et al., 2005; Li et al., 2014). Todavia, a aterogênese inicia com a agressão à parede arterial mediada por um ou mais fatores de riscos, o que facilita a penetração (principalmente) das lipoproteínas de baixa densidade (LDL-C) no espaço intimal. De uma forma geral, este processo é dependente de modificações oxidativas que ocorrem nas moléculas de LDL-C (LDL-ox) devido ao contato com espécies reativas de oxigênio e/ou nitrogênio, o que as tornam reativas e imunogênicas (Ross et al., 1999; Siti et al., 2015).

As moléculas de LDL-ox são retidas na camada íntima das artérias através de um processo mediado por citocinas inflamatórias sintetizadas pelo endotélio vascular, incluindo o fator de necrose tumoral (TNF-α), interleucina-1 (IL-1), interleucina-4 (IL-4), interleucina-6 (IL-6) e interferon gama (IFN-γ). Estas citocinas também promovem a expressão de moléculas de adesão leucocitária na superfície

endotelial, principalmente as moléculas de adesão vascular (VCAMs), às moléculas de adesão intercelular (ICAMs) e as E-seletinas, também denominadas moléculas de adesão de fase aguda; estas, por sua vez, são responsáveis pela atração de monócitos e linfócitos para a camada íntima da parede arterial (Blake and Ridker et al., 2001; Nikoforovm et al., 2013; Kirichenko et al., 2015).

Induzidos por proteínas quimiotáticas como a proteína-1 quimiotáxica dos monócitos (MCP-1), fator estimulante de colônia de macrófagos (M-CSF) e a interleucina-8 (IL-8), os monócitos migram para o espaço subendotelial se diferenciam em macrófagos. Após esse processo, os macrófagos reconhecem as moléculas de LDL-ox através de receptores “scavenger” e as fagocitam. Assim, repletos de inclusões lipídicas tornam-se células espumosas, característico das lesões macroscópicas iniciais da aterosclerose (Lind, 2003; Libby et al., 2012).

A placa aterosclerótica cresce lentamente diminuindo o fluxo sanguíneo, e como consequência, pode bloquear completamente a artéria. Esse processo é mediado por citocinas e fatores de crescimento que são secretados pelos macrófagos teciduais, permitindo a evolução da lesão ateromatosa para sua forma madura. Durante a maturação da placa aterosclerótica ocorre a migração de células musculares lisas da camada média das artérias para sua camada íntima, o que induz a produção da matriz extracelular que formará parte da capa fibrosa que reveste a placa aterosclerótica. Sob essa capa fibrosa, encontra-se um centro necrótico constituído por fragmentos celulares e lipídios liberados em consequência da morte das células espumosas (Lusis et al., 2000; Patel et al., 2008).

Além disso, os macrófagos presentes no centro necrótico da lesão secretam metaloproteinases, tais como colagenases e elastases que podem degradar a matriz extracelular da capa fibrosa acarretando a ruptura deste revestimento. Com a

ruptura da capa fibrosa há exposição de um conteúdo altamente trombogênico, iniciando o processo de coagulação, recrutamento de plaquetas e posteriormente formação de um trombo sobrejacente. Este processo também conhecido por aterotrombose é um dos principais determinantes da aterosclerose, podendo conduzir a eventos cardiovasculares agudos importantes como o infarto do miocárdio e o acidente vascular encefálico (Lusis et al., 2000; Schonbeck et al., 2000; Ouweneel et al.,2015).

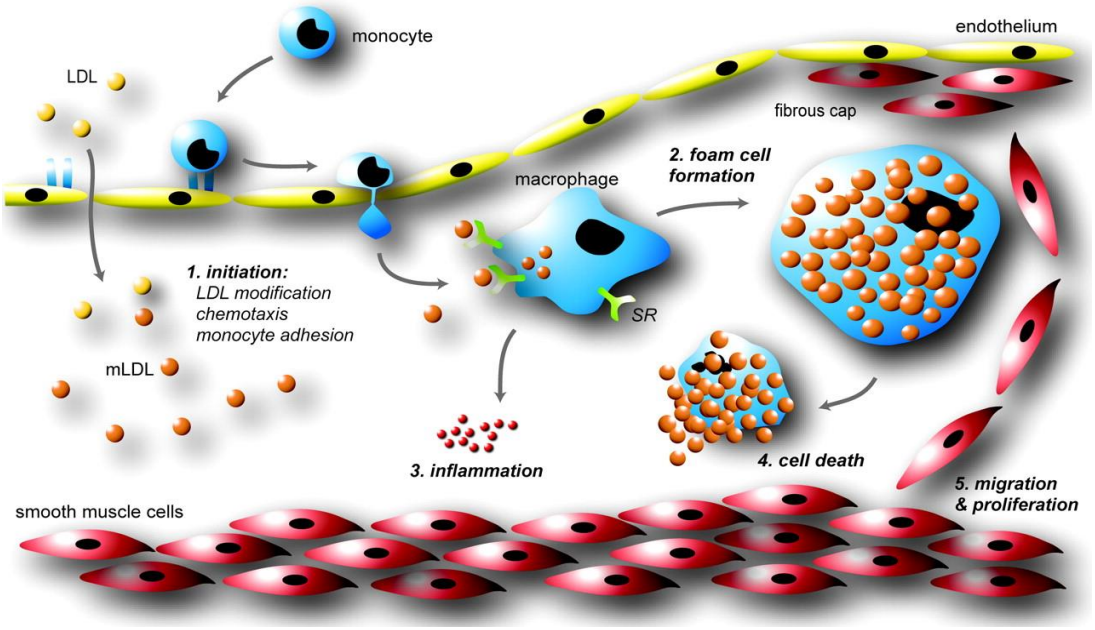

Figura 2. Representação esquemática da patogênese da aterosclerose. Madamanchi et al., 2005

1.3. Participação das espécies reativas de oxigênio (EROs) e nitrogênio (ERNs) na aterogênese

As EROs e ERNs são componentes químicos constituídos principalmente pelos radicais livres, dos quais os elétrons encontram-se desemparelhados, e a produção encontra-se aumentada durante o estresse oxidativo. As principais EROs

são o ânion superóxido ($O_2^{\bullet -}$) e o radical hidroxila (OH•), entretanto, o peróxido hidrogênio (H_2O_2) também aparenta ter um papel proeminente na doença aterosclerótica. Entre as ERNs destaca-se o radical óxido nítrico (NO•) e o peroxinitrito (ONOO-) (Nickening et al., 2002; Lubrano et al., 2015).

A formação de radicais livres constitui um processo regular e fisiológico durante os processos metabólicos. Por exemplo, estes atuam como mediadores endógenos quando em produção adequada, e possibilitam a geração de trifosfato de adenosina (ATP), fertilização do óvulo e ativação de genes. Por outro lado, quando sua produção é exacerbada pode conduzir a danos oxidativos em células, proteínas e lipídeos, o que contribui diretamente com a disfunção endotelial. Esse fator tem sido determinante no desenvolvimento da hipertensão arterial e sobretudo da aterosclerose (Vaziri et al., 2000; Vara et al., 2014; Husain et al., 2015).

Nos últimos anos, vários estudos demonstraram que as EROs e ERNs possuem um papel abrangente no desenvolvimento e progressão da doença aterosclerótica, principalmente devido às modificações oxidativas que ocorrem nas partículas de LDL-C. Além disso, há também importante interação destas espécies com monócitos e macrófagos, além de participarem da proliferação das células musculares lisas (Touyz et al., 2005; Siti et al., 2015).

Várias enzimas oxidantes foram identificadas em lesões ateroscleróticas jovens, incluindo a NADPH oxidase, xantina oxidase, lipoxigenases, mieloperoxidase e óxido nítrico sintase. Essas enzimas presentes em macrófagos e células do endotélio podem produzir uma ampla gama de EROs e ERNs. Alguns estudos têm comprovado que duas fontes particularmente importantes de espécies reativas são a NADPH oxidase e a óxido nítrico sintase (NOS) desacoplada. Ambas têm sido amplamente estudadas no sistema cardiovascular e no processo aterosclerótico,

especialmente por serem importantes fontes de $O_2^{\bullet-}$ (Guzik et al., 2000; Griendling et al., 2003; Li et al., 2014).

Ao longo do processo aterosclerótico, as isoformas mais relevantes do óxido nítrico sintase (NOS) são a endotelial (NOS-3 ou eNOS) e a induzida (NOS-2 ou iNOS). O desacoplamento da eNOS, devido a ausência de L-arginina ou tetrahidrobiopterina (BH4), um importante cofator para produção de NO, promove redução do oxigênio molecular (O_2) a $O_2^{\bullet-}$, ocorrendo alterações simultâneas na produção local desse radical livre e também do óxido nítrico (NO). Essa modificação conduz à formação de peroxinitrito (ONOO-), um eficiente oxidante que contribui diretamente para a disfunção endotelial e consequentemente para a aterogênese (Kawashima e Yokoyama, 2004). Além disso, quando existe um desequilíbrio redox, a NADPH oxidase presente nos vasos sanguíneos e no miocárdio também converte-se em uma fonte substancial de O_2-, promovendo estresse oxidativo sobrejacente a aterosclerose (Griendling et al., 2003; Vara et al., 2014).

De uma forma genérica, em todo sistema biológico há um equilíbrio entre a produção e a neutralização de EROs e ERNs. Esse equilíbrio é mantido por um eficiente sistema de defesa antioxidante, podendo ser dividido em enzimático e não enzimático. O sistema enzimático inclui as enzimas superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidade (GPX). Essas enzimas são extremamente importantes no controle e redução dos compostos oxidantes. Um importante exemplo da integração e eficiência deste sistema é quando a SOD promove a dismutação do O_2- em H_2O_2, o qual pode ser degradado em H_2O pela GPX ou pela enzima catalase (Nojiri et al., 2006; Lubrano et al., 2015).

Ademais, há também o sistema antioxidante não enzimático constituído por grande variedade de substâncias, que podem ser de origem endógena ou dietética.

Exemplos importantes destas substâncias são a glutationa, vitaminas, minerais e diversos compostos polifenólicos. Estudos conduzidos nos últimos anos demonstram que o consumo moderado de compostos polifenólicos podem apresentar vantagens adicionais na prevenção e desenvolvimento da aterosclerose, pois são capazes de melhorar a função endotelial, diminuir a pressão arterial e reduzir a agregação plaquetária (Stoclet et al. 2004; Quiñones et al., 2013).

1.4. Prevenção e tratamento das dislipidemias e aterosclerose

Inicialmente o controle das dislipidemias consistia em terapia nutricional e mudanças no estilo de vida. Atualmente sabe-se que a efetividade deste tipo de intervenção é variável e depende da adesão do paciente a dieta, atividade física regular, além da cessação do tabagismo e etilismo. Quando o tratamento não farmacológico é ineficaz, é necessário que haja uma intervenção medicamentosa. Nas últimas décadas importantes avanços foram obtidos com o desenvolvimento de drogas hipolipemiantes altamente eficazes, incluindo os inibidores da absorção de colesterol (p.ex. ezetimiba), os sequestradores de ácidos biliares (p.ex. colestiramina e colestipol), a niacina e seus derivados (p.ex. nicofuranose e niceritrol), os fibratos (p.ex. clofibrato e ciprofibrato), os inibidores da proteína de transferência de colesterol esterificado (CETP) (p.ex. anacetrapib e torcetrapib), e as estatinas (p.ex. sinvastatina, atorvastatina e rosuvastatina) (Tamargo et al ., 2007; Costet, 2010; Mannu et al., 2013).

Sem sombra de dúvidas, entre todos os hipolipemiantes supracitados, a classe das estatinas é a que apresenta melhores resultados em curto e longo prazo. Essa classe atua através da inibição competitiva da 3-hidroxi-3-metilglutaril coenzima A (HMG-CoA) redutase, enzima que catalisa a conversão do HMG-CoA

redutase em L-mevalonato, substrato para síntese de colesterol. Como consequência, há a indução da expressão de receptores para o colesterol LDL no fígado, o que por sua vez, aumenta o catabolismo do LDL-C e diminui a concentração plasmática de colesterol (Sposito et al., 2002; Silva et al., 2006; Gupta, 2015).

Apesar de vários estudos indicarem as estatinas como a terapia de maior eficácia clínica, grande parte da população mundial tem usado diversos tratamentos alternativos, incluindo as plantas medicinais, como terapia complementar. Isto ocorre devido - em parte - aos efeitos adversos decorrentes desses agentes. Nos últimos anos a adesão ao tratamento prolongado com diferentes estatinas tem diminuindo consideravelmente, devido ao aparecimento de alterações na função hepática, mialgia, e em menor proporção, a rabdomíolise (Silva et al., 2006; Kolovou et al., 2008; Magni et al., 2015).

Neste sentido, investimentos em novos estudos que visem buscar opções terapêuticas para a prevenção e tratamento das dislipidemias, junto às terapias convencionais, passam a ser de grande interesse da comunidade médica e das indústrias farmacêuticas. Assim, uma opção bem aceita provém dos produtos naturais, especialmente aqueles que são habitualmente utilizados pela população e fazem parte do arsenal cultural transmitido por gerações.

1.5. Importância dos compostos polifenólicos

As propriedades bioativas dos compostos polifenólicos são cada vez mais conhecidas. Alguns estudos populacionais sugerem que esses compostos podem ser bastante efetivos na proteção contra o desenvolvimento de várias doenças cardiovasculares, principalmente devido sua capacidade de reduzir o estresse

oxidativo, a disfunção endotelial e a inflamação (Fuhrman et al., 2001; Perez-Vizcaino et al., 2009; Quiñones et al., 2013). Atualmente, sabe-se que os compostos polifenólicos são normalmente sintetizados e armazenados no parênquima de diferentes espécies vegetais pela via do ácido xiquímico a partir de carboidratos ou pela via do acetato-polimalato (acetil-CoA e malonil-CoA). São quimicamente caracterizados por possuírem uma ou mais hidroxilas ligadas a um anel aromático, e seus principais representantes incluem os taninos, as lignanas, os derivados do ácido caféico, e principalmente os flavonóides (Arts e Hollman, 2005).

Os flavonóides são pigmentos de plantas, solúveis em água, que pertencem a um grande grupo de espécies (Geleijnse e Hollman, 2008). Mais de 6.000 diferentes flavonóides são descritos pela literatura e estão presentes em abundância em diversas espécies comestíveis, onde contribuem diretamente com os inúmeros efeitos benéficos dos vegetais, grãos e frutas (Scalbert *et al.*, 2005). Cerca de 80% dos flavonóides incluem as flavonas, os flavonóis e as flavanonas. São exemplos de flavonas a apigenina e a luteolina; dos flavonóis, o caempferol, a quercetina e seu análogo glicosilado isoquercitrina; e das flavononas, a naringenina (Ferro, 2006).

Nos últimos anos, vários estudos têm demonstrado que os flavonóides e seus derivados glicosilados são altamente eficazes em diminuir a produção de pró-oxidantes endógenos como a NADPH oxidase e também reduzir as partículas de LDL-ox, promovendo proteção contra a peroxidação lipídica (De Whaley et al., 1990; Sies et al., 2010; Yang et al., 2011). Além disso, diversos trabalhos recentemente publicados mostraram que compostos ricos em quercetina, isoquercitrina, kaempferol e miricitrina podem exibem várias atividades biológicas relevantes, tais como efeitos antioxidantes (Gasparotto Junior et al., 2012), antihipertensivos (Ojeda et al., 2010; Gasparotto Junior et al., 2011a), diuréticos (Gasparotto Junior 2011b;

2012), antiinflamatório (Gardi et al., 2015), e cardioprotetores (Stoclet et al., 2004).

Os efeitos cardioprotetores por parte destes compostos são atribuídos as suas propriedades fitoquímicas e pleiotrópicas que lhes permitem participar de inúmeras reações biológicas, modulando a atividade de várias enzimas e interferindo na sinalização dos processos celulares. Além disso, também foram observados que diversos compostos polifenólicos, incluindo os flavonóides, promovem aumento na expressão da eNOS, e consequentemente maior biodisponibilidade de NO, condição essa que contribui diretamente para a proteção do endotélio vascular (Ndiaye et al., 2003; Li et al., 2012; Abd-Elbaset et al., 2015).

Quando focamos nossos interesses em produtos naturais ricos em polifenóis destinados ao tratamento da doença aterosclerótica, observamos que apesar da ampla utilização de diferentes compostos no tratamento da dislipidemia, poucas espécies foram criticamente investigadas e validadas clinicamente como agentes hipolipemiantes e antiaterogênicos (Hasani-Ranjbar et al. 2010). Nesse sentido, os dados disponíveis sugerem a necessidade de uma investigação efetiva com o intuito de validar farmacologicamente a utilização desses produtos naturais. Além disso, um importante quesito nesse processo é apreciação da cultura popular como indicador etnobotânico na seleção da espécie a ser estudada e validada clinicamente.

1.6. *Cuphea carthagenensis* (Jacq.) J.F. Macbr.

Figura 3 - *Cuphea carthagenensis* (Foto do Horto de Plantas medicinais da Universidade Paranaense - UNIPAR)

A *Cuphea carthagenensis* (Jacq.) J.F. Macbr. (Lythraceae) é uma planta herbácea, de crescimento espontâneo, que ocorre preferencialmente em locais úmidos e banhados. Conhecida popularmente como sete sangrias, essa espécie é nativa de todo o Sul do Brasil (Lorenzi & Matos 2002). A infusão de *Cuphea carthagenensis* tem sido amplamente utilizada na medicina popular brasileira como diaforética, diurética, laxativa e especialmente no tratamento de hipertensão arterial, aterosclerose e doenças do aparelho circulatório (Vendruscolo and Mentz, 2006; Bolson et al., 2015).

Vários estudos em farmacologia experimental foram conduzidos com a *Cuphea carthagenensis* nos últimos anos. Schuldt et al. (2000) mostraram que o extrato butanólico das partes aéreas desta espécie promove relaxamento da aorta torácica de ratos. Biavatti et al. (2004) observaram uma significativa redução dos níveis séricos de lipídios de ratos normotensos, sugerindo um provável efeito benéfico na prevenção e no tratamento das doenças cardiovasculares. Além disso,

Schuldt et al. (2004) e Prando et al. (2015) constataram uma importante atividade antioxidante de diferentes extratos obtidos desta espécie. Além dos efeitos cardiovasculares supracitados, várias preparações obtidas desta espécie já mostraram atividade antibiótica sobre bactérias gram-positivas e gram-negativas (Duarte et al. 2002) e atividade antiviral sobre o vírus do herpes tipo I (HSV-1) (Andrighetti-Frohner et al. 2005).

Estudos fitoquímicos realizados previamente mostraram a presença abundante de compostos polifenólicos, tais como flavonóides, triterpenos, taninos e proantocianidinas (Gonzalez et al., 1994; Krepsky et al., 2010; 2012). Um estudo realizado recentemente por Prando et al. (2015) mostrou que os principais constituintes identificados no infuso desta espécie são os flavonóides, incluindo a quercetina e seus derivados glicosilados.

De forma contrária aos dados farmacológicos e fitoquímicos, estudos de segurança na utilização de diferentes preparações obtidas de *Cuphea carthagenensis* ainda são bastante restritos. Um estudo publicado por Biavatti et al. (2004) mostrou que a administração de diferentes doses do infuso obtido de *Cuphea carthagenensis* não acarretou quaisquer indícios de toxicidade após 90 dias de tratamento em ratos.

Apesar das informações acerca dos benefícios cardioprotetores desta espécie e seu extenso uso popular, inexistem dados na literatura que mostrem seus benefícios na prevenção e/ou evolução da doença aterosclerótica.

2. OBJETIVOS

2.1 Objetivos Gerais

Avaliar o perfil cardioprotetor de uma fração solúvel em etanol obtida das folhas de *Cuphea carthagenensis* (ES-CC) em coelhos Nova Zelândia submetidos à dieta rica em colesterol.

2.2 Objetivos Específicos

- Obter uma fração solúvel em etanol das folhas de *Cuphea carthagenensis* e investigar em detalhes os metabólitos secundários presentes nesta preparação;
- Identificar a localização e a distribuição anatômica dos compostos fenólicos presentes nas folhas de *Cuphea carthagenensis*;
- Induzir a hiperlipidemia e a aterogênese em coelhos da linhagem Nova Zelândia através da administração de uma dieta rica em colesterol;
- Mensurar a evolução do peso corporal e os níveis de colesterol total, frações HDL, LDL e VLDL, além dos níveis de triglicerídeos durante todo o período experimental;
- Avaliar o índice de aterogênese no arco aórtico, aorta torácica, aorta abdominal e aorta ilíaca após 60 dias de dieta rica em colesterol, bem como as propriedades hipolipemiantes e antiaterogênicas do ES-CC;
- Verificar a peroxidação lipídica induzido pela dieta rica em colesterol e a capacidade antioxidante do ES-CC.
- Investigar a atividade do sistema enzimático antioxidante no fígado dos animais tratados com dieta rica em colesterol, e avaliar as propriedades moduladoras do ES-CC sobre este sistema.

3. ARTIGO CIENTÍFICO

Atheroprotective effects of *Cuphea carthagenensis* (Jacq.) J.F. Macbr. in New Zealand rabbits fed with cholesterol-rich diet

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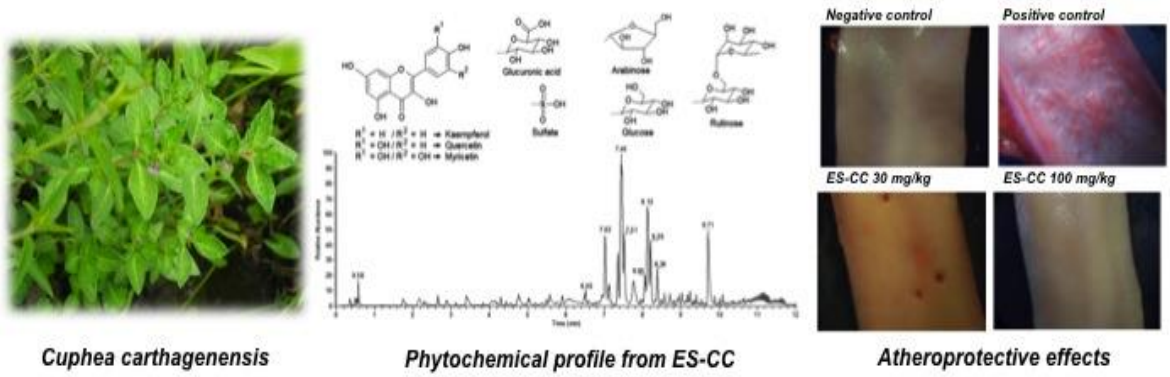
Graphical abstract

Atheroprotective effects of *Cuphea carthagenensis* (Jacq.) J.F. Macbr. In New Zealand rabbits fed with cholesterol-rich diet



Abstract

Ethnopharmacological relevance: Although *Cuphea carthagenensis* (Jacq.) J.F. Macbr. is used in Brazilian folk medicine in the treatment of atherosclerosis and circulatory disorders, no study evaluating these effects has been conducted. The aim of this study was to evaluate the possible hypolipemiant and antiatherogenic activity of the ethanol soluble fraction obtained from *Cuphea carthagenensis* (ES-CC) in an experimental atherosclerosis model using New Zealand (NZ) rabbits undergoing cholesterol-rich diet (CRD).

Material and methods: Dyslipidemia and atherogenesis were induced by administration of standard commercial diet increased of 1% cholesterol (CRD) for 8 weeks. ES-CC was orally administered at doses of 10, 30 and 100 mg/kg, once daily for four weeks, starting from the 4thweek of CRD diet. Body weight measurements were weekly carried out from the beginning of experiments for 8 weeks. Serum levels of triglyceride (TG), total cholesterol (TC) and their fractions (LDL-C, VLDL-C and HDL-C) were measured at the beginning of experiments and at weeks four and eight. After euthanasia of rabbits, aorta segments (aortic arc, thoracic, abdominal and iliac segments) were macroscopically and microscopically evaluated and the intima and media layers of the arteries were measured. Additionally, the antioxidant activity of ES-CC and its influence on the functioning of hepatic antioxidant enzymes were also determined.

Results: CRD induced dyslipidemia and major structural changes in the aortic wall. In addition, an increase in lipid peroxidation and a reduction of hepatic glutathione and serum nitrite levels were observed. Treatment with ES-CC was able to prevent the increase in TC, LDL-C, VLDL-C levels and triglycerides and promoted an increase in HDL-C levels in NZ rabbits. These effects were accompanied by a significant reduction in oxidative stress and modulation of the catalase and superoxide dismutase function. Moreover, the intima and media layers of the arterial segments were significantly reduced by ES-CC treatment.

Conclusions: This study demonstrated that ES-CC reduces serum lipids and hepatic oxidative stress when orally administered to NZ rabbits. In addition, it was able to prevent the development of CRD-induced atherosclerosis.

Keywords: *Cuphea carthagenensis;* lipid-lowering; anti-atherogenic; antioxidant

3.1 Introduction

The relationship between dyslipidemia and cardiovascular diseases has been well documented, especially for being one of the main causes of mortality in Western countries. Plasma lipoproteins such as very low density lipoprotein (VLDL-C) and low density lipoprotein cholesterol (LDL-C) influence three important aspects of atherosclerosis and atherothrombosis: endothelial function, platelet aggregation (primary coagulation) and secondary coagulation, leading to acute cardiovascular events such as myocardial infarction and stroke (Ouweneel and Van Eck, 2015).

Oxidative modifications that occur in LDL (oxLDL) particles are considered an essential process in the activation of the inflammatory pathway, leading to the formation of atheromatous plaque in the tunica intima of arteries. Atherogenesis starts up through the endothelial dysfunction due to oxidative stress induced by reactive oxygen species (ROS) and enzymes released during the inflammatory process. Therefore, this condition is directly related to risk factors, including diabetes, hypertension, smoking, obesity, and metabolic syndrome (Siti et al., 2015).

Considering the great impact of atherosclerosis on health services around the world, there has been a great interest in investigating the efficacy and safety of lipid-lowering and anti-atherogenic drugs based on the popular uses of natural products in recent decades (Bahmani et al., 2015; Salvamani et al., 2014; Hasani-Ranjbar et al., 2010). So, the idea would be not only to expand the therapeutic arsenal, but also to appreciate the local culture and give scientific support to phytomedicines used for hundreds of years. In recent years, *Cuphea carthagenensis* (Jacq.) J.F. Macbr. (Lythraceae), an important medicinal species native to the Southern States of Brazil and popularly known as “sete sangrias”, has attracted the attention of the scientific community for its expressive popular use in the treatment of hypertension,

atherosclerosis and circulatory disorders (Vendruscolo and Mentz, 2006; Bolson et al., 2015). The beneficial effects of *Cuphea carthagenensis* have been attributed to its ability to cause arterial vasodilation (Schuldt et al., 2000; Krepsky et al., 2012), decrease serum lipids (Biavatti et al, 2004), and reduce oxidative stress in rats (Prando et al., 2015; Schuldt et al., 2004). These effects can be, at least in part, due to the wide variety of *Cuphea carthagenensis* compounds, in which flavonoids including quercetin and their glycosylated derivatives are the most important (Krepsky et al., 2010; Prando et al., 2015). Moreover, other constituents of this plant such as triterpenes (Gonzalez et al., 1994), tannins, and proanthocyanidins have been detected (Krepsky et al., 2010; 2012).

Despite the widespread popular use of this plant species and studies indicating possible cardiovascular benefits, there are no data to prove its action in the prevention and treatment of the evolution of atherosclerotic diseases. The aim of the present study was to investigate the atheroprotective effects of an ethanol soluble fraction obtained from *Cuphea carthagenensis* (ES-CC) on New Zealand rabbits submitted to cholesterol-rich diet (CRD). First, we investigated in detail which secondary metabolites present in ES-CC could contribute to the possible cardioprotective effects of this species. In addition, we sought to identify the anatomical distribution of these compounds from *Cuphea carthagenensis* leaves using a botanical micro-chemical technique. Then, the lipid-lowering and anti-atherogenic properties of this extract were evaluated in different arterial branches from hypercholesterolemic rabbits. Finally, it was verified whether the reduction of ES-CC-induced oxidative stress could contribute to the cardioprotective effects in this animal model.

3.2 Material and methods

3.2.1. Drugs and chemicals

Cholesterol, simvastatin (SIMV) and standards of glycosides quercetin-3-*O*-β-glucopyranoside, quercetin-3-*O*-β-galactopyranoside, rutin [α-rhamnopyranosyl-(1→6)-β-glucopyranosyl-(1→3)-quercetin] and monosaccharides glucuronic acid, galacturonic acid, glucose, arabinose, xylose, rhamnose were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade solvents acetonitrile, methanol, *n*-propanol, ethyl acetate were purchased from J. T. Baker (Center Valley, PA, USA). All other reagents and/or solutions were obtained in analytical grade.

3.2.2. Plant material and preparation of Cuphea carthagenensis infusion

Cuphea carthagenensis leaves were collected on March 2014 from the botanical garden of the Paranaense University (UNIPAR) (Umuarama, Brazil) at 430 m of altitude above sea level (S23º47'55–W53º18'48). A voucher specimen of this species is cataloged at the Herbarium of the Paranaense University (HEUP) under number 2401.

The plant material was air-dried in an oven at 37 °C for 5 days and then cut and pulverized. Extracts were obtained by infusion in a similar manner to that popularly used in Brazil (Bolson et al., 2015). About 1L of boiling water was poured over 60 g of dried ground leaves; the container was sealed and the extraction was allowed to proceed until room temperature was reached (~ 6 h). The infusion was treated with 3 volumes of EtOH, which produced a precipitate and an ethanol soluble fraction (ES-CC; yield 10.34%). All preparations were freeze-dried and maintained at room temperature until

3.2.3. Phytochemical investigation

2.2.4. Liquid chromatography-mass spectrometry analysis

Cuphea carthagenensis sample was investigated for its phytochemical composition by liquid chromatography-mass spectrometry (LC-MS), using Acquity^{TM} ultra performance liquid chromatography (UPLC – Waters) coupled to a high-resolution mass spectrometry (HR-MS). The separation was developed with a C18 column, HSS T3 (Waters) of 100 x 2.1 mm with particle of 1.7 µm, using ultra-pure water (type 1) and acetonitrile, both containing 0.1 % formic acid (v/v). Temperature was 60^{o}C and the gradient was the increase in the organic solvent content as follows: initial 0%, then 30% (7 min), 60% (12 min), 0% (15 min). Additional 2 min were allowed for system re-equilibration. Detection was provided by photodiode array detector (PDA) at 200-400 nm and HR-MS at 100 - 2000 *m/z*. The sample was prepared at 2 mg/mL in ultra-pure water and the injection volume was 5 µL.

Mass spectrometry was developed in LTQ-Orbitrap XL (Thermo Scientific), with electrospray ionization in the positive and negative modes using nitrogen at flow rate of 60 arbitrary units (a.u.) in the sheath gas and 20 a.u. in the auxiliary gas with source temperature of 350^{o}C. Energies for positive ionization were: spray at 4 kV, tube lens at 80 V and capillary at 30 V and, for negative ionization: spray at 3.5 kV, tube lens at -110 V and capillary at -20 V. Mass resolution was set at 15000 FWHM (at 400 *m/z*) in LC-MS mode and mass accuracy was obtained by external calibration (100 - 2000 *m/z*). Data acquisition was obtained in total ion current (TIC) and the compound identification was accompanied by fragmentation obtained by collision-induced dissociation (CID) using helium and energy at 20 eV.

2.2.5. Thin layer chromatography (TLC): monosaccharide analysis

TLC analysis was used to investigate the monosaccharide composition of glycosides. About 5 mg of sample was fractionated by liquid/liquid partition in 2 mL of water/*n*-butanol (1:1, v/v) in order to isolate flavonol glycosides from free glycans. The organic layer was removed, dried in nitrogen stream, and then hydrolyzed in 1M trifluoroacetic acid, 100 ^{o}C for 8 h. The hydrolyzate was fractionated with *n*-butanol to remove aglycones and the aqueous layer was removed and dried under nitrogen stream and resuspended in 200 µL of water, used for TLC analysis.

TLC was developed in 7 cm long silica-gel plates (Merck). Sample and standards were applied on plate and developed with ethyl acetate, *n*-propanol, acetic acid and water (4:2:2:1, v/v). After chromatography, the plate was dried at 100^{o}C and stained by orcinol-H_2SO_4 reagent, at 100^{o}C (Sassaki et al., 2008).

2.2.6. Microchemical analyses

For the microchemical analysis, free-hand cross-sections and longitudinal sections were prepared with fresh *Cuphea carthagenensis* leaves. The Hoepfner-Vorsatz test modified by Reeve (1951) (aqueous 10% sodium nitrate, aqueous 10% acetic acid, aqueous 10% urea and, 2 N NaOH) was used to stain phenolic compounds. Hoepfner-Vorsatz test reagents first form a nitrous derivative of the phenolic compound, and then a colored salt with the addition of base.

2.2.7. Pharmacological studies

2.2.8. Animals

Male New Zealand rabbits weighing 1.8 – 2.0 kg were used throughout the study. Animals received food and water *ad libitum*, being obtained from the State

University of Maringá (UEM) and housed at the Paranaense University (UNIPAR) under controlled ambient temperature (20 ± 2°C), relative humidity (50 ± 10%) and 12 h light/12 h dark cycle (lights on 07:00 h). All experimental procedures performed in this study have been carried out in accordance with Guidelines for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institute of Health and previously approved by the Institutional Ethics Research Committee of the Paranaense University (UNIPAR, Brazil; authorization number 25454/2014).

2.2.9. Experimental groups and induction of hyperlipidemia and atherogenesis

Dyslipidemia and atherogenesis were induced by administration of standard commercial diet (Nutricoelho, Purina®) supplemented with 1% cholesterol (cholesterol-rich diet – CRD) for 8 weeks. CRD was offered *ad libitum* to groups of rabbits from 6 months of age. The animals were used in the study after confirmation of hypercholesterolemia. Four weeks after starting the diet, the animals were randomly assigned to one of five experimental groups (n=5-6) as follows:

1) Negative control: New Zealand rabbits receiving standard commercial diet and treated for 4 weeks with vehicle;

2) Positive Control: New Zealand rabbits receiving CRD and treated for 4 weeks with vehicle;

3) ES-CC 10: New Zealand rabbits receiving CRD and treated for 4 weeks with ES-CC obtained from *Cuphea carthagenensis* at dose of 10 mg/kg;

4) ES-CC 30: New Zealand rabbits receiving CRD and treated for 4 weeks with ES-CC obtained from *Cuphea carthagenensis* at dose of 30 mg/kg;

5) ES-CC 100: New Zealand rabbits receiving CRD and treated for 4 weeks with ES-CC obtained from *Cuphea carthagenensis* at dose of 100 mg/kg;

6) SIMV 5: New Zealand rabbits receiving CRD and treated for 4 weeks with simvastatin at dose of 5 mg/kg.

ES-CC was orally administered once daily for four weeks, starting from the 4th week of treatment with CRD. All parameters described below were measured during and/or after the treatment period.

2.2.10. Measurement of body weight and serum lipid profile

Body weight measurements were carried out using analytical scale weekly from the beginning of experiments until 8 weeks. The levels of triglyceride (TG), total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) were measured using biochemical test kits with semi auto analyzer by colorimetric method at the beginning of experiments and at weeks four and eight. Serum levels of low-density lipoprotein cholesterol (LDL-C) and very low-density lipoprotein cholesterol (VLDL-C) were calculated using the formula of Friedewald et al. (1972).

2.2.11. Processing of arterial branches and pathological analyses

2.2.12. Macroscopic analysis

Aorta segments (aortic arc, thoracic, abdominal and iliac segments) were removed from each animal after euthanasia and placed in 10% buffered formalin. After 48 hours of fixation, each aorta was longitudinally opened, rinsed in 70% alcohol and immersed in Herxheimer's solution [containing Sudan-IV (5g), 70% ethyl alcohol (500 mL) and acetone (500 mL) at room temperature for 15 minutes. Tissues were transferred to 80% ethanol for 20 minutes and washed in tap water for 1 hour to remove excess staining. Luminal surface was then assessed for sudanophilic lesions. Image data acquisition and analysis were carried out with Motic Images Plus

software version 2.0.

2.2.13. Histopathological evaluation

After euthanasia of rabbits, aorta segments (aortic arc, thoracic, abdominal and iliac segments) were gently taken, washed with ice-cold sterile physiological saline and immediately stored in neutral buffered 10% formalin. Tissue samples were dehydrated in alcohol, cleared with xylene and embedded in paraffin. Samples were sectioned (5 mm), stained with haematoxylin and eosin and examined under light microscope. The intima and media layers were measured. Image data acquisition and analysis were carried out with Motic Images Plus software version 2.0.

2.2.14. Evaluation of the antioxidant system under the atheroprotective effects of ES-CC

2.2.15. Evaluation of serum lipid peroxidation (TBARS)

Thiobarbituric acid levels (TBARS) were measured using commercial assay kits (Nanjing Jiancheng Institute, Nanjing, China) on a spectrophotometer (DU7400, Beckman Co., Fullerton, CA, USA), according to manufacturer's instruction. This method was used to obtain the spectrophotometric measurement of the color produced during the reaction of thiobarbituric acid with malondialdehyde (indicator of peroxidation of polyunsaturated fatty acids in cell membranes subsequent to reactions with reactive oxygen species) at 535 nm. The TBARS level was expressed as mmol/L.

2.2.16. Serum nitrate/nitrite (NOx) determination

Plasma nitrite concentration was enzymatically determined by reducing nitrate using nitrate reductase enzyme (Schmidt et al., 1989). Plasma samples obtained from NZ rabbits were deproteinized with zinc sulfate (30 mmol) and diluted at 1:1 with Milli-Q water. For the conversion of nitrate into nitrite, samples were incubated at 37°C for 2 hours in the presence of nitrate reductase expressed in *E. coli*. After the incubation period, samples were centrifuged (800 *g*, 10 minutes) to remove bacteria. Then, 100 µL of supernatant were mixed with an equal volume of Griess reagent (1% sulfanilamide in 10% phosphoric acid/0.1% alpha-naphthyl-ethylenediamine in Milli-Q water) in a 96-well plate and read at 540 nm in a plate reader. Standard nitrite and nitrate curves (0-150 mM) were simultaneously performed.

2.2.17. Preparation of liver homogenate

Hepatic samples were homogenized in potassium phosphate buffer (0.1 M, pH 6.5), in a 1:10 dilution. Part of the homogenate was used to determine GSH levels and part was centrifuged at 9000 × g for 20 min. The supernatant was used to evaluate glutathione-S-transferase (GST), superoxide dismutase (SOD), and catalase (CAT) activities.

2.2.18. Determination of reduced glutathione (GSH) levels

To determine reduced glutathione levels, 100 µL of homogenate was mixed with 80 µL of 12.5% trichloroacetic acid and centrifuged at 6000 rpm, during 15 min, at 4°C. Then, 20 µL of supernatant was added to 280 µL of TRIS buffer (0.4 M, pH 8.9) and 5 µL of DTNB (0.01 M), as described by Sedlak and Lindsay (1968), with

minor modifications. The results were interpolated into a standard GSH curve (0.375 – 3 µg) and expressed as µg/g of tissue.

2.2.19. Investigation of glutathione S-transferase (GST) activity

GST activity was measured using method described by Habig et al. (1974). Supernatant samples obtained from liver homogenates were diluted at 1:80 in potassium phosphate buffer (0.1 M, pH 6.5). Reactions were performed in the presence of 100 µL of diluted supernatant and 200 µL of reagent solution [CDNB (3 mM), GSH (3 mM), and potassium phosphate buffer (0.1 M, pH 6.5)] at room temperature. The conjugation of CDNB with GSH was monitored at 340 nm for 180 s. Specific activity was calculated using an extinction coefficient of $9.6 \cdot mM^{-1} \cdot cm^{-1}$ for GSH, and the results were expressed as $mmol \cdot min^{-1} \cdot mg\ of\ protein^{-1}$.

2.2.20. Determination of superoxide dismutase (SOD) activity

SOD activity was measured through the ability of SOD to inhibit pyrogallol autoxidation, according to Gao et al. (1998). Supernatant from hepatic homogenate was diluted at 1:10 in potassium phosphate buffer (0.1 M, pH 6.5). About 60 µL of dilution was added to 1327.5 µL of Tris EDTA buffer solution (0.4 M, pH 8), and mixed with 75 µL of pyrogallol solution (15 mM). The reaction was incubated for 30 min at room temperature and stopped with the addition of 37.5 µL of 1N HCl. The absorbance of the resulting supernatant was measured at 405 nm. The amount of SOD that inhibited pyrogallol oxidation by 50% (relative to the control) was defined as one SOD unit and the enzymatic SOD activity was expressed as $U\ SOD \cdot mg\ of\ protein^{-1}$.

2.2.21. Determination of catalase (CAT) activity

CAT activity was evaluated using technique proposed by Aebi (1984). Briefly, 5 µL of 1:10 supernatant dilution (in 0.1 M potassium phosphate buffer, pH 6.5) were mixed with hydrogen peroxide solution (Tris EDTA buffer, pH 8.0; ultrapure water; and 30% hydrogen peroxide) and read at 240nm. Results were expressed as mmol/min/mg of protein.

2.2.22. Statistical analyses

Results were expressed as mean ± standard error mean (S.E.M.) of 5-6 animals per group. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Bonferroni or Student's*t*-test, when applicable. *P*-values less than 0.05 were considered statistically significant. Graphs were drawn and statistical analysis was carried out using GraphPad Prism software version 5.0 for Mac OS X (GraphPad Software, San Diego, CA, USA).

3.3 Results

3.3.1. Phytochemical investigation

3.3.2. Chromatography and spectrometry analysis

Quercetin-3-sulfate is the main compound reported in the aerial parts of *C.carthagenensis*, followed by other flavonol glycosides (Krepsky et al., 2012). However, in our previous investigation on the phytochemical composition of *C. carthagenensis*, high amounts of flavonol glycosides were observed (Prando et al., 2015). In the present investigation, aerial parts of *C.carthagenensis* were extracted by infusion and after LC-MS analysis, a variety of flavonol glycosides were identified. These flavonols were quercetin or kaempferol, with lower amounts of myricetin

attached by mono- or disaccharides composed of arabinose, glucose, rhamnose and glucuronic acid, as observed on TLC analysis (data not shown).

Flavonol-glycosides gave characteristic UV-spectra, with two absorbance regions (λ_{max}), at ~250-260 nm (Band B) and at ~350-370 nm (Band A). Several peaks have appeared on the chromatogram with similar UV-spectra, being further identified according to their MS^1 and MS^2 profile, from protonated and deprotonated ions (Souza et al., 2008; Souza et al., 2009) (Figure 1). A low abundant peak was found at 6.52 min, with 479.08310 *m/z* $[M-H]^-$ and a main radical fragment at 316.02226 *m/z* consistent with myricetin-glucoside. Quercetin-hexoside at 463.08865 *m/z* $[M-H]^-$ and 465.10284 $[M+H]^+$ (Rt 7.03) was found with Rt different from that of standard quercetin-3-*O*-β-glucopyranoside. Characteristically, flavonol-3-*O*-glycosides produce a pronounced radical ion provided by homolytic cleavage between aglycone and glycan moiety (Souza et al., 2008). However, peak at 7.03 min produced a main fragment at 301.03542 *m/z*, a regular ion (from heterolytic cleavage) being consistent with quercetin-5-*O*-β-glucopyranoside, as previously described (Krepsky et al., 2012). Rutin was not observed in the previous investigation, however, it was present the extract, giving rise the ions at 609.14631 *m/z* $[M-H]^-$ and 611.16068 $[M+H]^+$ (Rt 7.37), as observed in the standard.

The main compound/peak found in the extract from *C. carthagenensis* was found at 7.45 min, giving the ion at 477.06742 *m/z*. This compound has been reported in our previous work (Prando et al. 2015), but it was not properly identified. A main fragment produced appeared at 301.03533 *m/z*, being consistent with the regular quercetin ion. The neutral loss observed was 176.03209, exactly the expected from the loss of an uronic acid residue. Furthermore, the TLC analysis

confirmed the presence of glucuronic acid, therefore the peak at 7.45 min was identified as quercetin-glucoronide.

The peak at 7.53 min gave the ion with 463.08854 *m/z*, with a main fragment at 300.02742 *m/z* (radical ion from quercetin), being confirmed as quercetin-3-*O*-β-glucopyranoside, being supported by the authentic standard. Quercetin-3-sulfate was also found, but at lower abundance than values previously reported (Krepsky et al., 2012). It appeared at 7.74 min with ion at *m/z* 380.99239 with fragment at *m/z* 301.03545. Quercetin-pentoside found by Krepsky and coworkers was identified as quecertin-3-*O*-arabinofuranoside. A peak consistent with this structure was found at 8.14 min, yielding ion at *m/z* 433.07725 and fragment at *m/z* 300.02754. Kaempferol-diglycoside also appeared at *m/z* 593.15150 (Rt 8.22), being consistent with kaempferol-rutinoside. Kaempferol-glucoside has appeared at 8.39 min with *m/z* 447.09368 and fragment at *m/z* 284.03263. Free quercetin was also found in the present extract, appearing at 9.71 min, with *m/z* 301.03560, confirmed with authentic standard. This phytochemical composition is summarized in Table 1.

3.3.3. Microchemical analyses

Figure 2 shows the results of the microchemical test performed with fresh *Cuphea carthagenensis* leaves. The color reaction produced showed the massive presence of phenolic compounds. From a frontal view, the presence of phenolic compounds was observed in the epidermis of the leaf blade (Figure 2B), but more frequently on midrib (Figure 2A). In cross- section, the leaf showed phenolic compounds in the mesophyll and in the midrib. In the mesophyll, these compounds were more distributed in the palisade parenchyma (Figure 2C). The midrib showed few cells with phenolic compounds spread in the ground parenchyma. In cross-

section, the petiole evidenced the presence of phenolic compounds (Figure 2D) in both epidermis (more evidenced in the adaxial side) (Figure 2E), and dispersed in the vascular bundle (more evidenced in the xylem) or near it in the ground parenchyma (Figures 2D and 2F).

3.3.4. Pharmacological studies

3.3.5. ES-CC induces lipid-lowering effects on New Zealand rabbits

The effects of the oral administration of ES-CC obtained from *Cuphea carthagenensis* and simvastatin (SIMV) on the serum lipid levels of New Zealand rabbits are showed in Table 2. Total serum cholesterol of negative controls (baseline 54 ± 4.9 mg/dL) was increased to 237 ± 34 mg/dL in positive controls (CRD) after 60 days. At the same time, the SIMV values in treated rabbits were significantly lowered to 146 ± 28 mg/dL when compared to positive controls ($p<0.05$). Similarly, significant reduction in the TC levels was also evident in CRD rabbits treated with ES-CC from *Cuphea carthagenensis*. In animals that received ES-CC (30 and 100 mg/kg), CT levels were reduced to 164±25 and 155 ±30 mg/dL, respectively ($p<0.05$). Likewise, rabbits treated with ES-CC presented an expressive reduction in serum LDL-C at doses of 30 and 100 mg/kg (122 ± 15 and 117 ± 17 mg/dL, respectively; positive control: 185 ±20 mg/dL; $p<0.05$). In addition, the VLDL-C values were similarly reduced in animals treated with ES-CC (30 and 100 mg/kg) with reduction estimated in 30%. Moreover, a significant increase in the HDL-C levels was evident in CRD rabbits that received ES-CC (30 and 100 mg/kg). In this case, the HDL-C levels increased to 8.2 ± 0.2 and 8.6 ± 0.4 mg/dL, respectively (positive control: 7.2 ± 0.3 mg/dL; $p<0.05$). Moreover, the oral administration of ES-CC (30 and 100 mg/kg) resulted in significant reduction in triglyceride levels when compared to positive control group (positive

control: 190 ± 28; ES-CC 30: 140 ± 31; ES-CC100: 147± 25 mg/dL; $p<0.05$). In addition, the triglyceride levels measured in groups treated with ES-CC were close to those found in the SIMV group (135 ± 36 mg/dL). The body weight of animals fed with CRD and treated with ES-CC or SIMV showed no significant changes when compared with positive or negative control groups (data no shown).

3.3.6. ES-CC induces antiatherogenic effects on aorta segments obtained from New Zealand rabbits

3.3.7. Macroscopic evaluation

Sudan-positive lesions, indicative of stained lipids, were found in all aorta branches, but not in a similar manner along segments under study. The intensity of lesions was more expressive in the thoracic segment (TS), and was used as an index of disease severity (Figure 3).Sudan-positive red lesions (average area) in TS of negative controls (~100 Sq μm) were increased to 53148 ± 6226Sqμm in positive controls. On the other hand, the area for rabbits treated with SIMV was significantly lowered to 1602 ± 810Sq μm when compared to positive controls ($p<0.05$). Similarly, a significant reduction in Sudan-positive lesions was also evident in CRD rabbits treated with ES-CC from *Cuphea carthagenensis*. In animals that received ES-CC (10, 30 and 100 mg/kg), Sudan-positive lesions were reduced to 30266 ± 2795, 19672 ± 4917, and 2020 ± 990Sq μm, respectively ($p<0.05$).

3.3.8. Histomorphometric analysis

Morphometric measures of the intima and media layer of aorta segments (aortic arch [AA], thoracic [TS], abdominal [AS] and iliac [IS] segments) are showed in Figures 4 and 5. Histopathological examination of all aorta segments showed an

important thickness in intima layer after 8 weeks of CRD (AA: 137 ± 26 μm; TS: 98 ± 8 μm; AS: 115 ± 12 μm; IS: 74 ± 3 μm) when compared to negative controls (AA: 68 ± 5 μm; TS: 57 ± 4 μm; AS: 43 ± 2 μm; IS: 35 ± 2 μm; $p<0.05$). Treatment with ES-CC (30 and 100 mg/kg) was able to prevent thickening, with values close to groups that received standard commercial diet (Figures 4A and F, and Figure 5A and F). Similarly, New Zealand rabbits treated with SIMV also showed intima layer values close to those obtained for negative controls, preventing the increase of thickening observed in positive controls.

Similarly, the media layer of the aortic arch and abdominal and iliac segments showed significant thickening in positive controls after 8 weeks of CRD (AA: 1948 ± 277 μm; AS: 746 ± 46 μm; IS: 648 ± 31 μm) when compared to negative controls (AA: 1251 ± 122 μm; AS: 456 ± 44 μm; IS: 488 ± 21 μm; $p<0.05$) (Figures 4B and G, and Figure 5B and G). On the other hand, treatments with ES-CC or SIMV were able to prevent thickening in the media layer only in the aortic arch ($p<0.05$). The values found at dose of 30 and 100 mg/kg were 1269 ± 35 and 1263 ± 100 μm (close to those found in SIMV or negative control) (Figure 4G).

3.3.9. Atheroprotective effects of ES-CC on New Zealand rabbits may involve the antioxidant system

3.3.10. Treatment with ES-CC increases NO bioavailability, reduces lipid peroxidation, and increases glutathione levels

The effects of the oral administration of ES-CC obtained from *Cuphea carthagenensis* and SIMV on nitrite, T-BARS and glutathione levels of New Zealand rabbits are showed in Figure 6 (A-C). Treatment with CRD for 8 weeks was able to elevate serum T-BARS levels and significantly reduce hepatic glutathione and nitrite

levels. Treatment with ES-CC (100 mg/kg) was able to reduce TBARS levels in approximately 27% in CRD rabbits. Additionally, serum nitrite levels increased from 21 ± 1.9 to 50 ± 3.1 μM after treatment with ES-CC, while hepatic glutathione levels increased by approximately 42%.

3.3.11. Treatment with ES-CC regulates the activity of antioxidant enzymes in rabbits with experimental atherosclerosis

The effects of the oral administration of ES-CC obtained from *Cuphea carthagenensis* and SIMV on the hepatic CAT and SOD activity of New Zealand rabbits are showed in Figure 6 (D-F). The CAT and SOD activity of negative controls (baseline 186 ± 2.0and 37 ± 2.1 μmol/min/mg, respectively) was changed to 240 ± 7.7 and 29 ± 0.8 μmol/min/mg in positive controls (CRD) after 60 days. Four weeks after treatment, ES-CC (100 mg/kg) significantly reduced the CAT (172 ± 4.7 μmol/min/mg) and increased the SOD (35 ± 1.2 μmol/min/mg) activities in liver tissues ($p<0.05$). On the other hand, four weeks after treatment with ES-CC, no significant change was observed in the GST activity.

4. Discussion

Atherogenesis is characterized by a remodeling of blood vessels due to the accumulation of lipid, inflammatory cells and fibrous elements, leading to the formation of fatty streaks and atherosclerotic plaques (Ouweneel and Van Eck, 2015). Arteries of medium and large caliber such as aorta, coronaries, and cerebral arteries are more affected than micro-arterioles, leading to acute cardiovascular events such as heart attack, stroke and renal failure (Gottlieb et al., 2005). In recent years, due to the high impact of this disease on health, several animal models have

become relevant tools for studies on dyslipidemia in humans, sharing important features of lipid metabolism and pathophysiology of atherosclerosis. Currently, lagomorphs are considered as the main pre-clinical model for studies on lipoprotein metabolism and consequent comorbidities. Rabbits and humans share lipid features that are not observed in rodents, including LDL-C particle in greater abundance in plasma, activity of hepatic LDL-C receptor, presence of VLDL-C receptor on macrophages, cholesterol ester transferase protein (CETP), HDL-C heterogeneous particle, and sensitivity to dietary cholesterol. In most cases, this model is based on dietary-induced hypercholesterolemia and precursor lesions that lead to the development of atherosclerosis, which can be viewed after one week of exposure (Fan et al. 2015).

Classically, it is known that CRD raises plasma levels of lipoproteins, primarily LDL-C and VLDL-C, which are considered one of the main risk factors for the development of dyslipidemia and atherosclerosis. CRD increases oxidative stress, induces endothelial dysfunction, contribute to the contraction of smooth muscle, and also increases arterial blood pressure through a process that involves intracellular and extracellular Ca(2+) mobilization (Wilde et al., 2000; Kruth, 2001). In our study, New Zealand rabbits fed with CRD for 8 weeks presented significant increase in serum lipoproteins, including TC, LDL-C and VLDL-C. Similarly, significant increase in oxidative stress and important alterations in the function of antioxidants enzymes such as SOD and CAT were observed. Furthermore, these alterations were accompanied by significant atherosclerotic lesions in different branches of the aorta, including a high number of lesions in the aortic arch and thoracic segment, in part due to shear stress caused by deformation of the vascular structure (points of branches and bends) in these regions (Li et al., 2014).

When we consider the ethnopharmacological research proposed in this work, relevant results were obtained. It was shown that ES-CC obtained from *Cuphea carthagenensis* was able to reduce triglyceride, TC, LDL-C and VLDL-C levels and increase HDL-C levels after 4 weeks of treatment. In addition, it was also observed that ES-CC administration was able to cause a significant antiatherogenic effect, especially at dose of 100 mg/kg, reducing the number of macroscopic lesions in the thoracic segment of the aorta as well as the thickness of the tunica intima in all arterial segments evaluated.

In these important results, we investigated which molecular mechanisms could be influencing the lipid-lowering activity and antiatherogenic effects of ES-CC. Literature data suggest that the oxidative changes that occur in LDL-C particles (oxLDL) form a starting point in endothelial dysfunction that leads to atherosclerosis. In fact, oxLDL has high ability to bind to the extracellular matrix, thereby increasing their retention within the intima of arteries, promoting functional changes in endothelial cells, lymphocytes, macrophages (forming foam cells), and smooth muscle cells (Nicolletti et al., 2000; Griendling et al., 2003). The activation of these cells triggers the interaction of a broad spectrum of cytokines, adhesion molecules, growth factors, and cell proliferation, contributing to the formation of atherosclerotic lesions. Furthermore, local inflammatory response can also be directly induced by oxidative imbalance (Siti et al., 2015). In this context, several enzymes have been identified in atherosclerotic lesions, playing a prominent role in LDL-C oxidation. These include nicotinamide adenine dinucleotide phosphate (NADP), xanthine oxidase, 12/15-lipoxygenase, NO synthase and myeloperoxidase. These enzymes present in macrophages and endothelial cells produce reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS includes superoxide anion (O_2^-),

hydroxyl radical (OH), and hydrogen peroxide (H_2O_2); and key representatives of the RNS include nitric oxide (NO) and peroxynitrite ($ONOO^-$) (Selemidis et al., 2008).

In biological systems, there is a balance between ROS production and neutralization. This balance is maintained by the presence of natural antioxidants and antioxidant enzymes such as SOD, CAT and GST. These enzymes play a central role in the degradation of O_2^- and H_2O_2, and a change in the expression of these molecules may directly influences the onset and evolution of the atherosclerotic process (Siti et al., 2015). In addition, another enzyme that deserves attention in this process is the endothelial nitric oxide synthase (eNOS). This enzyme, which produces NO, is a key element in the protective function of the endothelium. In contrast to these regulatory functions under physiological conditions, disturbances in the redox system can result in the production of peroxides and free radicals that damage all cell components, including proteins and lipids. These alterations significantly contribute to endothelial dysfunction, mainly due to rapid inactivation of NO by excess free radicals. In the second step, eNOS is uncoupling in such a way that it is unable to produce NO (Wohlfart et al., 2008; Li et al., 2014).

In our study, it was observed that animals fed with CRD for 8 weeks showed a significant increase in lipid peroxidation, accompanied by a significant reduction in serum nitrite and glutathione levels. Moreover, a significant increase in hepatic CAT activity accompanied by a reduction in SOD activity was also observed. Recent clinical data have shown that in the early stages of the atherosclerotic disease, there is a homeostatic up-regulation of antioxidant enzyme systems in response to increased free radicals to prevent vascular damage. As soon as free radicals achieve chronically elevated levels, this compensation ceases (Lubrano and Balzan, 2015). It is probable that a few weeks after the starting of CRD, the animals had presented a

significant increase in SOD activity in order to reduce aggression induced mainly by O_2^-; and after an extended period (8 weeks), these values presented a regulation decrease. On the other hand, the fact of CAT activity remains high may be due to a direct and significant involvement of H_2O_2 during the chronicity of the atherosclerotic process (Vara and Pula, 2014). In this sense, ES-CC administration (100 mg/kg) was able to alleviate oxidative damage induced by CRD, increasing the bioavailability of nitric oxide and hepatic GSH levels. Moreover, the CAT and SOD activity in rabbits treated with ES-CC was similar to that of animals that received diet free of cholesterol.

Although data presented are quite compelling, secondary metabolites present in ES-CC that would be responsible for these activities remain not entirely clear. In recent years, several studies have shown that polyphenols, especially flavonoids and their glycosylated derivatives, are highly effective in reducing LDL oxidation and protect against lipid peroxidation (De Whaley, 1990; Agati et al., 2012). Part of this effect can be attributed to the presence of ortho-dihydroxy and hydroxyl groups in C-5 and C-3 positions, and carbonyl in C-4 position, which allows the formation of chelates with divalent ions and increases their inhibitory effects on lipid peroxidation (Cholbi et al., 1991). In fact, secondary metabolites present in ES-CC were characterized in detail, showing the strong presence of different flavonoids, including several quercetin glycosides. Moreover, through microchemical analysis, the diffusion of polyphenols along *Cuphea carthagenensis* leaves could be observed, suggesting that these metabolites may play a central role in the antiatherosclerotic activities of ES-CC.

5. Conclusion

In this work, ethanol supernatant infusion from *Cuphea carthagenensis* (ES-CC) was obtained and its phytochemical profile was characterized in detail. It was shown that ES-CC has several secondary metabolites that may be responsible for reducing serum lipids and oxidative stress when orally administered in New Zealand rabbits. In addition, ES-CC showed high efficacy as anti-atherogenic agent, reducing and preventing atherosclerotic lesions induced by CRD. This study showed for the first time positive evidences for the validation of the ethnopharmacological use of *Cuphea carthagenensis* and features ES-CC as a potential candidate for the development of a new herbal medicine for prevention and/or treatment of dyslipidemias and atherosclerosis.

Conflict of Interest

The authors declare that there are no conflicts of interest in this study.

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Table 1. Phytochemical composition of *Cuphea carthagenensis* obtained by LC-MS/MS

Rt	$MS^1(-) \rightarrow MS^2(-)$	$MS^1(+) \rightarrow MS^2(+)$	Tentative identification
6.50	479.083 → 316.022	481.097 → 319.044	Myricetin-glucoside
7.02	463.088 → 301.035	465.103 → 303.050	Quercetin-5-*O*-β-glucopyranoside
7.37	609.146 → 300.027	611.160 → 465.102 → 303.050	Rutin
7.44	477.067 → 301.035	479.082 → 303.049	Quercetin-glucoronide
7.51	463.088 → 300.027	465.103 → 303.050	Quercetin-3-*O*-β-glucopyranoside
7.74	380.992 → 301.035	-	Quercetin-3-sulfate
8.12	433.077 → 300.027	435.092 → 303.050	Quecertin-3-*O*-arabinofuranoside
8.20	593.151 → 284.032	595.165 → 449.107 → 287.055	Kaempferol-rutinoside
8.38	447.093 → 284.032	449.108 → 287.055	Kaempferol-glucoside
9.71	301.035	303.050	Quercetin

Table 2.

Effects of the oral administration of the ES-CC obtained from *Cuphea carthagenensis* and simvastatin (SIMV) on serum lipid levels from New Zealand rabbits

Group	TC (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)	VLDL-C (mg/dL)	TG (mg/dL)
N. Control	54 ± 4.9	10 ± 0.2	27 ± 4.9	23 ± 2.1	82 ± 16
P. Control	237± 34[a]	7.2 ± 0.3[a]	185 ± 20[a]	81 ± 10[a]	190 ± 28[a]
ES-CC (10)	208 ± 31[a]	7.8 ± 0.8[a]	166 ± 33[a]	78 ± 9.2[a]	166 ± 35[a]
ES-CC (30)	164 ± 25[ab]	8.2 ± 0.2[ab]	122 ± 15[ab]	57 ± 6.9[ab]	140 ± 31[ab]
ES-CC (100)	155 ± 30[ab]	8.6 ± 0.4[ab]	117 ± 17[ab]	56 ± 7.1[ab]	147 ± 25[ab]
SIMV (5)	146 ± 28[ab]	8.6 ± 0.3[ab]	110 ± 15[ab]	54 ± 8.2[ab]	135 ± 36[ab]

Values are expressed as mean ± S.E.M. (n=5-6 in each group) in comparison to the negative control (a; $p < 0.05$) or positive control (b; $p < 0.05$) using one-way ANOVA followed by Bonferroni test. TC (total cholesterol); HDL-C (high-density lipoprotein cholesterol); LDL-C (low-density lipoprotein cholesterol); VLDL-C (very low-density lipoprotein cholesterol); TG (triglyceride).

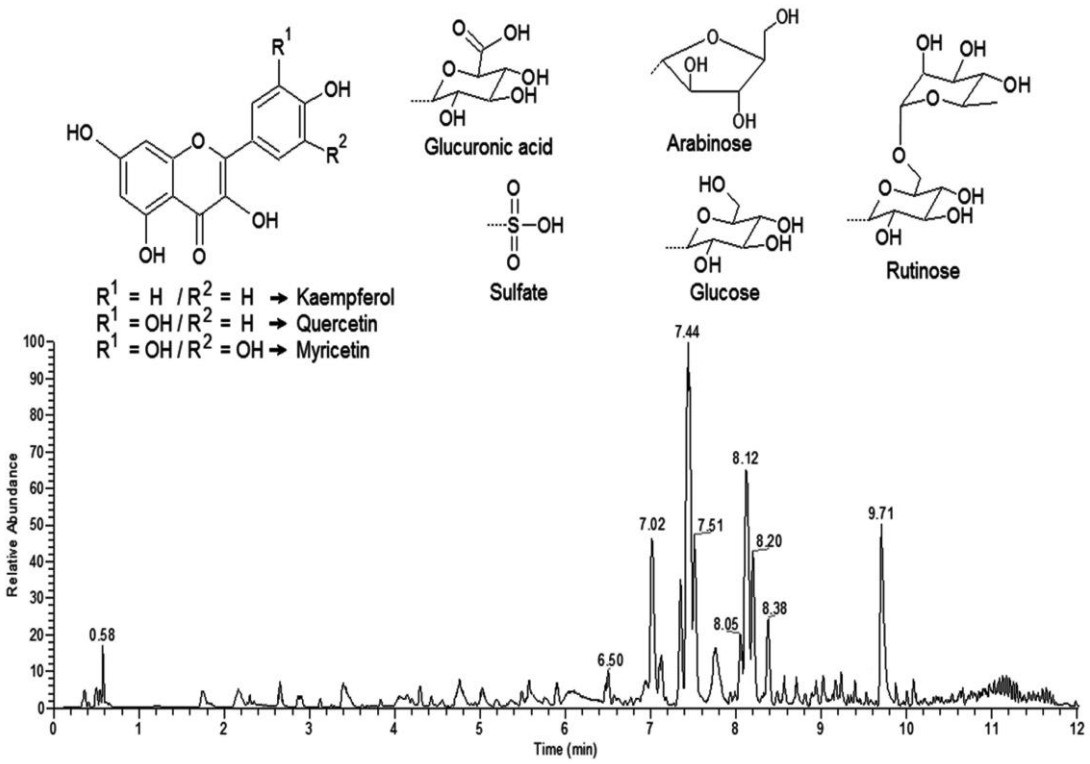

Figure 1

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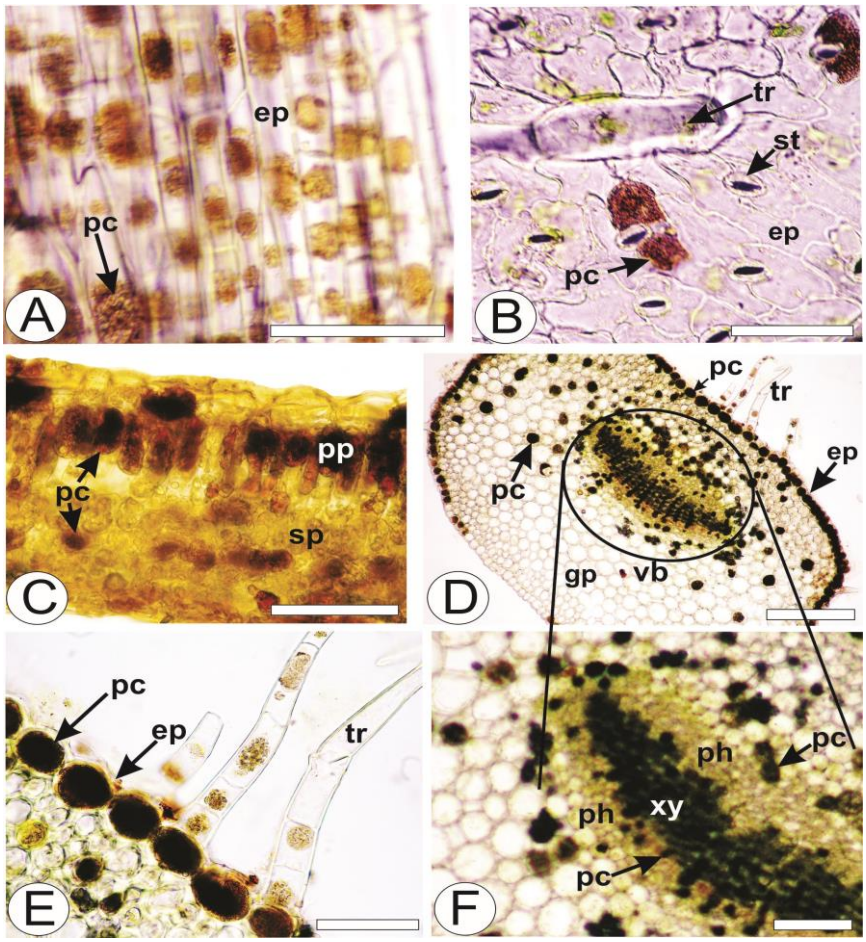

Figure 2

Barboza et al.

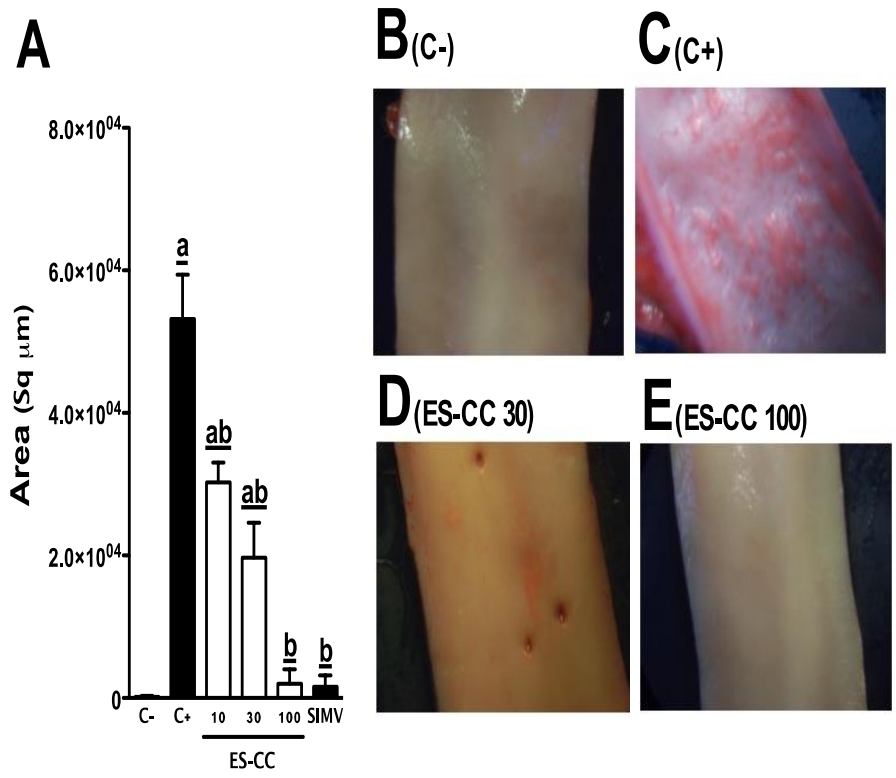

Figure 3

Barboza et al.

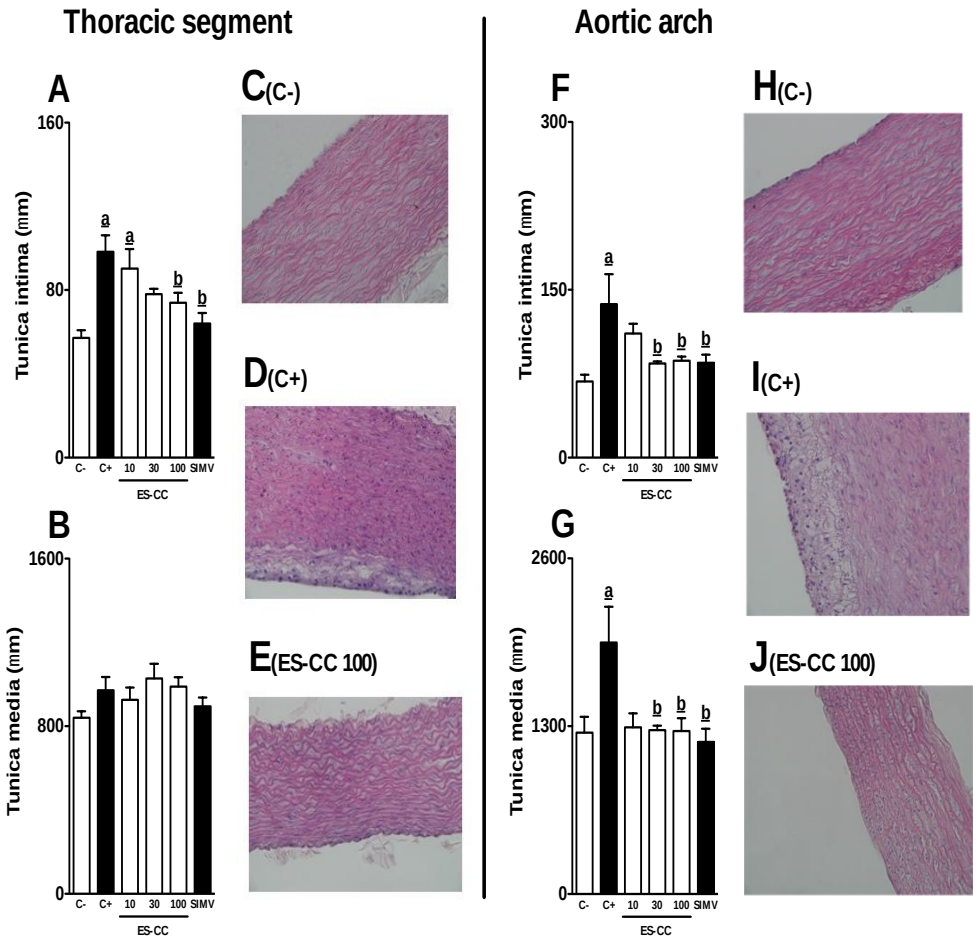

Figure 4

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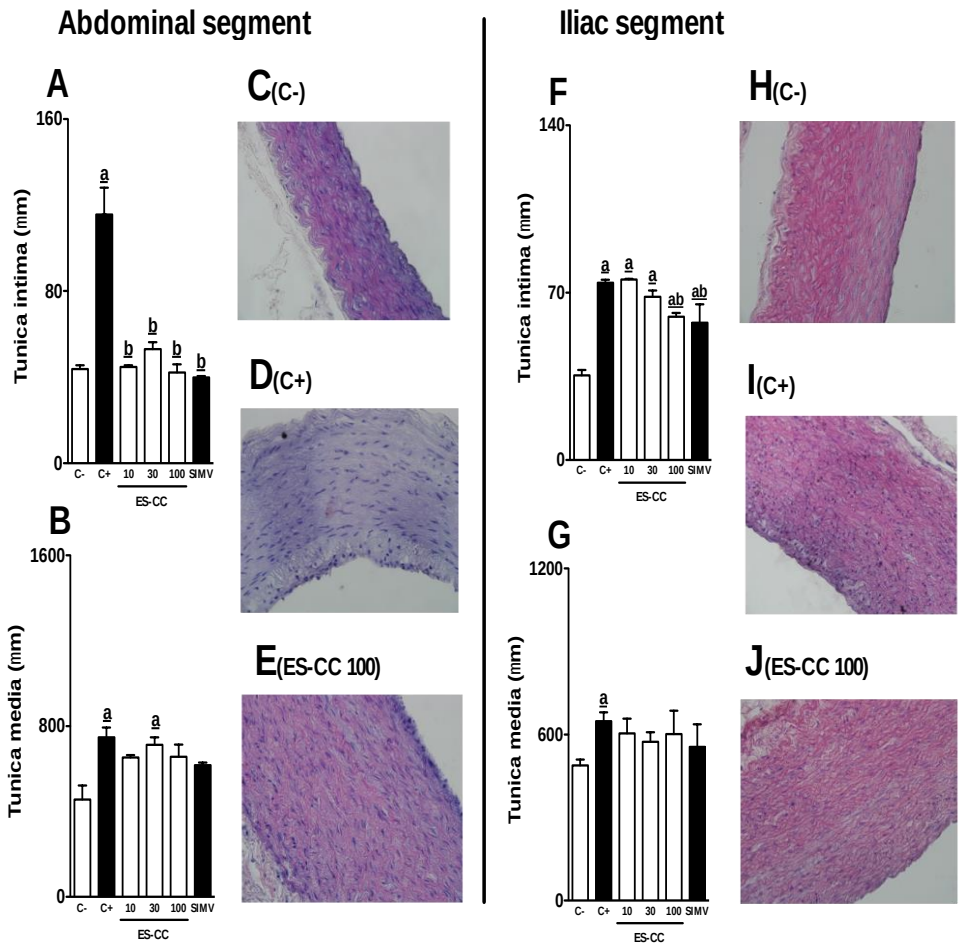

Figure 5

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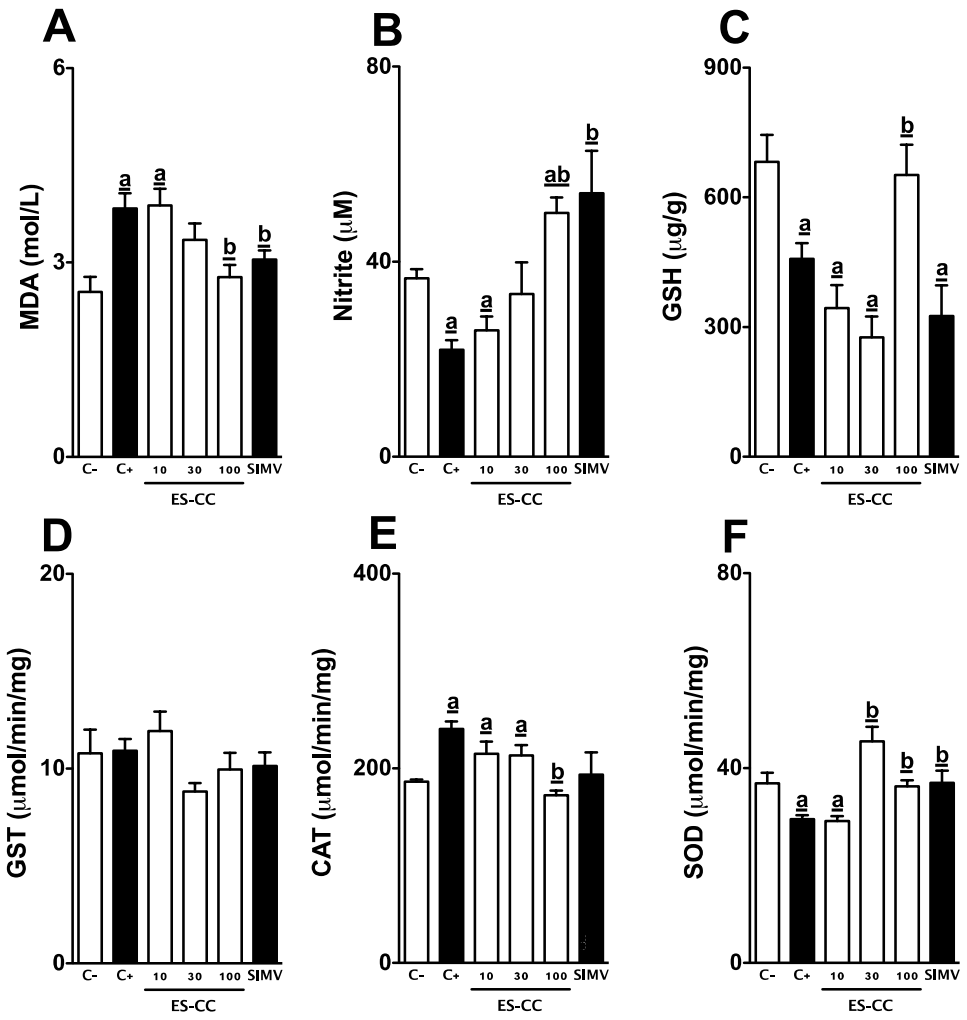

Figure 6

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4. CONSIDERAÇÕES FINAIS

Neste trabalho obtivemos uma fração solúvel em etanol a partir das folhas de *Cuphea carthagenensis* (ES-CC) e caracterizamos em detalhes os metabólitos secundários responsáveis por sua atividade biológica. Complementarmente, identificamos através de um estudo microquímico a localização anatômica dos compostos fenólicos presentes nas folhas desta espécie como importante ferramenta farmacognóstica para controle de qualidade desta espécie. Além disso, mostramos que o ES-CC é capaz de produzir significativos efeitos hipolipemiantes e antiaterogênicos em coelhos Nova Zelândia, modulando a atividade enzimática antioxidante hepática e reduzindo o estresse oxidativo e a evolução das lesões ateroscleróticas induzidas pela dieta rica em colesterol.

Este estudo traz luz às evidências de eficácia do ES-CC como agente arterioprotetor, e apresenta esta preparação como um potencial candidato ao desenvolvimento de um novo fitofármaco para o tratamento das dislipidemias e aterosclerose.

5. REFERÊNCIAS BIBLIOGRÁFICAS ADICIONAIS

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6. ANEXOS

ANEXO 1. Normas da revista Journal of Ethnopharmacology

JOURNAL OF ETHNOPHARMACOLOGY
An Interdisciplinary Journal Devoted to Indigenous Drugs
ELSEVIER

AUTHOR INFORMATION PACK

Article structure

Subdivision - numbered sections
Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

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State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

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Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

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A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

Results
Results should be clear and concise.

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This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

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Please supply, as a separate list, the definitions of field-specific terms used in your article.

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If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

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The author should divide the abstract with the **headings *Ethnopharmacological relevance*, *Materials and Methods*, *Results*, and *Conclusions*.**
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Acknowledgements

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