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EFEITOS DO EXTRATO AQUOSO DE FOLHAS DE *Syzygium cumini* SOBRE A OXIDAÇÃO E GLICAÇÃO DE LIPOPROTEÍNAS DE BAIXA DENSIDADE

DISSERTAÇÃO DE MESTRADO

Matheus Mülling dos Santos

Santa Maria, RS, Brasil
2014

EFEITOS DO EXTRATO AQUOSO DE FOLHAS DE *Syzygium cumini* SOBRE A OXIDAÇÃO E GLICAÇÃO DE LIPOPROTEÍNAS DE BAIXA DENSIDADE

Matheus Mülling dos Santos

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EFEITOS DO EXTRATO AQUOSO DE FOLHAS DE *Syzygium cumini* SOBRE A OXIDAÇÃO E GLICAÇÃO DE LIPOPROTEÍNAS DE BAIXA DENSIDADE

Elaborada por

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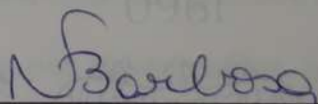

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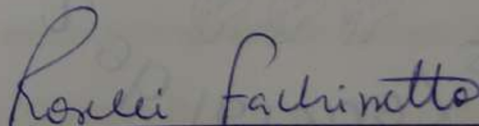

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Santa Maria, 28 de Agosto de 2014.

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LISTA DE ABREVIATURAS

AGEs: Produtos finais de glicação avançada

apoB-100: Apolipoproteína B-100

AVC: Acidente vascular cerebral

DC: Dieno conjugado

DCV: Doenças carviovasculares

glyLDL: LDL glicada

LDL: Lipoproteína de baixa densidade

MCP-1: Proteína quimiostática de monócitos-1

M-CSF: Fator estimulador de colônia de macrófagos

MDA: Malondialdeído

MG: Metilglioxal

MG-H1: hydroimidazolone N_{δ}-(5-hydro-5-methyl -4-imidazolon-2-yl)-ornithine

mm-LDL: LDL minimamente modificada

nLDL: LDL nativa

OMS: Organização Mundial de Saúde

oxLDL: LDL oxidada

PUFAs: Ácidos graxos poliinsaturados

RESUMO

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

Efeitos do extrato aquoso de folhas de *Syzygium cumini* sobre a oxidação e glicação de liporoteínas de baixa densidade

AUTOR: Matheus Mülling dos Santos
ORIENTADORA: Nilda Berenice de Vargas Barbosa
LOCAL E DATA DA DEFESA: Santa Maria, 28 de agosto de 2013

Modificações estruturais nas lipoproteínas de baixa densidade (LDL) são consideradas uma das principais causas para o desenvolvimento da aterosclerose. Dentre essas modificações, destacam-se as causadas por oxidação e/ou glicação. A glicação é favorecida em indivíduos diabéticos devido à hiperglicemia e torna o Diabetes mellitus um dos principais fatores de risco para a aterosclerose. Tendo em vista as relações entre a hiperglicemia e a aterosclerose, bem como o papel do estresse oxidativo em ambas as condições, este trabalho teve como objetivo avaliar o efeito *in vitro* do extrato aquoso de folhas de *Syzygium cumini* (*S.c*Ext), uma planta utilizada popularmente no tratamento do diabetes, sobre parâmetros de oxidação e glicação em LDL isoladas de plasma humano. Os resultados obtidos demonstraram que o *S.c*Ext exibiu um potente efeito antioxidante sobre a peroxidação lipídica induzida por $CuSO_4$ em LDL, soro e plasma humanos avaliada através da formação de dienos conjugados e da produção de espécies reativas ao ácido tiobarbitúrico (TBARS). Além da ação antioxidante exibida contra a peroxidação lipídica, a análise da perda da fluorescência do triptofano indicou que o *S.c*Ext é também capaz de reduzir os danos oxidativos causados pelo $CuSO_4$ à porção protéica da LDL. Diferentemente dos parâmetros de oxidação, o *S.c*Ext não exibiu potencial antiglicante sobre a glicação de LDL induzida pelo metilglioxal (MG), que foi avaliada através da mobilidade eleroforética na eletroforese em gel de agarose e através da análise fluorimetrica da formação de produtos finais de glicação avançada (AGEs). De forma geral, os dados obtidos mostram que o *S.c*Ext pode ser considerado um agente antiaterogênico promissor devido à sua atividade antioxidante sobre a oxidação de LDL *in vitro*.

Palavras-Chave: LDL, aterosclerose, oxidação, glicação, estresse oxidativo, *Syzygium cumini*.

ABSTRACT

Dissertation of Master's Degree
Graduate Course in Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

Effect of *Syzygium cumini* aqueous-leaf extract on oxidation and glycation of low density lipoproteins

AUTHOR: Matheus Mülling dos Santos
ADVISOR: Nilda Berenice de Vargas Barbosa
PLACE AND DATE OF THE DEFENSE: Santa Maria, 28thaugust, 2014.

Structural modifications of low density lipoproteins (LDL) are considered one of the main causes to atherosclerosis development. Among these modifications, highlights the modifications caused by oxidation and/or glycation, the latter being favored in diabetic subjects due to the hyperglycemia status and consequent increased availability of glucose, making diabetes mellitus one of the risk factors of atherosclerosis. Considering the relationships established between atherosclerosis and hyperglycemia, as well as the role of oxidative stress in both conditions, this work aimed to evaluate the effect *in vitro* of the *Syzigium cumini* aqueous-leaf extract (*S.c*Ext), a plant popularly used to treat diabetes, on parameters of oxidation and glycation in low density lipoproteins isolated from human plasma. The results obtained demonstrated that *S.c*Ext exhibited a potent antioxidant effect on $CuSO_4$-induced lipid peroxidation in human LDL, serum and plasma assayed by conjugated dienes formation and thiobarbituric acid reactive substances (TBARS) production. Besides the antioxidant activity exhibited against lipid peroxidation, the assay of loss of tryptophan fluorescence showed that *S.c*Ext is also able to diminish the oxidative damage caused by $CuSO_4$ in the protein moiety of LDL. In contrast to oxidation parameters, *S.c*Ext did not modify the LDL glycation induced by methylglyoxal (MG), which was evaluated by eletrophoretic mobility in agarose gel eletroforesis and by the fluorimetric analysis of the formation of advanced glycation end products (AGEs). In general, the data obtained here suggest that *S.c*Ext can be considered a promising antiatherogenic agent due its antioxidant activity on LDL oxidation *in vitro*.

Key words: LDL, atherosclerosis, oxidation, glycation, oxidative stress, *Syzygium cumini*.

APRESENTAÇÃO

No item **INTRODUÇÃO**, está descrito uma sucinta revisão bibliográfica sobre os temas abordados nessa dissertação.

O **DESENVOLVIMENTO** está apresentado sob a forma de um manuscrito, o qual se encontra alocado no item **MANUSCRITO**.

As seções Materiais e Métodos, Resultados e Discussão encontram-se no próprio manuscrito e representam a íntegra deste estudo.

Os itens **CONCLUSÕES** e **PERSPECTIVAS** são encontrados no final desta dissertação e apresentam interpretações e comentários gerais sobre a investigação desenvolvida.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem no item **INTRODUÇÃO**, uma vez que o manuscrito contém as suas próprias referências.

1. INTRODUÇÃO

Segundo dados da Organização Mundial de Saúde (OMS), as doenças cardiovasculares (DCV) são atualmente a principal causa de morte em todo o mundo. Em 2008, estimou-se que cerca de 17,3 milhões de pessoas tenham morrido em decorrência de DCV, o que representa 30% do total de mortes no mundo (WHO, 2011a). Dentre essas, estima-se que em torno de 7,3 milhões ocorreram devido à doença cardíaca coronária e 6,2 milhões devido à ocorrência de acidente vascular cerebral (AVC) (WHO, 2011b). Um terço dos óbitos decorrentes de DCV ocorre de maneira precoce, em indivíduos na faixa etária de 35 a 64 anos (Ishitani et. al, 2006).

O infarto agudo do miocárdio bem como o AVC são considerados as principais manifestações das doenças cardiovasculares. Ambas as manifestações costumam ocorrer em decorrência de um processo vascular denominado aterosclerose. A aterosclerose pode ser definida como uma doença inflamatória crônica caracterizada pela formação de ateromas em artérias de grande e médio calibre (Libby et al., 2002). Esses ateromas são placas contendo basicamente lipídeos e tecido fibroso que se acumulam no endotélio arterial causando uma diminuição gradativa do lúmen do vaso sanguíneo, a qual pode culminar com a obstrução total do mesmo e com consequente prejuízo no fornecimento de sangue tecidual (Montecucco et al., 2009) (Figura 1). Além disso, o trombo formado pode se desprender e se alojar em artérias de menor calibre, ocluindo-as e causando um infarto. A trombose oriunda de uma placa aterosclerótica é considerada uma das principais causas dos eventos cardiovasculares que ocorrem de forma súbita (Lybby, 2002).

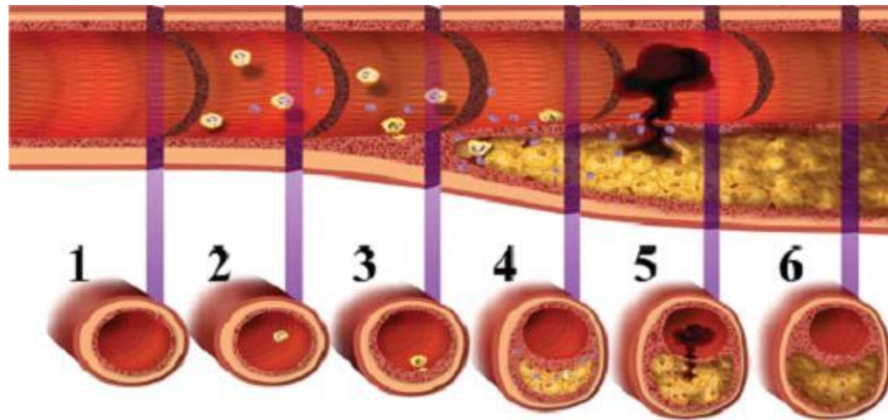

Figura 1: Progressão da lesão aterosclerótica. 1- Artéria em seu estado normal. 2- Acúmulo inicial de lipídeos na camada mais interna da artéria. 3- Estágios iniciais da placa. 4- Formação de uma placa complexa. 5- Ruptura da placa aterosclerótica com formação de trombo. 6- Reabsorção do trombo com consequente aumento no tamanho da placa aterosclerótica. (http://radlivres2010-1.blogspot.com.br/2010/07/aterosclerose-e-sua-relacao-com.html acessado em 18/08/2014)

Dados epidemiológicos vêm demonstrando que a aterosclerose é uma doença diretamente ligada ao envelhecimento, cujo aparecimento prematuro pode estar relacionado a diferentes condições clínicas como: excesso de lipoproteínas de baixa densidade (LDL) (Figura 2), diabetes, hipertensão, tabagismo, entre outras (Smith et al., 2004). Uma das principais dificuldades para o diagnóstico da aterosclerose se deve ao fato da mesma ser uma doença silenciosa. Várias evidências sugerem que o processo de formação da placa aterosclerótica inicia na infância, porém, a doença progride de maneira assintomática até a idade adulta (Françoso e Coates, 2002).

Durante as últimas décadas, inúmeros estudos vêm tentando elucidar os mecanismos celulares e moleculares envolvidos na aterogênese, a ponto de serem criadas diferentes hipóteses para explicar tal evento. Uma das hipóteses que tenta explicar como ocorre o processo aterogênico é a chamada hipótese da modificação oxidativa (Steinberg et al., 1989).

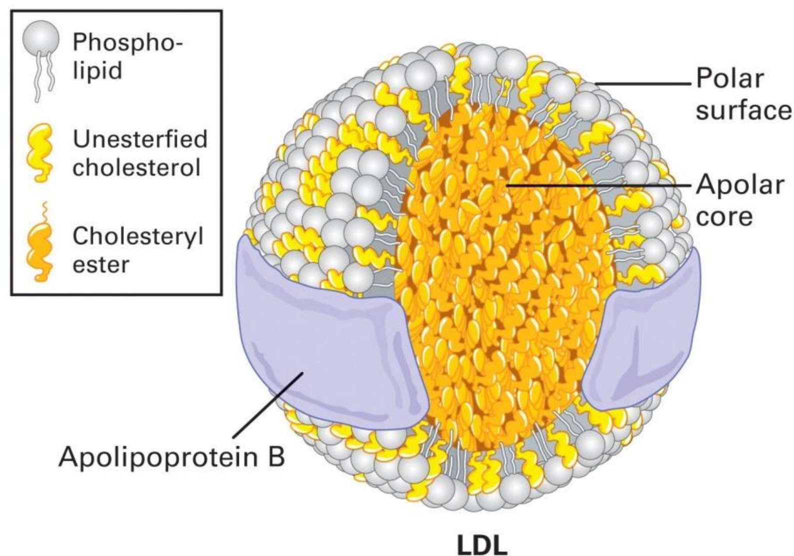

Figura 2: Lipoproteína de baixa densidade (LDL).
(http://healthyprotocols.com/2_ldl.htm acessado em 15/08/2014)

De acordo com a hipótese da modificação oxidativa, as moléculas de LDL oxidada se tornam aterogênicas através (I) do recrutamento de monócitos circulantes para a túnica íntima da artéria, (II) da inibição da capacidade dos macrófagos residentes abandonarem este espaço, (III) da sua internalização pelos receptores scavengers dos macrófagos com consequente formação de células espumosas e (IV) da citotoxidade e indução de perda da integridade endotelial (Steinberg et al., 1989).

Os mecanismos envolvidos na modificação da LDL ainda não estão completamente elucidados; no entanto, um dos eventos iniciais é a peroxidação lipídica de folfolipídeos e ésteres de colesterol, os quais possuem ácidos graxos poliinsaturados (PUFAs). Inicialmente ocorre a remoção de um hidrogênio seguida pelo rearranjo das duplas ligações formando um dieno conjugado (DC). Este por sua vez reage com o oxigênio e desencadeia uma sequência de reações culminando com a formação de hidroperóxidos lipídicos. Pode então haver a clivagem das

ligações carbono-carbono dos hidroperóxidos lipídicos dando origem a fragmentos como o malondialdeído (MDA) (Yamaguchi et al., 2002). Os produtos formados na peroxidação lipídica podem interagir com resíduos de lisina presentes na apolipoproteína B-100 (apoB-100) causando modificações oxidativas também na porção protéica da LDL (Steinbrecher et al., 1987) (Figura 3).

Figura 3: Oxidação da LDL (Adaptado de Yamaguchi et al., 2002).

Essas modificações oxidativas na apoB-100 são responsáveis por alterar a carga elétrica da proteína, deixando a apoB-100 com elevada carga negativa. Essa carga negativa causa uma diminuição da afinidade da LDL pelo seu receptor e um aumento na afinidade pelos receptores *scavenger* (CD-36, CD-68 e SR-A) dos

macrófagos (Brown et al., 1983). Quando ligadas a esses receptores, as moléculas de LDL são internalizadas nos macrófagos formando as chamadas células espumosas que são as células predominantes na lesão aterosclerótica inicial conhecida como estria gordurosa (Osterub et al., 2003). Diferentemente dos receptores de LDL que são inibidos por retroalimentação, os receptores *scavenger* dos macrófagos não são regulados negativamente com o aumento no conteúdo de colesterol nos macrófagos. Dessa forma, os macrófagos continuam capturando moléculas de LDL oxidada (oxLDL), com conseqüente acúmulo de uma quantidade significativa de lipídeos na parede arterial (Osterub et al., 2003).

Diversos trabalhos têm sugerido que o aumento de oxLDL é um fator pró-aterogênico por desencadear um processo inflamatório (Witztum, 1993). Quando oxidada, a LDL pode induzir a ativação de células endoteliais e musculares-lisas; a secreção de mediadores inflamatórios e a expressão de moléculas de adesão (Witztum e Steinberg, 1991).

Antes de ocorrer a oxidação da apoB-100, quando apenas a porção lipídica é afetada e a apoB-100 ainda se encontra íntegra, a LDL é conhecida como “minimamente modificada” (mm-LDL). Nesse estágio, por não haver modificação na estrutura da apoB-100, as moléculas de LDL ainda são reconhecidas pelos receptores de LDL e não pelos receptores *scavenger* (Mashima e cols., 2001). As mm-LDL podem induzir a expressão da proteína quimiostática de monócitos-1 (MCP-1) e também do fator estimulador de colônia de macrófagos (M-CSF) pelas células endoteliais que então iniciam o recrutamento de monócitos para a parede arterial e promovem a diferenciação de monócitos em macrófagos (Cushing et al., 1990; Villa-Colinayo et al., 2000). A figura 3 ilustra o processo de oxidação da LDL e formação das células espumosas (foam cells).

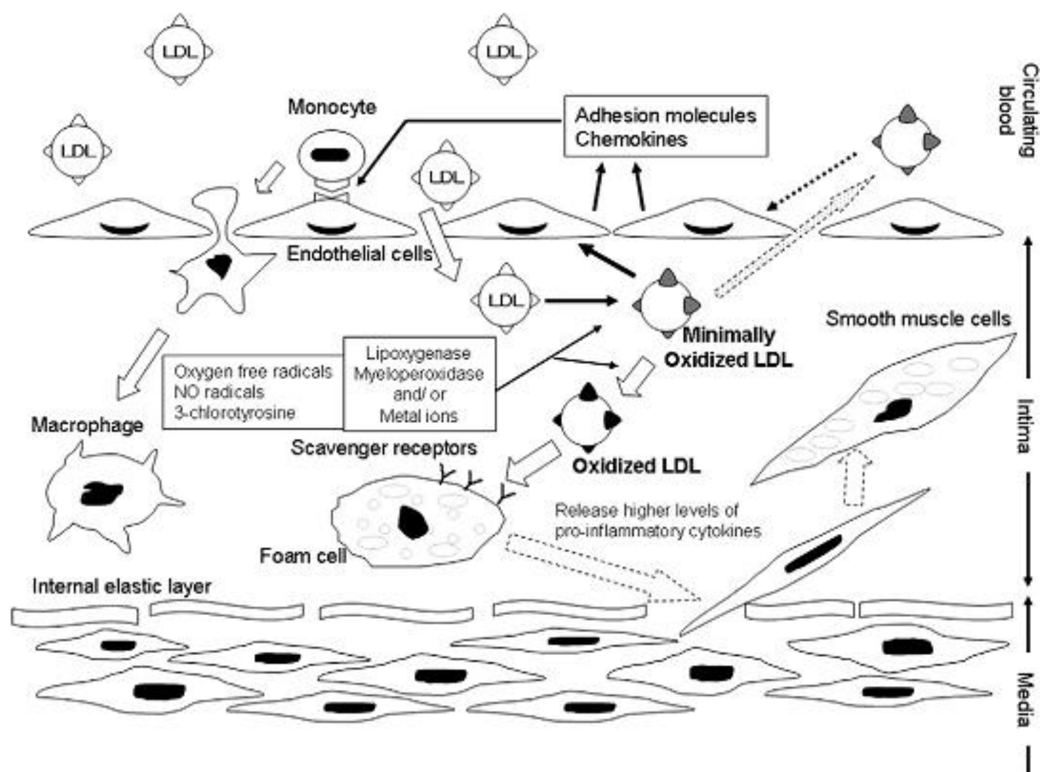

Figura 3: Oxidação da LDL e formação de células espumosas (foam cells) (Hioshida e Hisugi, 2010).

A importância da oxidação da LDL no desenvolvimento da aterosclerose torna a mesma uma das inúmeras doenças relacionadas ao estresse oxidativo (Dhalla et al., 2000). Nesse contexto, muitas pesquisas demonstram o papel benéfico do consumo de antioxidantes no combate à aterogênese, principalmente antioxidantes oriundos de produtos naturais como frutas e vegetais (Cherubini et al., 2005; Kardinaal et al., 1993; Rimm et al., 1993; Smith et al., 1989).

Dentre os fatores de risco para o desenvolvimento da aterosclerose, destaca-se o Diabetes mellitus, uma doença que acomete um número grande de pessoas no mundo e tem como evento chave um quadro de hiperglicemia crônica (D'Agostino et al., 2004). Segundo Lyons et al. (1986) o risco para o desenvolvimento de aterosclerose é aumentado aproximadamente três vezes em pacientes diabéticos. Alem disso, diversos trabalhos vêm demonstrando o papel da hiperglicemia como

precursora de eventos oxidativos em diferentes tecidos, dentre eles a peroxidação lipídica, a qual contribui para a formação da ox-LDL (Jain et al., 1989).

Além da oxidação da LDL, outro tipo de reação extremamente importante no que diz respeito ao processo aterogênico é a glicação não-enzimática da lipoproteína, favorecida em condições hiperglicêmicas pela alta disponibilidade de glicose. Nas reações de glicação não-enzimática, os grupos carbonila de carboidratos reagem com os grupamentos amino de proteínas formando inicialmente bases de *Schiff.* Estas, por sua vez, sofrem rearranjos espontâneos formando os chamados produtos de Amadori, sendo esses denominados os produtos iniciais da glicação (Yeboah et al., 1999). Posteriormente, os produtos de Amadori podem ser degradados e formar compostos α-dicarbonílicos tais como o glioxal, o metilglioxal e o 3-deoxiglucosona, cujos grupamentos carbonila são ainda mais reativos frente aos grupos amino de proteínas e como resultado de tal interação, ocorre a formação dos produtos finais de glicação avançada (AGEs) (Onorato et al., 1998) (Figura 4).

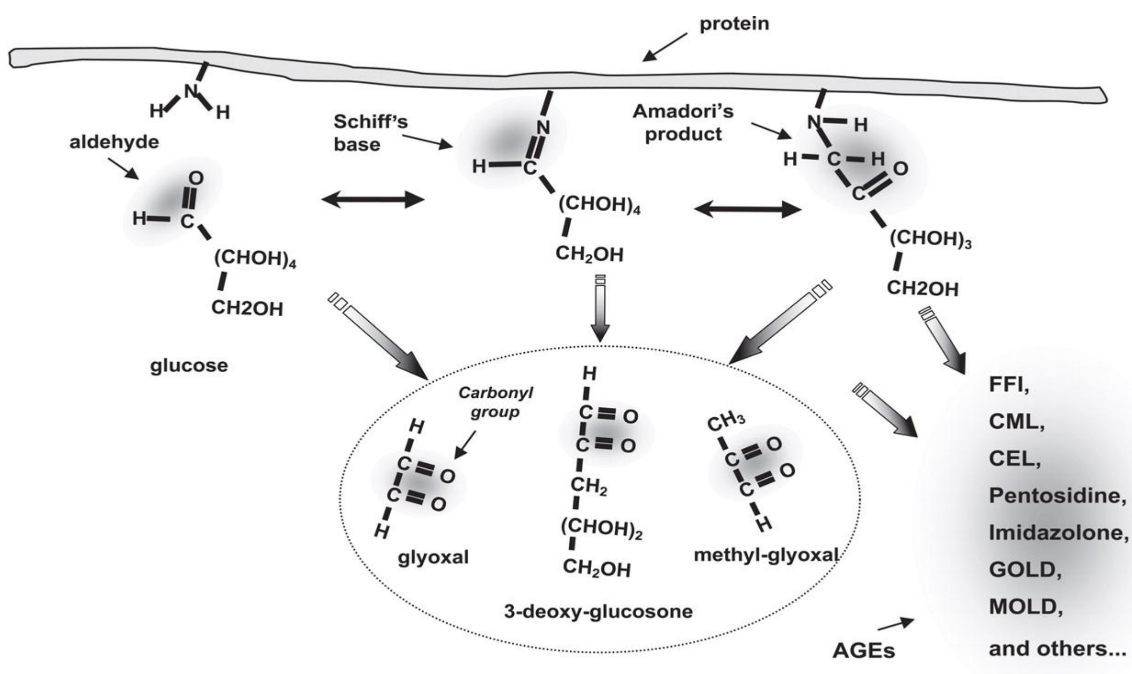

Figura 4: Possíveis vias para formação de AGEs (Basta et al., 2004).

Dentre os compostos α-dicarbonílicos precursores da formação de AGEs, destaca-se o metilglioxal (MG) que pode ainda ser formado em condições hiperglicêmicas através da degradação de trioses fosfato durante a glicólise (O'Brien et al., 2010). A concentração plasmática de MG é aumentada de duas a cinco vezes em pacientes diabéticos (McLellan et al., 1994). A reação de glicação entre os resíduos de arginina da LDL com MG leva à formação do AGE hydroimidazolone N_{δ}-(5-hydro-5-methyl -4-imidazolon-2-yl)-ornithine (MG-H1), o qual é considerado o AGE presente em maior quantidade nos sistemas fisiológicos (Thornalley, 2005) (Figura 5)

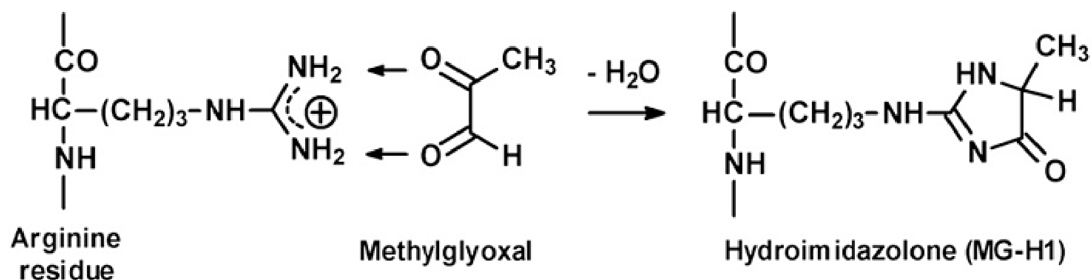

Figura 5: Reação entre resíduos de lisina e metilglioxal formando o AGE MG-H1 (Rabbani, N. et al., 2011).

Pesquisas recentes vêm demonstrando que muitas reações de glicação necessitam de condições pró-oxidantes para ocorrer, sendo por isso, muitas vezes utilizado o termo “glicooxidação” para designar tal processo (Vlassopoulos et al., 2013). Além disso, a LDL glicada (glyLDL) parece ser mais suceptível à oxidação que a a LDL nativa (nLDL) (Hunt et al., 1990; Kawamura et al., 1994). Esses fatos fazem com que muitas vezes seja difícil distinguir potenciais agentes antiglicantes de antioxidantes. Porém, modelos de glicação com compostos carbonilicos como o

metilglioxal são modelos de glicação não-oxidativa (Baynes, 1991; Cervantes-Laurean et al., 1996). Desta forma, o modelo de glicação induzida pelo MG oferece uma vantagem em relação a modelos onde a glicação é induzida pela glicose, por permitir a verificar a ação de inibidores das reações de glicação que atuam independentemente de sua ação antioxidante.

Considerando o conhecimento obtido sobre o papel oxidante/glicante do processo aterogênico, a busca por substâncias com potencial antioxidante e antiglicante vêm crescendo muito nas últimas décadas. Neste contexto, destacam-se os produtos naturais, por serem normalmente de fácil acesso à população e amplamente usados na medicina tradicional.

A *Syzygium cumini,* conhecida popularmente como "jambolão", é uma planta pertencente à família Asteraceae que é utilizada na medicina popular brasileira no tratamento da hiperglicemia (Oliveira et al., 2005). Na literatura, diversos trabalhos têm demonstrado a eficácia de diferentes partes e preparações da planta em modelos experimentais relacionados com condições diabetogênicas. Existem evidências que o tratamento com extrato aquoso de sementes e/ou etanólico de folhas da planta reduz os níveis de glicose sanguínea e a hipercolestorolemia em ratos diabéticos (Mahapatra et al., 1985; Vikrant et al., 2001). Além destas, outras propriedades descritas na literatura incluem: a ação antioxidante (Banerjee et al., 2005), antiinflamatória (Chaudhuri et al., 1990), antifúngica (Braga et al., 2007), anti-HIV (Kusumoto et al., 1995), antiulcerogênica (Ramirez et al., 2003), entre outras. No entanto, não há estudos acerca do efeito de nenhuma parte da planta frente à oxidação e/ou glicação de lipoproteínas de baixa densidade e consequentemente sobre o seu potencial como modulador do processo aterogênico.

2. OBJETIVOS

2.1 Objetivo Geral

O objetivo geral deste trabalho é avaliar o efeito do extrato aquoso de folhas da planta *Syzygium cumini* (*S.c*Ext) sobre parâmetros de oxidação induzida por $CuSO_4$ na porção lipídica e protéica de LDL, bem como sobre parâmetros de glicação induzida por metilglioxal nas mesmas.

2.2 Objetivos Específicos

- Avaliar o efeito do *S.c*Ext sobre a peroxidação lipídica inzudida por $CuSO_4$ em LDL, soro e plasma humanos através da formação de dienos conjugados e produção de TBARS.
- Analisar o efeito do *S.c*Ext sobre o dano oxidativo induzido por $CuSO_4$ na porção protéica da LDL utilizando a fluorescência do triptofano.
- Verificar a suceptibilidade da LDL isolada de plasma pré-incubado com o *S.c*Ext à oxidação lipídica e protéica.
- Avaliar o efeito do *S.c*Ext sobre a glicação da LDL induzida pelo metilglioxal através da eletroforese em gel de agarose e formação de AGEs fluorescentes.

3. DESENVOLVIMENTO

O desenvolvimento referente a essa dissertação está apresentado sob a forma de um manuscrito. Os itens Materiais e Métodos, Resultados, Discussão e Referências encontram-se no próprio manuscrito.

MANUSCRITO: Efeitos do extrato aquoso de folhas de *Syzygium cumini* sobre a oxidação e glicação de lipoproteínas de baixa densidade *in vitro*

Effects of aqueous-leaf extract of *Syzygium cumini* on oxidation and glycation of low density lipoproteins *in vitro*

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Abstract

Structural modifications elicited by oxidation and glycation of low density lipoproteins (LDL) are considered crucial in the atherosclerosis development. Glycated LDL (glyLDL) levels are elevated in diabetic patients and it is known that diabetes mellitus is a risk factor to atherosclerosis. In view of the importance of LDL glycation and oxidation in the pathogenesis of atherosclerosis, the antioxidant and anti-glycant effect of many synthetic and natural products have been studied. In this work we evaluated the effects of *Syzygium cumini* aqueous-leaf extract (*S.c*Ext) on oxidation and glycation of human LDL *in vitro*. Our results show that the *S.c*Ext was effective in blunting LDL, plasma and serum oxidation evaluated by different parameters, namely: conjugated dienes formation, thiobarbituric acid reactive substances (TBARS) levels and tryptophan (Trp) fluorescence. On the order hand, the data obtained in the assays evaluating glycation reactions suggest that the *S.c*Ext does not exhibit anti-glycant effect. These findings indicate that *S.c*Ext has potential as antiatherogenic agent.

Key words: LDL, atherosclerosis, oxidation, glycation, oxidative stress, *Syzygium cumini*

1. Introduction

Cardiovascular diseases (CVD) are considered the major cause of death in the world (WHO, 2014). The atherosclerosis, a chronic inflammatory process characterized by formation of atherosclerotic plaque in large and medium size arteries, has been pointed as the underlying cause of coronary heart disease and most strokes (Steinberg and Witztum, 2002). Several studies have suggested that modifications in the structure of low density lipoprotein (LDL) are crucial in the atherosclerosis development (Stocker and Keaney, 2004; Nishi et. al., 2002; Matsuura et al., 2006), especially the oxidative modifications of LDL to form oxidized LDL (oxLDL) (Chisolm and Steinberg, 2000). OxLDL acts as an atherogenic particle by inducing endothelial cell activation, secretion of inflammatory mediators and expression of adhesion molecules; events that promote the recruitment of monocytes and their differentiation into macrophages in the intima of the arteries (Witztum and Steinberg, 1991). It is well known that macrophages are able to cause oxidation of LDL via oxidant enzyme systems, such as myeloperoxidase, NADPH oxidase and nitric oxide synthase (NOS) (Parhami et al, 1993). The total oxidation of LDL culminates with an increase in the apoB-100 negative charge, which promotes the recognition of oxLDL by scavenger receptors on macrophages and its internalization to form the foam cells, an important hallmark of the atherosclerotic lesion (Enriksen et al., 1981).

A growing body of evidence from experimental and clinical studies shows a close association between atherosclerosis and Diabetes Mellitus (DM). In fact, there is evidence that the risk for atherosclerosis manifestations is increased by approximately three-fold in diabetic patients (Lyons et al., 1986). DM is a metabolic disorder characterized by chronic hyperglycemia, resulting from defects in insulin

secretion, insulin action or both (WHO, 1999). Another important type of LDL modification, which is favored under hyperglycemic condition, is the LDL glycation with consequent formation of glycated LDL (glyLDL) (Veiraiah, 2005). Protein glycation is a complex series of Mailard reactions where glucose or others reactive carbonyl compounds such glyoxal (GO) and methylglyoxal (MG) react with protein amino groups to form initially reversible Shiff bases (Thornalley et al., 2001). Over time, the Shiff bases are able to form irreversible Amadori products and in the last stage can be formed the advanced glycation end products (AGEs), which trigger pathological events as oxidative stress and pro-inflammatory responses (Lee et al., 2009).

MG can be formed via oxidation of reducing sugars under hyperglycemic conditions and through triose phosphate intermediates in glycolysis (O'Brien et al., 2010). The plasma concentration of MG is increased two- to five fold in diabetic patients (McLellan et al., 1994). Glycation of LDL by MG is directed to the arginine residues present in apoB-100 and culminates with the formation of the hydroimidazolone N_{δ}-(5-hydro-5-methyl -4-imidazolon-2-yl)-ornithine residues (MG-H1) which are considered the major AGE in physiological systems (Thornalley, 2005).

LDL glycation and oxidation are closely linked and glycated LDL has been shown to be more sensitive to oxidation than native LDL (nLDL) (Hunt et al., 1990; Kawamura et al., 1994). However, MG-mediated protein glycation is considered a nonoxidative model (Pashikanti et al., 2010).

Considering the important role played by oxidative stress in the atherosclerosis pathogenesis, numerous *in vitro* and *in vivo* studies have been demonstrated the efficacy of antioxidants therapies (Bocan et al., 1992; Fruebis et al., 1994; Shaish et al., 1995;

Portella et al., 2008; de Bem et al., 2009; Barcelos et al, 2011). This scenario highlights the use of medicinal plants, which are promising sources of potent antioxidants and widely used around the world.

Syzygium cumini, popularly known in Brazil as "jambolão" belongs to the family Asteraceae and the hot infusion of its leaves is popularly used for Brazilian people in the treatment of hyperglycemia (Oliveira et al., 2005). Literature findings show that different parts and preparations of *S. cumini* exhibit antihyperglycemic (Ayyanar et al., 2013), antioxidant (Banerjee et al., 2005), anti-inflammatory (Chaudhuri, 1990), antifungal (Braga et al., 2007), anti-HIV (Kusumoto et al., 1995) and antiulcer properties (Ramirez et al., 2003). However, there is no data about the effects of *S. cumini* on the glycation and/or oxidation LDL. Taken the close relationship established among LDL, atherosclerosis and hyperglycemia, this study was undertaken to evaluate the possible antiatherogenic potential of *S. cumini* aqueous-leaf extract on oxidation and glycation parameters in human LDL.

2. Materials and Methods

2.1 Plant material

S. cumini leaves were collected from botanical garden of Universidade Federal de Santa Maria (UFSM). The leaves were then used to prepare the hot infusion: 30 g of leaves in 100 ml of distilled watter at 100ºC. Afterwards, the hot infusion was submitted to lyophilization process. The product was stored at –20ºC and resuspended in distilled for the use in the different assays.

*2.2 HPLC of S.cumini Extrac (S.c*Ext)

High performance liquid chromatography (HPLC-DAD) was performed with the HPLC system (Shimadzu, Kyoto, Japan), Prominence Auto Sampler (SIL-20A), equipped with Shimadzu LC-20AT reciprocating pumps connected to the degasser DGU 20A5 with integrator CBM 20A, UV-VIS detector DAD (diode) SPD-M20A and Software LC solution 1.22 SP1. Reverse phase chromatographic analyses were carried out under gradient conditions using C_{18} column (4.6 mm x 250 mm) packed with 5 μm diameter particles; the mobile phase was water containing 2% acetic acid (A) and methanol (B), and the composition gradient was: 5% of B until 2 min and changed to obtain 25%, 40%, 50%, 60%, 70% and 100% B at 10, 20, 30, 40, 50 and 80 min, respectively, following the method described by Laghari et al. (2011) with slight modifications. The lyophilized aqueous extract of leaves of *Syzygium cumini* was analyzed, dissolved in water at a concentration of 5 mg/mL. The presence of six phenolics compounds was investigated, namely: gallic, chlorogenic and caffeic acids and the flavonoids quercetin, rutin and kaempferol. The identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. The flow rate was 0.5 ml/min, injection volume 40 μl and the wavelength were 254 nm for gallic acid, 325 nm for

caffeic and chlorogenic acids, and 365 nm for quercetin, rutinan and kaempferol. All the samples and mobile phase were filtered through 0.45 µm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.020 – 0.200 mg/ml for kaempferol, quercetin and rutin; and 0.025 – 0.250 mg/ml for gallic, caffeic and chlorogenic acids. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to 400 nm). Calibration curve for gallic acid: $Y = 10237x + 1454.8$ ($r = 0.9998$); caffeic acid: $Y = 11372x + 1366.0$ ($r = 0.9996$); rutin: $Y = 11473x - 1375.7$ ($r = 0.9999$); quercetin: $Y = 12135x - 1392.6$ ($r = 0.9997$) and kaempferol: $Y = 129623x - 1343.9$ ($r = 0.9991$). All chromatography operations were carried out at ambient temperature and in triplicate.

2.3 LDL isolation

LDL isolation was performed by discontinuous density-gradient ultracentrifugation using fresh human plasma as described by Silva et al. (1998), with minor modifications. Plasma of non-fasted healthy normolipidemic voluntary donors was collected and pooled with EDTA (1 mg/ml). To prevent LDL aggregation was added sucrose at final concentration of 0.5 %. Solid KBr (0.326 g/ml) was used to adjust the EDTA-plasma to a density of 1.22 g/ml. Five milliliters of this mixture (EDTA-plasma-KBr) was placed in a centrifuge tube and 5 ml of a sodium chloride solution (density 1.006 g/mL) was overlaid on the top of the mixture. Ultracentrifugation was run at 65,000 rpm for 2 h at 4ºC using a Himac CP80MX ultracentrifuge. LDL particles were collected aspirating the yellow/orange band at the center of saline layer. Isolated LDL was submitted to exhaustive dialysis with 10 mM phosphate buffer (pH 7.4) during 24 h at 4ºC. After dialysis, isolated LDL was stored at -20ºC for less than 2 weeks.

Protein quantification of the isolated LDL was performed by the method described by Lowry et. al. (1951).

2.4 LDL oxidation

2.4.1 Conjugated dienes formation

Isolated LDL (50 µg protein/ml) was incubated at 37ºC with different concentrations (0.25-1.5 µg/ml) of *S.c*Ext in a medium containing phosphate buffer (10 mM, pH 7.4). After 5 minutes, the oxidation was initiated by the addition of $CuSO_4$ (10 µM). In another set of experiments, we verified the effect of *S.c*Ext (1.0 µg/ml) after the initiation of the conjugated dienes formation. For this, *S.c*Ext (1.0 µg/ml) was added at 60 and 70 minutes after $CuSO_4$ (10 µM) addition. We also evaluated the conjugated dienes formation in LDL isolated from human plasma incubated with *S.c*Ext. Human plasma was incubated without or with 200, 400 or 600 µg/ml of *S.c*Ext during 5 h at 37 ºC. Afterwards, LDL was isolated as described in the section 2.2. Conjugated dienes formation was evaluated spectrophotometrically by changes in the absorbance at 234 nm as described previously (Esterbauer et. al., 1989). Absorbance was measured every 20 minutes.

2.4.2 Lag phase determination and oxidation rate

Several parameters can be obtained from the conjugated dienes formation vs. time profile. One of them is the lag phase which is commonly determined graphically by the intercept of the tangents to the slow and fast increase of the dienes absorption. Another parameter is the maximum oxidation rate, given by the peak of the first derivative, i.e., change of absorbance at 234 nm as a function of time (Gieseg and Esterbauer, 1994).

2.4.3 LDL-tryptophan fluorescence

A single band centered at approximately 332 nm is observed in the fluorescence spectra of native LDL. This is attributed to the tryptophan (Trp) residues in ApoB-100 (Giessauf et al., 1995). So, loss of Trp fluorescence is considered a marker of oxidative damage in the protein moiety of LDL. In this study, LDL-Trp fluorescence measurement was performed incubating LDL (50 µg protein/ml) at 37ºC in the presence or absence of $CuSO_4$ (10 µM) and different concentrations of *S.c*Ext (0.25-8.0 µg/ml) in a medium containing phosphate buffer (10 mM, pH 7.4). We also evaluated the loss of Trp fluorescence in LDL isolated from human plasma incubated with *S.c*Ext. Human plasma was incubated without or with 200, 400 or 600 µg/ml of *S.c*Ext during 5 h at 37 ºC. After, LDL was isolated as described in the section 2.2.The fluorescence (excitation at 282 nm and emission at 331 nm) was measured every 20 minutes in a Shimadzu espectrofluorometer as previously described (Giessauf et al., 1995; Reyftmann et al., 1990). The results were expressed as the percentage of the decrease in Trp fluorescence. The time required for reaching half Trp fluorescence (t1/2) was also calculated.

2.4.4 TBARS production

Lipid peroxidation levels of LDL were evaluated by thiobarbituric acid reactive substances (TBARS), following the method described by Okhawa et. al (1979) with minor modifications. LDL (50 µg protein/ml) was incubated for 2 h at 37ºC in the presence or absence of $CuSO_4$ (10 µM) and different concentrations of *S.c*Ext (0.25-2.0 µg/ml) in a medium containing phosphate buffer (10 mM, pH 7.4). Afterwards, 300 µl of SDS 8.1 %, 500 µl of phosphoric acid and 500 µl of TBA 0.8 % were added. TBARS formation was also evaluated in the LDL samples isolated from human plasma incubated without or with 200, 400 or 600 µg/ml of *S.c*Ext for 5 h at 37 ºC.

The color reaction was performed by the incubation for 1 h at 95ºC. TBARS production was measured spectrophotometrically at 532 nm and the results were expressed as percentage of control.

1.5 Serum oxidation

Conjugated dienes formation was used to analyze the serum oxidation. To evaluate the serum oxidation levels, venous blood was draw from nonfasted healthy normolipidemic voluntary donors into tubes containing no anticoagulant and centrifuged for 10 minutes at 1000 x g. The serum (diluted 100-fold) was incubated in the presence or absence of different concentrations of *S.c*Ext (0.25-1.0 µg/ml) and $CuSO_4$ (30 µM) in a medium containing phosphate buffer (10 mM, pH 7.4). In another set of experiments, we verified the effect of *S.c*Ext (1.0 µg/ml) after the initiation of the conjugated dienes formation. For this, *S.c*Ext (1.0 µg/ml) was added at 50 and 60 minutes after $CuSO_4$ (30 µM) addition. Conjugated dienes formation was monitored by changes in the absorbance at 245 nm as previously described (Schnitzer et. al., 1998).

2.6 Plasma oxidation

TBARS levels were used to evaluate the plasma oxidation. Venous blood was draw from nonfasted healthy normolipidemic voluntary donors into tubes containing anticoagulant (heparin) and centrifuged for 10 minutes at 1000 x g. Fresh plasma (diluted 1:4) was incubated for 3 h at 37ºC in the presence or absence of $CuSO_4$ (100 µM) and different concentrations of *S.c*Ext (4.0-24.0 µg/ml) in a medium containing phosphate buffer (10 mM, pH 7.4). Then, 300 µl of SDS 8.1 %, 500 µl of phosphoric acid and 500 µl of TBA 0.8 % were added. The color reaction was performed by the

incubation for 1 h at 95ºC. TBARS production was measured spectrophotometrically at 532 nm and the results were expressed as percentage of control.

2.7 LDL glycation

2.7.1 AGEs formation by LDL glycation

Fluorescent AGEs formation was performed incubating the isolated LDL (1.4 mg protein/ml) and methylglyoxal (10 mM) for 72 h at 37ºC in the presence or absence of a medium containing phosphate buffer (10 mM, pH 7.4), 10 mM of aminoguanidine (positive control) and different concentrations of *S.c*Ext (0.1-1.0 mg/ml). Afterwards, the samples were dialyzed for 24 h at 4ºC against 2 l of 10 mM phosphate buffer (pH 7.4). The samples were diluted (1:10) in phosphate buffer and the fluorescence (λ_{EX} = 370 nm, λ_{EM} = 440 nm) was measured using a Shimadzu spectrofluorometer as previously described (Pashikanti et al, 2010). The results were expressed as fluorescence units (F.U).

2.7.2 Electrophoresis

Electrophoresis was performed with the same samples used for AGEs measurement (see 2.7.1). The electrophoresis was conducted with agarose gel (0.7 %) for 60 minutes at 100 V and 400 mA (Younis et. al., 2009).

2.8 Statistical analyzes

Data are expressed as means ± SD. Statistical analysis was performed using a one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test when appropriate. Differences were considered significant when $p < 0.05$.

3. Results

3.1 Effects of S.cExt on LDL oxidation

3.1.1 Conjugated dienes formation

*S.c*Ext was able to delay the conjugated dienes formation $CuSO_4$-induced, causing a marked increase in the lag phase in a concentration dependent manner (p<0.05, Fig. 1A and 1B). Indeed, the addition of *S.c*Ext, 60 and 70 min, after the start of conjugated dienes formation blocked the progression of the oxidation elicited by $CuSO_4$ (Fig. 1C). *S.c*Ext also reduced the maximum oxidation rate, at all tested concentrations, when compared to $CuSO_4$ (p<0.05, Table 1). In the experiments performed with LDL isolated from plasma incubated with *S.c*Ext, the lag phase was significantly increased by *S.c*Ext (400 and 600 µg/ml) when compared to LDL exposed to $CuSO_4$ alone (p<0.05, Fig. 4A and 4B).

3.1.2 TBARS production

The levels of lipid peroxidation in isolated LDL, evaluated by TBARS production, were significantly increased by $CuSO_4$. *S.c*Ext, at concentrations of 0.5 to 2.0 µg/ml, was able to blunt this effect induced by $CuSO_4$ (p<0.05, Fig. 2). Similarly, TBARS content was decreased in the LDL isolated from plasma incubated with 400 and 600 µg/ml of *S.c*Ext when compared to the values found in LDL exposed to $CuSO_4$ alone (p<0.05, Fig. 4C).

3.1.3 LDL-Trp Fluorescence

The data of Fig. 3 show that *S.c*Ext was effective in reducing the loss of LDL-Trp fluorescence $CuSO_4$-induced. *S.c*Ext increased significantly the required time for reaching half Trp fluorescence ($t_{1/2}$) in concentrations up to 0.75 µg/ml and for the

concentrations 2 to 8 μg/ml the Trp $t_{1/2}$ obtained was higher than the time period of the experiment (180 min) ($p<0.05$). Similar to previous results obtained with conjugated dienes formation and TBARS production, the incubation of human plasma with 400 and 600 μg/ml of *S.c*Ext prevented partially the loss of LDL-Trp fluorescence and the Trp $t_{1/2}$ was significantly higher than LDL incubated with $CuSO_4$ alone ($p<0.05$, Fig. 5A and 5B).

3.2 Serum Oxidation

In this set of experiment, the *S.c*Ext was also able to delay the conjugated dienes formation induced by $CuSO_4$, causing a significant increase in the lag phase in a concentration dependent manner ($p<0.05$, Fig. 6A and 6B). However, when *S.c*Ext was added after the start of conjugated dienes formation, it was not effective in blocking the progression of the oxidation (Fig. 6C). Here, *S.c*Ext also caused a significant reduction in the maximum oxidation rate when compared to $CuSO_4$ ($p<0.05$, Table 2). This effect was verified in all concentrations tested.

3.3 Plasma Oxidation

The levels of human plasma oxidation $CuSO_4$-induced were evaluated by TBARS production measurement. In this parameter was possible observe that all tested concentrations of *S.c*Ext were able to blunt the plasma oxidation induced by $CuSO_4$ ($p<0.05$, Fig. 7).

3.4 Effect of S.cExt on LDL glycation

3.4.1 AGEs formation

As expected, the incubation of LDL with MG (10 mM) for 72 h caused an increase in fluorescent AGEs formation, which was completely blocked by AG, used as an anti-glycant control. On the order hand, the *S.c*Ext was not able to reduce the AGEs levels induced by LDL glycation ($p<0.05$, Fig. 8)

3.4.2 Electrophoresis

Incubation of LDL with MG (10 mM) for 72 h culminated with an increase in LDL eletroforetic mobility, which was represented by higher distance traveled into agarose gel when compared to the control. This increased dislocation of LDL was prevented by AG treatment. Different, no effect of *S.c*Ext (0.1-1.0 mg/ml) was observed in this parameter (Fig. 9).

3.5 S.cExt composition by HPLC

HPLC fingerprinting of *Syzygium cumini* lyophilized aqueous extract revealed the presence of the gallic acid (t_R = 14.72 min; 3.46%; peak 1), chlorogenic acid (t_R = 25.81 min; 2.09%; peak 2), caffeic acid (t_R = 34.27 min; 1.57%; peak 3), rutin (t_R = 39.64 min; 4.95%; peak 4), quercetin (t_R = 54.43 min; 3.37%; peak 5) and kaempferol (t_R = 47.28 min; 0.62%; peak 6) (Fig. 10).

4. Discussion

Several lines of evidence suggest that modifications in the structure of low density lipoproteins implicate in an increase risk to development of atherosclerosis (Witzum, 1994). LDL oxidation which culminates in the formation of oxLDL is a key factor in the atherosclerosis development principally via stimulation of macrophage foam cell formation (Ross, 1999). Elevated levels of cooper in plasma have been associated with existing atherosclerosis and it represents a risk factor to the development of disease (Ferns et al., 1997). Thus, the use of cooper ions to induce LDL peroxidation *in vitro* is considered a valid method to study the events that occur in atherosclerotic artery wall (Smith et al., 1992). There are several assays to evaluate the extension of LDL peroxidation (Esterbauer et. al., 1992). However, due to its convenience and objectivity, the formation of conjugated dienes (CD) monitored by the changes in absorbance at 234 nm is the most used method to monitor LDL oxidation *in vitro.* After the initiation of lipid peroxidation, there is a rearrangement in the double bonds of the lipids which leads to the formation of CD. In view of this, CD formation is considered an early event in the chain reaction (Kappus, 1985). In this study, we demonstrated that the *S.c*Ext delayed the LDL conjugated dienes in a concentration dependent manner (Fig. 1A). This effect is clearly observed analyzing the increase in the lag phase (Fig 1B). The lag phase determination represents the resistance of LDL to oxidation expressed by the time of the consumption of the antioxidants. It is determined graphically by the intercept of the tangents to the slow and fast increase of the diene absorption. This parameter is widely used to test antiathrogenic compounds (Witzum, 1994; Gieseg and Esterbauer, 1994). We also investigated the effect of *S.c*Ext after the start of CD formation. The results expressed in Fig 1C demonstrate the efficacy of *S.c*Ext, at 1 µg/ml, in blocking the

progression of CD formation in LDL at two different times (50 and 60 minutes). Another parameter that can be obtained from dienes absorbance vs. time profile is the oxidation rate which is given by the peak of first derivative, i.e., change of A_{234} as a function of time (Gieseg and Sterbauer, 1994). In this parameter, our results showed that *S.c*Ext, at all tested concentrations, was able to prevent the reduction of oxidation rate caused by $CuSO_4$ (Table 1). To better mimic the physiological conditions and to verify a possible direct interaction between *S.c*Ext and LDL, we incubated human plasma with different concentrations of *S.c*Ext during 5 h at 37 ºC and after LDL was isolated and submitted to oxidation. Herein, we showed that the exposure of plasma to *S.c*Ext (400 and 600 μg/ml) caused a significant increase in lag phase of oxidized LDL (Fig. 4A and 4B).

Since CD formation is an early event in lipid oxidation reactions, TBARS production can be used as a tool to verify the levels of malondialdehyde (MDA), an end product of the chain reaction (Okhawa et. al., 1979). In this study, *S.c*Ext exhibited a potent effect in inhibiting MDA formation in LDL. Similarly to the effect on CD formation, LDL isolated from plasma incubated with 400 and 600 μg/ml of *S.c*Ext was more resistant to MDA formation $CuSO_4$-induced.

Beside lipid oxidation in isolated LDL, we also evaluated the effect of *S.c*Ext on the oxidation of LDL protein moiety by the loss of Trp fluorescence. It has been reported that the fluorescence spectrum of native LDL displays a single band centered at approximately 332 nm which is assigned to the Trp residues present in apoB-100. So, the loss of Trp fluorescence is considered a marker of oxidative damage in the protein core of LDL (Reyftmann et al., 1990). Here, *S.c*Ext exhibited a potent effect in preventing the loss of Trp fluorescence causing a considerable increase in the required time for reaching half Trp fluorescence ($t_{1/2}$). In the end of the

experimental period (180 minutes), *S.c*Ext at 8 μg/ml maintained about 95% of Trp fluorescence (Fig. 3). Moreover, LDL isolated from plasma incubated 5 h with *S.c*Ext (400 and 600 μg/ml) was more resistant to the loss of Trp fluorescence than native LDL (Fig. 5). These observations demonstrate that *S.c*Ext can protect both lipid and protein core of LDL from $CuSO_4$-induced oxidation.

In the literature, there are controversial studies about the use of antioxidants as an alternative therapy for atherogenesis (Mashima et al., 2001). This conflict may contribute for our limited understanding about LDL oxidation in atherogenic process and about how antioxidants could act in preventing CVD. However, several findings demonstrate that the use of natural antioxidants and herbal medicine can inhibit the formation of oxLDL and consequently decrease the development and/or progression of atherosclerosis, attributing the effects to its phytochemical composition (Ahmadvand et al., 2012; Jeong et al., 2008; Lee et al., 2006). Among the most studied phytochemical constituents, polyphenolic compounds have demonstrated to possess various pharmacological properties, including antioxidant, anti-inflamatory, anticarcinogenic and antiatherogenic activities (Graziano et al., 1992; Surh, 2003). Indeed, *in vitro* studies have demonstrated that polyphenolic compounds from different plants can inhibit LDL oxidation (Teissedre et al., 1996; Waterhouse et al., 1996; Yoshida et al., 1999). Here, HPLC analyses showed the presence of six polyphenolic compounds in *S.c*Ext, namely: Gallic acid, chlorogenic acid, caffeic acid, rutin, quercetin and kaempferol (Fig. 10). It is plausible assume that the potent effect of *S.c*Ext in inhibiting LDL oxidation could be attributed, at last in part, to its polyphenolic composition. However, more studies are needed to verify if the effect is offered by a specific compound or by a synergic effect among them. In this way, Wu et al. (2010) demonstrated that the polyphenol epigallocatechin gallate (EGCG) binds

to LDL and protect it from oxidation and glycation in samples isolated from plasma incubated during 3 h with EGCG. Here we showed that LDL isolated from plasma incubated during 5 h with *S.c*Ext (400 and 600 µg/ml) was more resistant to CuSO4-induced lipid and protein oxidation than native LDL (Fig. 4 and 5). We suggest that probably one or more polyphenolic compound of *S.c*Ext can bind to LDL, similarly to EGCG, and confer the protection against the oxidation.

LDL *in vivo* is present in the interstitial space of the artery wall, a place where there is various antioxidants from plasma/serum (Schwarz et al., 1991; Witztum and Steinberg, 1991). In view of this, Spranger et al. (1998) suggested that LDL oxidation *in vivo* can be more adequately tested *in vitro* using whole plasma/serum. In this work, we evaluated the effect of *S.c*Ext on conjugated dienes formation in human serum and TBARS production in human plasma. Similarly to isolated LDL, *S.c*Ext caused a significant delay in CD $CuSO_4$-induced increasing the lag phase in a concentration dependent manner (Fig. 6A and 6B). On the other hand, no effect was observed when *S.c*Ext was added after the initiation of CD formation (Fig. 6C). It is plausible assume that the endogenous antioxidants have been responsible for the reduction in CD content. In human plasma, similarly to isolated LDL, *S.c*Ext caused a significant protection against TBARS production. The higher concentration of *S.c*Ext was able to maintain lipid peroxidation at basal levels (Fig. 7).

Previous studies have been reported that hyperglycemia can accelerate LDL oxidation and glycation with consequent AGEs formation (Wu et al., 2009). Similarly to oxLDL, the surface charge of glyLDL is also more negative and apoB-100 conformation is altered, impairing its recognition by the classical LDL receptor (Jenkins et al., 2004). The negative residual charges are responsible to increase the LDL eletrophoretic mobility. In accordance, the Fig. 8 clearly shows the increase in

eletrophoretic mobility of LDL MG-glycated in comparison with native LDL. In contrast to oxidative parameters, *S.c*Ext did not alter the glycation of LDL induced by MG. LDL samples incubated with AG, a classical inhibitor of glycation process, were maintained in a position similar to native LDL in agarose gel. Additionally, we evaluated the AGEs formation based in the fact that the fluorescence at 370 nm of excitation and 440 nm of emission is a general measure to detect the presence of AGEs (Pashikanti, S. et al., 2010). Corroborating with the results obtained in agarose gel electrophoresis, *S.c*Ext was not effective in blunting MG-induced AGEs formation (Fig. 8).

Protein glycation mediated by glucose can occur in both oxidative and nonoxidative conditions (Fu et al., 1994). Because of this, many potential antiglicant candidates are difficult to distinguish from general antioxidants. In contrast, reactive carbonyl compounds like MG mediate nonoxidative glycation reactions (Baynes, 1991; Cervantes-Laurean et al., 1996). Thus, this model offers an important advantage on glucose-induced glycation because it allows us to identify pure glycation inhibitors, which can act independent of its antioxidant potential. This fact may explain the reason by which *S.c*Ext did not show any effect on MG-induced LDL glycation.

In conclusion, the findings obtained in this work showed that *S.c*Ext posses a strong antioxidant capacity in inhibiting human LDL, serum and plasma oxidation induced by $CuSO_4$. *S.c*Ext was also able to maintain the integrity of LDL apoB-100. LDL isolated from plasma incubated with *S.c*Ext was more resistant to lipid and protein oxidation suggesting a possible bind of one or more *S.c*Ext constituents with LDL. On the other hand, *S.c*Ext was not effective in protecting LDL from glycation induced by MG. Although this result indicates that *S.c*Ext did not exhibit anti-glycant

effect, further studies are needed to verify if the antioxidant capacity of *S.c*Ext could inhibit LDL glycation in an oxidative model. Taken together, our data suggest that *S.c*Ext may be considered a promising antiatherogenic agent.

5. References

Ahmadvand, H. et al. Effects of olive leaves extract on LDL oxidation induced-$CuSO_4$ in vitro. **Pak J Pharm Sci** 25:571-575, 2012.

Ayyanar, M. et al. Syzygium cumini (L.) Skeels., a novel therapeutic agent for diabetes: Folk medicinal and pharmacological evidences. **Comp Ther M** 21:232-243, 2013.

Banerjee, A. et al. In vitro study of antioxidant activity of Syzygium cumini fruit. **Food Chem** 90:727-733, 2005.

Barcelos, R.P. et al. Thiosemicarbazone derivates protects from AAPH and Cu^{2+} -induced LDL oxidation. **Life Sci** 89:20-28, 2011.

Baynes, J.W. Role of oxidative stress in development of complications in diabetes. **Diabetes** 40: 405-412, 1991.

Bocan, T.M. et al. Antiatherosclerotic effects of antioxidants are lesion-specific when evaluated in hypercholesterolemic New Zealand white rabbits. Exp Mol Pathol 57:70-83, 1992.

Braga, F.G. et al. Antileishmanial and antifungal activity of plants used in traditional medicine in Brazil. **J Ethnopharmacol** 111:396-402, 2007.

Cervantes-Laurean, D. et al. Glycation and glycoxidation of histones by ADP-ribose. **J Biol Chem** 271:10461-10469; 1996.

Chaudhuri, A.K.N. Antiinflammatory and related actions of Syzigium cumini seed extract. **Phytother R** 4:5-10, 1990.

Chisolm, G.M and Steinberg, D. The oxidative modification hypothesis of atherogenesis: an overview. Free Radical Bio Med 28:1815-1826, 2000.

de Bem, A.F. et al. Diphenyl diselenide, a simple glutathione peroxidase mimetic, inhibits human LDL oxidation *in vitro*. Atherosclerosis 201:92-100, 2008.

Esterbauer, H. et al. The role of lipid peroxidation and antioxidants in oxidative modifications of LDL. **Free Radic Biol Med** 13:241-290, 1992.

Esterbauer, H. et. al. Continuous monitoring of in vitro oxidation of human low density lipoprotein. **Free Radical Res Com** 6:67-75, 1989.

Ferns, G.A.A. et al. The possible role of copper ions in atherogenesis: the Blue Janus. **Atherosclerosis** 133:139-52, 1997.

Fruebis, J. et al. A comparison of antiatherogenic effects of probucol and of a structural analogue of probucol in low density lipoprotein receptor-deficient rabbits. **J Clin Invest** 94:392-398, 1994.

Fu, M.X. Et al. Glycation, glycoxidation, and cross-linking of collagen by glucose: kinetics, mechanisms, and inhibition of late stages of the Maillard reaction. **Diabetes** 43:676-683, 1994.

Gaziano, J. M. et al. Dietary antioxidants and cardiovascular disease. **Ann N Y Acad Sci** 669:249-259, 1992.

Gieseg, S.P and Esterbauer, H. Low density lipoprotein is saturable by pro-oxidant copper. **FEBS Lett** 343:188-194, 1994.

Giessauf, A. et al. Early destruction of tryptophan residues of apolipoprotein B is a vitamin E- independent process during copper-mediated oxidation of LDL. **Biochim Biophys Acta** 1256:221-232, 1995.

Hunt, J.V. et al. Auto-oxidative glycosylation and possible involvement of peroxides and free radicals in LDL modification by glucose. **Diabetes** 39:1420-1424, 1990.

Jenkins, A.J. et al. Lipoproteins, glycoxidationand diabetic angiopathy. **Diabetes-Metab Res** 20:349-368, 2004.

Jeong, T.S. et al. Low density lipoprotein (LDL)-antioxidant flavonoids from roots of Sophora flavescens. **Biol Pharm Bull** 31:2097-2102, 2008.

Kappus, H. Lipid peroxidation: mechanisms, analysis, enzymology and biological relevance. In: Sies, H., ed. Oxidative stress. **London: Academic Press** pp. 273-310, 1985.

Kawamura, M. et al. Pathophysiological concentrations of glucose promote oxidative modification of low density lipoprotein by a superoxide-dependent pathway. **J Clin Invest** 94:771-778, 1994.

Kusumoto, I.T. et al. Screening of various plant extracts used in ayurvedic medicine for inhibitory effects on human immunodeficiency virus type I (HIV-I) protease. **Phytother R** 12:488-493, 1995.

Laghari, A.H. et al. Determination of free phenolic acids and antioxidant activity of methanolic extracts obtained from fruits and leaves of *Chenopodium album*. **Food Chem** 126:1850-1855, 2011.

Lee, J. H. et al. LDL-antioxidant pterocarpans from roots of Glycine max (L.) Merr. **J Agric Food Chem** 54:2057-2063, 2006.

Lee, O. et al. Fructose and carbonyl metabolites as endogenous toxins. **Chem Biol Interact** 178:332-339, 2009.

Lowry, O.H. et al. Protein measurement with the Folin phenol reagent. **J Biol Chem** 193:265-275, 1951.

Lyons, T.J. et al. Glycosylation of low-density lipoprotein in patients with type I (insulin dependent) diabetes: correlations with other parameters of glycemic control. **Diabetologia** 29:685-691, 1986.

Mashima, R. et al. Oxidants and antioxidants in atherosclerosis. **Curr Opin Lipidol** 12:411-418, 2001.

Matsuura, E. et al. Oxidative modification of low-density lipoprotein and imunne regulation of atherosclerosis. **Prog Lipid Res** 45:466-486, 2006.

McLellan, A.C. et al. Glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications. **Clin Sci** 87:21-29, 1994.

Nishi, K. et al. Oxidized LDL in carotid plaques and plasma associates with plaque instability. **Atheroscler Thromb Vasc Biol** 22:1649-1654, 2002.

O'Brien, P.J. et al. Fructose-derived endogenous toxins, in: O'Brien, P.J. and Bruce, W.R. (Eds.). Endogenous Toxins: Targets for Disease Treatment and Prevention. **Wiley-VCH Verlag GmbH & Co. KGaA**, Weinheim, pp. 173-212, 2010.

Okhawa, H. et al. Assay for lipid peroxides in animal tissue by thiobarbituric acid reaction. **Anal Biochem** 95:351-358, 1979.

Oliveira, A.C.P. et al. Effect of the extracts and fractions of Baccharis trimera and Syzygium cumini on glycaemia of diabetic and non-diabetic mice. **J Ethnopharmacol** 102:465-469, 2005.

Parhami, F. et al. Minimally modiied low density lipoprotein-induced inlammatory responses in endothelial cells are mediated by cyclic adenosine monophosphate. **J Clin Invest** 92:471-478, 1993.

Pashikanti, S. et al. Rutin metabolites: Novel inhibitors of nonoxidative advanced glycation end products. **Free Radic Biol Med** 48:656-663, 2010.

Portella, R.L. et al. Oximes as inhibitors of low density lipoprotein oxidation. **Life Sci** 83:878-885, 2008.

Ramirez, R.O. et al. The gastroprotective effect of tannins extracted from duhat (Syzygium cumini Skeels) bark on HCl/ethanol induced gastric mucosal injury in Sprague-Dawley rats. **Cl Hemorh M** 29:253-261, 2003.

Reyftmann, J.P. et al. Sensitivity of tryptophan and related compounds to oxidation induced by lipid autoperoxidation. Application to human serum low- and - high density lipoproteins. **Biochim Biophys Acta** 1042:159-167, 1990.

Ross, R. Atherosclerosis – An inflammatory disease. **N Engl J Med** 340:115-126, 1999.

Schnitzer, E. et al. Lipid oxidation in unfractionated serum and plasma. **Chem Phys Lipids** 92:151-170, 1998.

Schwartz, C. et al. The pathogenesis of atherosclerosis: an overview. **Clin Cardiol** 14:11-16, 1991.

Shaish, A. et al. Beta-carotene inhibits atherosclerosis in hypercholesterolemic rabbits. **J Clin Invest** 96:2075-2082, 1995.

Silva, L.E. et al. Inhibition of mammalian 15-lipoxygenase-dependent lipid peroxidation in low-density lipoprotein by quercetin and quercetin monoglucosides. **Arch Biochem Biophys** 349:313-320, 1998.

Smith, C. et al. Stimulation of lipid per-oxidation and hydroxyl-radical generation by the contents of human atherosclerotic lesions. **Biochem J** 286:901-905, 1992.

Spranger, T. et al. How different constituents of human plasma and low density lipoprotein determine plasma oxidizability by copper. **Chem Phys Lipids** 91:39-52, 1998.

Steinberg, D. and Witztum, J.L. Is the oxidative modification hypothesis relevant to human atherosclerosis? Do the antioxidant trials conducted to date refute the hypothesis? **Circulation** 105:2107-2111, 2002

Stocker, R. and Keaney, J.F. Role of oxidative modifications in atherosclerosis. **Physiol Rev** 84:1381-1478, 2004.

Surh, Y. J. Cancer chemoprevention with dietary phytochemicals. **Nat Rev Cancer** 3:768-780, 2003.

Teissedre, P.I. et al. Inhibition of in vitro human LDL oxidation by phenolic antioxidants from grapes and wines. **J Sci Food Agric** 70:55-61, 1996.

Thornalley, P.J. Dicarbonyl intermediat es in the Maillard reaction. **Ann N Y Acad Sci** 1043:111-117, 2005.

Thornalley, P.J. et al. Suppression of the accumulation of triosephosphates and increased formation of methylglyoxal in human red blood cells during hyperglycaemia by thiamine in vitro. **Biochem J** 129:543-549, 2001.

Veiraiah, A. Hyperglycemia, lipoprotein glycation, and vascular disease. **Angiology** 56:431-438, 2005.

Waterhouse, A.L. et al. Antioxidants in chocolate. **Lancet** 348:834, 1996.

WHO (World Health Organization). Definition, diagnosis and classification of Diabetes Mellitus and its complications. WHO/NCD/NCS, 1999.

WHO (World Health Organization). The top 10 causes of death. Fact sheet 310, may 2014. http://www.who.int/mediacentre/factsheets/fs310/en/

Witztum, J.L. and Steinberg, D. Role of oxidized low density lipoprotein in atherogenesis. **J Clin Invest** 88:1785-1792, 1991.

Witztum, J.L. The oxidation hypothesis of atherosclerosis. **Lancet** 344:793-795, 1994.

Wu, C-H. et al. Epigallocatechin gallate (EGCG) binds to low-density lipoproteins (LDL) and protects them from oxidation and glycation under high-glucose conditions mimicking diabetes. **Food Chem** 121:639-644, 2010.

Wu, C-H. et al. Low-density-lipoprotein (LDL)-bound flavonoids increase the resistance of LDL to oxidation and glycation under pathophysiological concentrations of glucose in vitro. **J Agric Food Chem** 57:5058-5064, 2009.

Yoshida, H., et al. Inhibitory effect of tea flavonoids on the ability of cells to oxidize low density lipoprotein. **Biochem Pharmacol** 58:1695-1703, 1999.

Younis, N. et al. Glycation of LDL in non-diabetic people: Small dense LDL is preferentially glycated both in vivo and in vitro. **Atherosclerosis** 202:162-168, 2009.

Figure 1

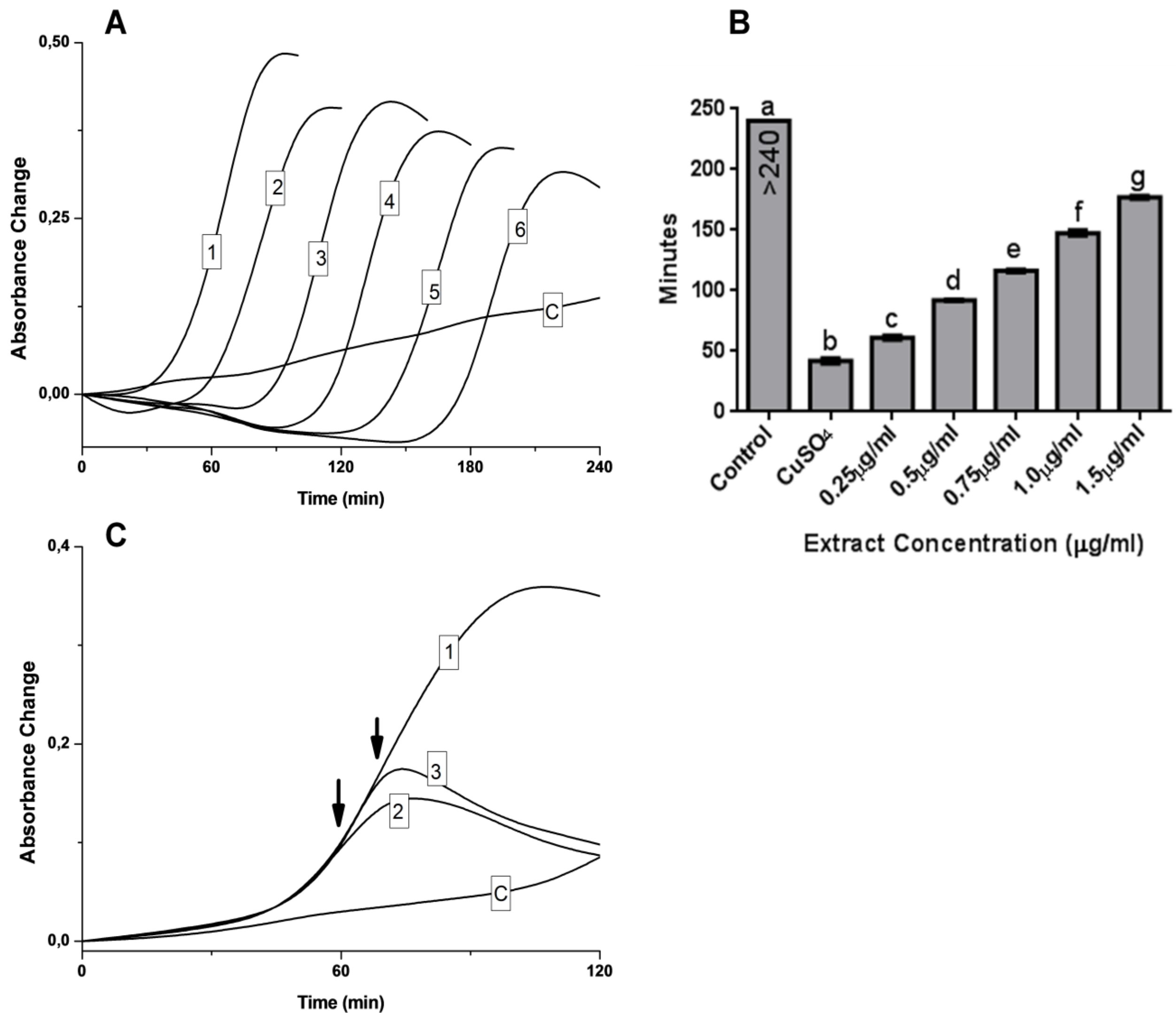

Fig. 1. Effect of *S.c*Ext on conjugated dienes formation in human LDL. **(A)** Representative figure of the dienes formation. The incubation medium without *S.c*Ext (1) or with 0.25 μg/ml (2), 0.50 μg/ml (3), 0.75 μg/ml (4), 1.0 μg/ml (5) or 1.5 μg/ml (6) of *S.c*Ext and $CuSO_4$. Letter C represents the control without *S.c*Ext and $CuSO_4$. **(B)** Effect of *S.c*Ext on lag phase values for conjugated dienes formation in isolated human LDL. **(C)** *S.c*Ext added after the initiation of conjugated dienes formation. The incubation medium without *S.c*Ext (1) or with *S.c*Ext 1.0 μg/ml added at 60 minutes (2) and *S.c*Ext 1.0 μg/ml added at 70 minutes (3). Letter C represents the control without *S.c*Ext and $CuSO_4$. The values are expressed as mean ± SD of four independent experiments in duplicate. Different letters indicate statistically significant differences ($p<0.05$).

Figure 2

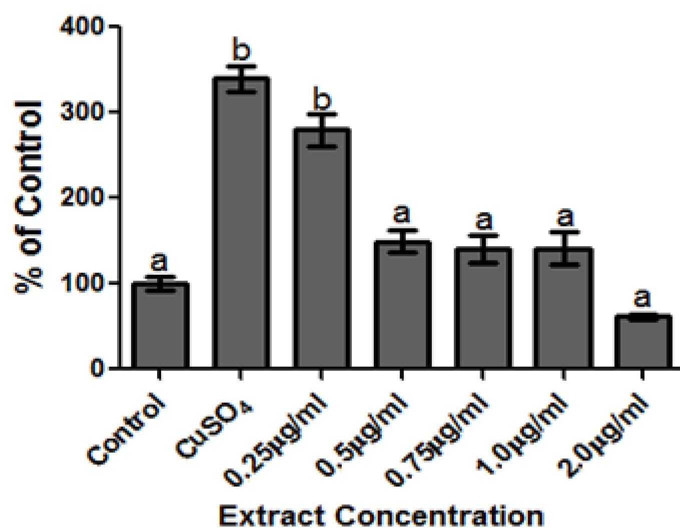

Fig. 2. . Effect of *S.c*Ext concentrations on time-dependent changes in TBARS production during LDL oxidation induced by $CuSO_4$. LDL (50 µg protein/ml) was incubated for 2 h at 37 °C in the presence or absence of 10 µM $CuSO_4$ and *S.c*Ext. The values are expressed as mean ± SD of four independent experiments in duplicate. Different letters indicate statistically significant differences ($p<0.05$).

Figure 3

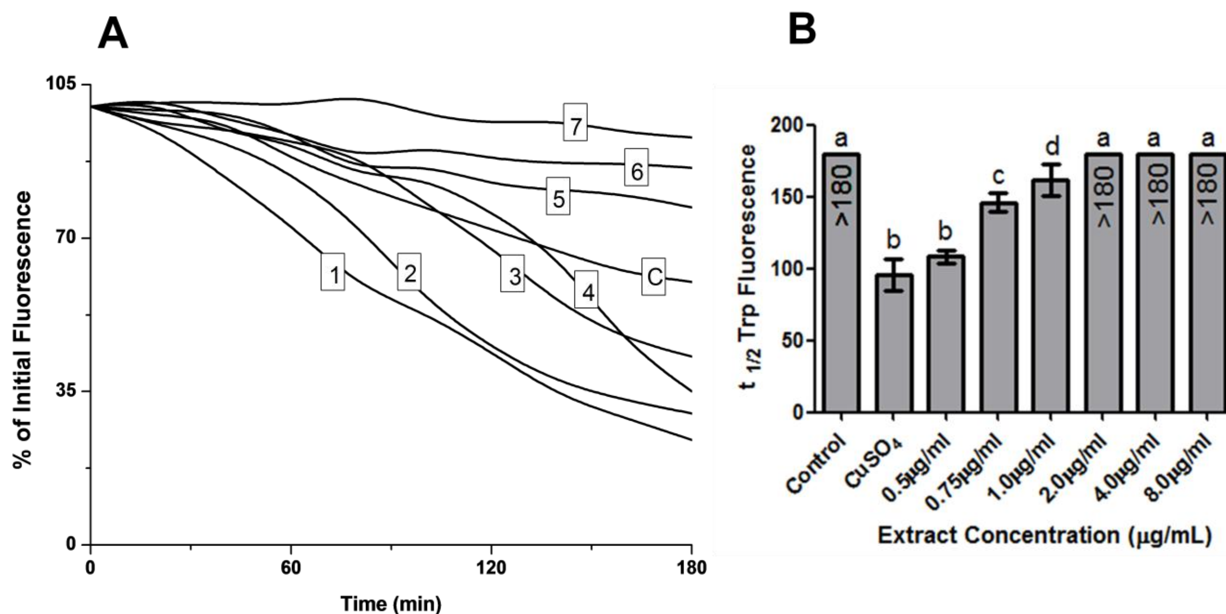

Fig. 3. Effect of *S.c*Ext on Trp fluorescence in human LDL. **(A)** Representative figure of loss in Trp fluorescence. The incubation medium without *S.c*Ext (1) or with 0.50 µg/ml (2), 0.75 µg/ml (3), 1.0 µg/ml (4), 2.0 µg/ml (5), 4.0 µg/ml (6) or 8.0 µg/ml (7) of *S.c*Ext and $CuSO_4$. Letter C represents the control without *S.c*Ext and $CuSO_4$. **(B)** Oxidation $t_{1/2}$ values for Trp fluorescence expressed as the time (minutes) required to cause 50% of loss of the LDL tryptophan. The values are expressed as mean ± SD of four independent experiments in duplicate. Different letters indicate statistically significant differences ($p<0.05$).

Figure 4

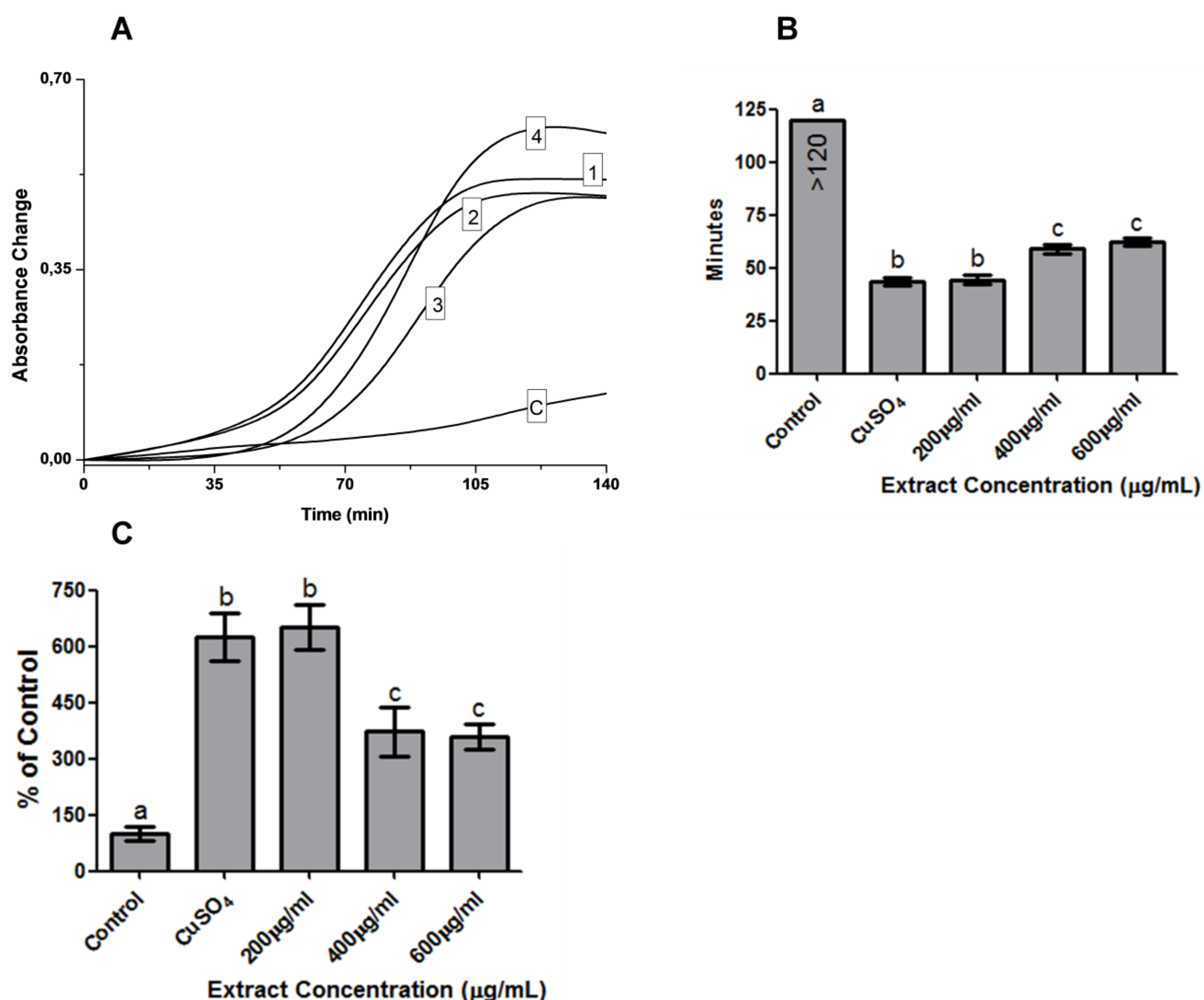

Fig. 4. Oxidative parameters of human LDL isolated from plasma incubated for 5 h with *S.c*Ext . **(A)** Representative figure of the dienes formation. The incubation medium without *S.c*Ext (1) or with 200 µg/ml (2), 400 µg/ml (3),or 600 µg/ml (4) of *S.c*Ext and $CuSO_4$. Letter C represents the control without *S.c*Ext and $CuSO_4$. **(B)** Effect of incubation of human plasma with *S.c*Ext on lag phase values for conjugated dienes formation in isolated human LDL. **(C)** TBARS levels of human LDL isolated from plasma incubated with *S.c*Ext. The values are expressed as mean ± SD of four independent experiments in duplicate. Different letters indicate statistically significant differences ($p<0.05$).

Figure 5

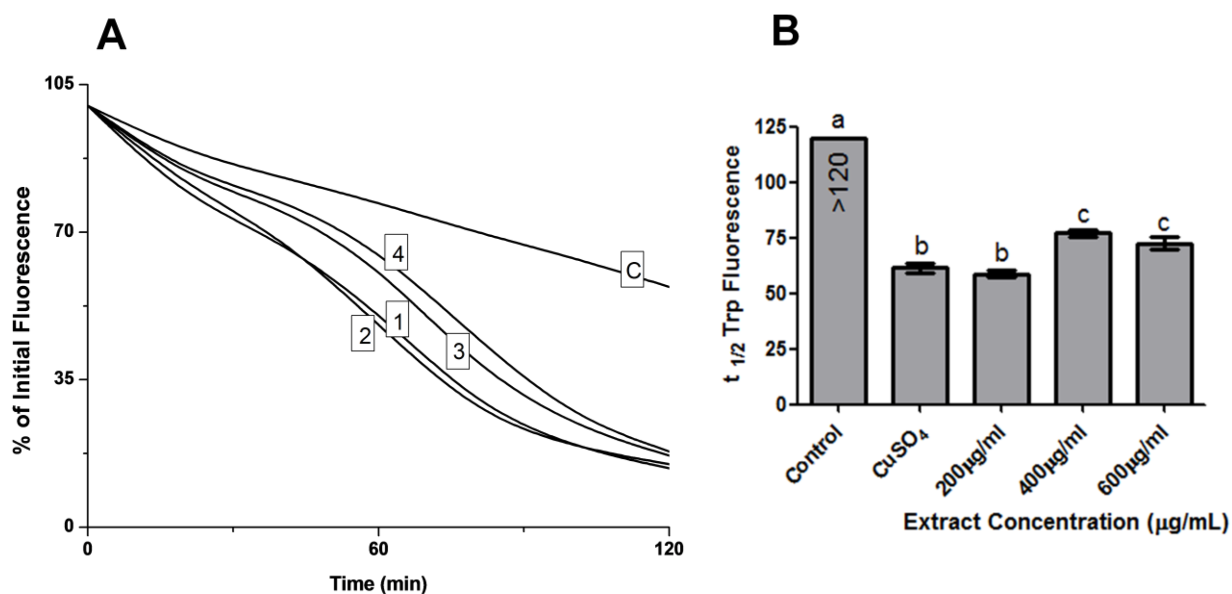

Fig. 5. Loss of Trp fluorescence of human LDL isolated from plasma incubated for 5 h with *S.c*Ext. **(A)** Representative figure of Trp fluorescence. The incubation medium without *S.c*Ext (1) or with 200 µg/ml (2), 400 µg/ml (3),or 600 µg/ml (4) of *S.c*Ext and $CuSO_4$. Letter C represents the control without *S.c*Ext and $CuSO_4$. **(B)** Oxidation $t_{1/2}$ values for Trp fluorescence expressed as the time (minutes) required to cause 50% of loss of the LDL tryptophan. The values are expressed as mean ± SD of four independent experiments in duplicate. Different letters indicate statistically significant differences ($p<0.05$).

Figure 6

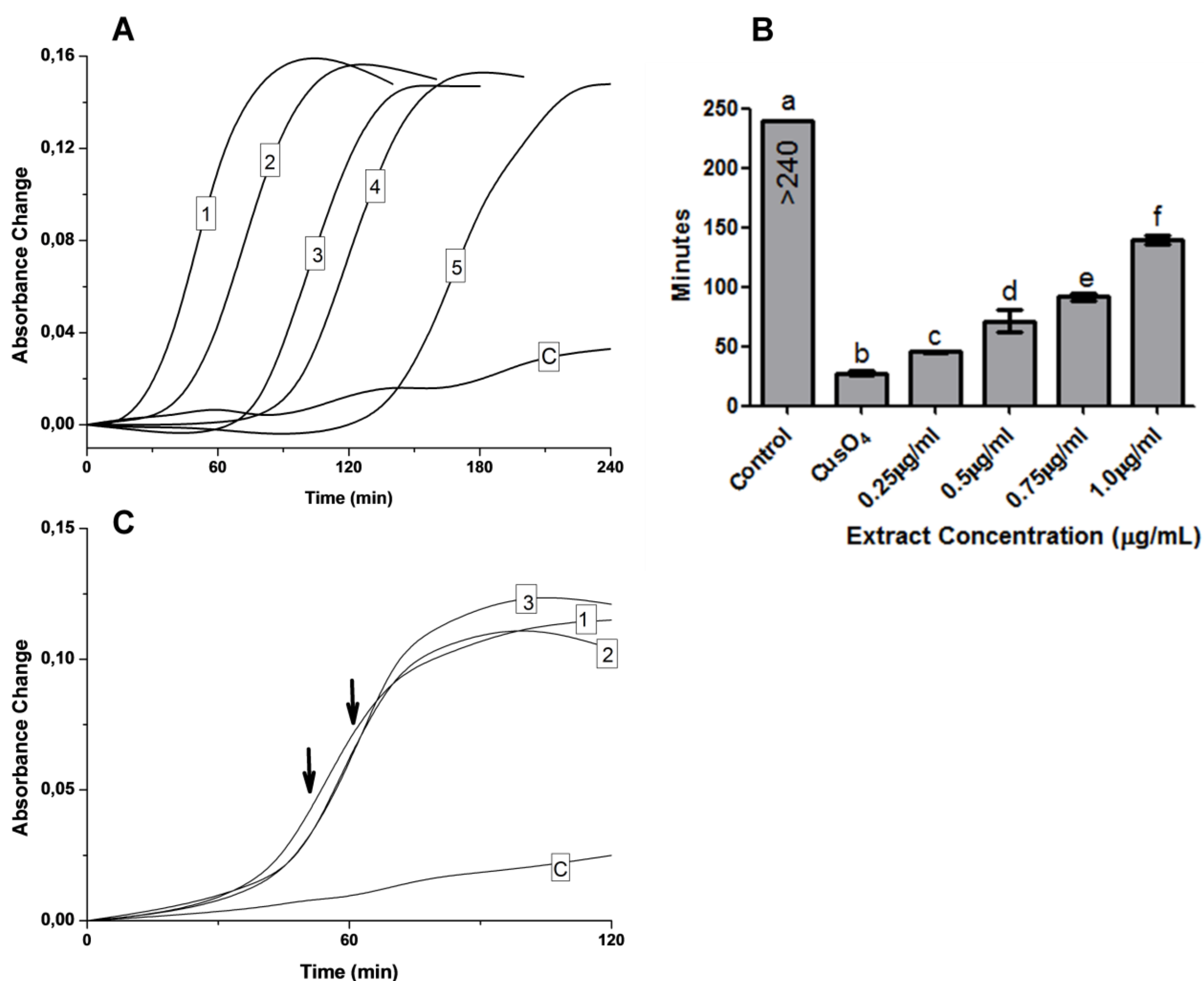

Fig. 6. Effect of *S.c*Ext on conjugated dienes formation in human serum. **(A)** Representative figure of the dienes formation. The incubation medium without *S.c*Ext (1) or with 0.25 µg/ml (2), 0.50 µg/ml (3), 0.75 µg/ml (4) or 1.0 µg/ml (5) of *S.c*Ext and $CuSO_4$. Letter C represents the control without *S.c*Ext and $CuSO_4$. **(B)** Effect of *S.c*Ext on lag phase values for conjugated dienes formation in human serum. **(C)** *S.c*Ext added after the initiation of conjugated dienes formation. The incubation medium without *S.c*Ext (1) or with *S.c*Ext 1.0 µg/ml added at 50 minutes (2) and *S.c*Ext 1.0 µg/ml added at 60 minutes (3). Letter C represents the control without *S.c*Ext and $CuSO_4$. The values are expressed as mean ± SD of four independent experiments in duplicate. Different letters indicate statistically significant differences ($p<0.05$).

Figure 7

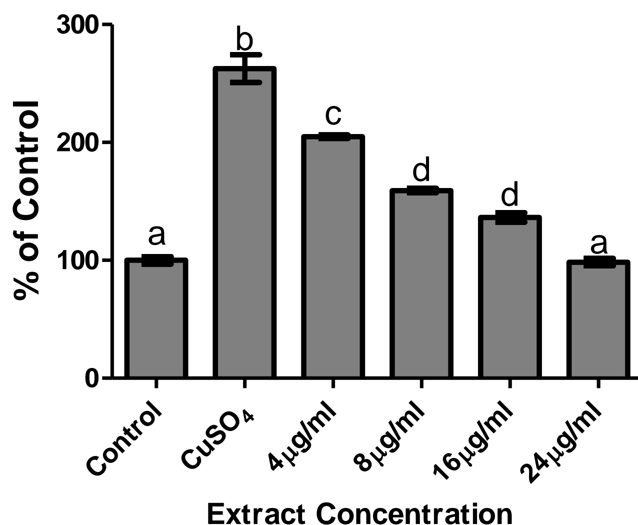

Fig. 7. Effect of *S.c*Ext concentrations on time-dependent changes in TBARS production during plasma oxidation induced by $CuSO_4$. Plasma (diluted 1:4) was incubated for 3 h at 37 °C in the presence or absence of 10 μM $CuSO_4$ and *S.c*Ext. The values are expressed as mean ± SD of four independent experiments in duplicate. Different letters indicate statistically significant differences ($p<0.05$).

Figure 8

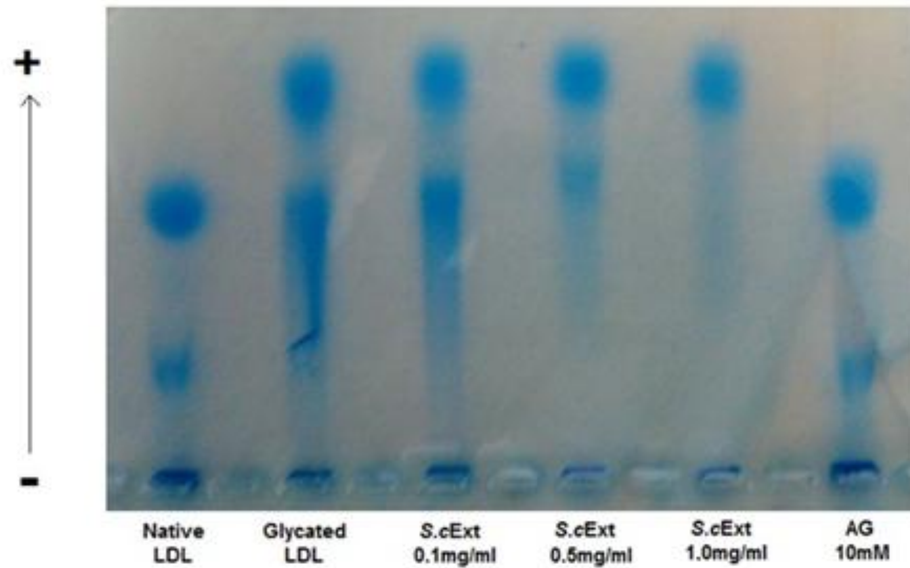

Fig. 8. Effect of *S.c*Ext concentrations on LDL glycation evaluated by agarose gel eletrophoresis. LDL (50 μg protein/ml) was incubated for 48 h at 37 °C in the presence or absence of 10 mM of MG and *S.c*Ext or 10 mM aminoguanidine (positive control).

Figure 9

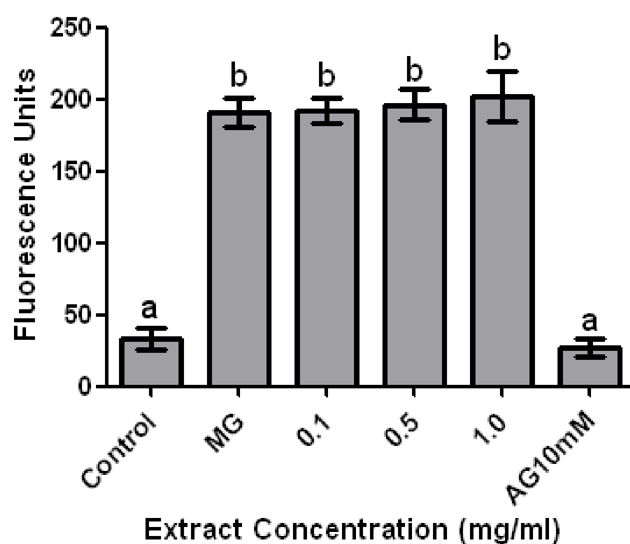

Fig. 9. Effect of *S.c*Ext concentrations on fluorescent AGEs formation. LDL (50 μg protein/ml) was incubated for 48 h at 37 °C in the presence or absence of 10 mM of MG and *S.c*Ext or 10 mM aminoguanidine (positive control). The values are expressed as mean ± SD of four independent experiments in duplicate. Different letters indicate statistically significant differences ($p<0.05$)

Figure 10

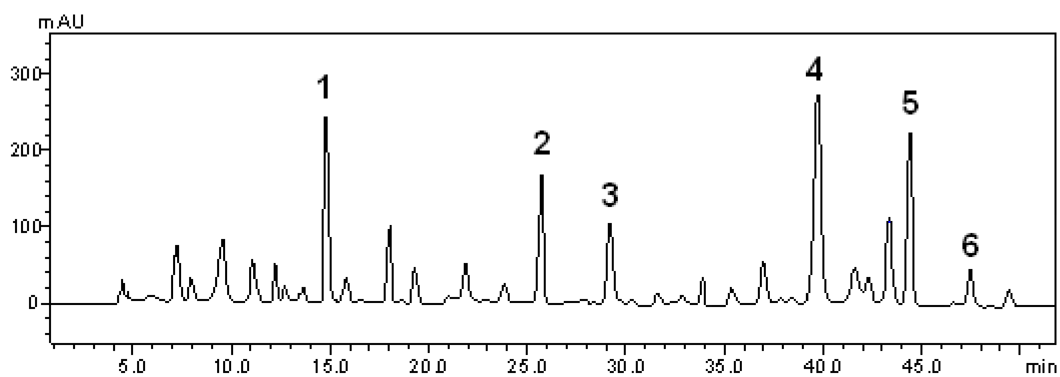

Fig. 10. Representative high performance liquid chromatography profile of *Syzygium cumini*, detection UV was at 325nm. Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), rutin (peak 4), quercetin (peak 5) and kaempferol (peak 6). Chromatographic conditions are described in the Methods section.

Table 1. Effect of *S.c*Ext on the oxidation rate for conjugated dienes formation in human LDL.

	Oxidation Rate (Δabs/20min)
Control	ND
$CuSO_4$	0.313±0.006 **a**
0.25 μg/ml	0.220±0.001 **b**
0.50 μg/ml	0.297±0.006 **c**
0.75 μg/ml	0.287±0.006 **d**
1.00 μg/ml	0.240±0.010 **e**
1.50 μg/ml	0.257±0.006 **f**

LDL was oxidized in the presence of $CuSO_4$ 10 μM with or without different concentrations of *S.c*Ext. The values are expressed as mean± S.D. of three independent experiments in duplicate. Different letters indicate statistically significant differences ($p<0.05$). ND: not determined.

Table 2. Effect of *S.c*Ext on the oxidation rate for conjugated dienes formation in human serum.

	Oxidation Rate (Δabs/20min)
Control	ND
$CuSO_4$	0.079±0.013 **a**
0.25 μg/ml	0.066±0.007 **b**
0.50 μg/ml	0.055±0.001 **c**
0.75 μg/ml	0.054±0.002 **d**
1.00 μg/ml	0.050±0.005 **e**

LDL was oxidized in the presence of $CuSO_4$ 30 μM with or without different concentrations of *S.c*Ext. The values are expressed as mean± S.D. of three independent experiments in duplicate. Different letters indicate statistically significant differences ($p<0.05$). ND: not determined.

4. CONCLUSÕES

De acordo com os resultados obtidos neste trabalho é possível inferir que o *S.cExt:*

- Reduz a formação de dienos conjugados (DC) e aumenta o período de fase lag de maneira concentração-dependente na oxidação de LDL e soro humanos induzida por $CuSO_4$.
- É capaz de bloquear a propagação da formação de DC na LDL.
- Diminui a formação de TBARS induzida por $CuSO_4$ em LDL e plasma humanos.
- Previne danos oxidativos à porção protéica da LDL.
- Quando incubado com o plasma, torna a LDL menos suceptível à oxidação lipídica e protéica.
- Não possui efeito sobre a glicação da LDL induzida por metilglioxal.

Este conjunto de conclusões mostram a eficácia do do *S.cExt* nos modelos experimentais avaliados e apontam o *S.cExt* como um agente antiaterogênico promissor. No entanto, mais estudos com o intuito de elucidar os mecanismos envolvidos na proteção contra a oxidação da LDL bem como os efeitos do *S.cExt* em outros modelos de glicação são necessários.

5. PERSPECTIVAS

Tendo em vista os resultados obtidos nesse trabalho, temos como perpectivas

- Avaliar os efeitos do *S.cExt* sobre a formação de células espumosas em cultura de macrófagos.
- Verificar se o *S.cExt* inibe a oxidação da LDL induzida por outros agentes pró-oxidantes.
- Investigar o efeito do *S.cExt* sobre enzimas e marcadores inflamatórios *in vivo*
- Avaliar os efeitos do *S.cExt* em outros modelos de glicação.

6. REFERÊNCIAS BIBLIOGRÁFICAS

Banerjee, A. et al. In vitro study of antioxidant activity of *Syzygium cumini* fruit. **Food Chem** 90:727-733, 2005.

Basta, G. et al. Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes. **Carviovasc Res** 63:582-592, 2004.

Baynes, J.W. Role of oxidative stress in development of complications in diabetes. **Diabetes** 40: 405-412, 1991.

Braga, F.G. et al. Antileishmanial and antifungal activity of plants used in traditional medicine in Brazil. **J Ethnopharmacol** 111:396-402, 2007.

Brown, M.S. et al. Lipoprotein metabolism in the macrophage: implication for cholesterol deposition in atherosclerosis. **Ann Rev Biochem** 52:223-261, 1983.

Cervantes-Laurean, D. et al. Glycation and glycoxidation of histones by ADP-ribose. **J Biol Chem** 271:10461-10469; 1996.

Chaudhuri, A.K.N. Antiinflammatory and related actions of *Syzigium cumini* seed extract. **Phytother R** 4:5-10, 1990.

Cherubini, A. et al. Role of antioxidants in atherosclerosis: epidemiological and clinical update. **Curr Pharm Des** 11:2017-2032, 2005.

Cushing, S.D et al. Minimally modified low density lipoprotein induces monocyte chemotact protein 1 in human endothelial cells and smooth muscle cells. **Proc Natl Acad Sci USA** 87:5134-5138, 1990.

D'agostino, R.B.Jr. et al. Cardiovascular disease risk factors predict the development of type 2 diabetes: the insulin resistance atherosclerosis study. **Diabetes Care** 27:2234-2240, 2004.

Dhalla, N. et al. Role of oxidative stress in cardiovascular diseases. **J Hypertens** 18:655-673, 2000.

Françoso, L. A.; Coates, V. Anatomicopathological evidence of the beginning of atherosclerosis in infancy and adolescence. **Arq Bras Cardiol** 78:131-142, 2002.

Hioshida, H & Kisugi, R. Mechanisms of LDL oxidation. **Clin Chim Acta** 411:1875-1882, 2010.

Hunt, J.V. et al. Auto-oxidative glycosylation and possible involvement of peroxides and free radicals in LDL modification by glucose. **Diabetes** 39:1420-1424, 1990.

Ishitani, L.H. wt al. Desigualdade social e mortalidade precoce por doenças cardiovasculares no Brasil. **Revista Saúde Pública** 40:684-691, 2006.

Jain, S.K et al. Erythrocyte membrane lipid peroxidation and glycosylated hemoglobin in diabetes. **Diabetes** 38:1539-1543, 1989.

Kardinaal, A. F. et al. Antioxidants in adipose tissue and risk of myocardial infarction: the EURAMIC Study. **Lancet** 342:1379-1384, 1993.

Kawamura, M. et al. Pathophysiological concentrations of glucose promote oxidative modification of low density lipoprotein by a superoxide-dependent pathway. **J Clin Invest** 94:771-778, 1994.

Kusumoto, I.T. et al. Screening of various plant extracts used in ayurvedic medicine for inhibitory effects on human immunodeficiency virus type I (HIV-I) protease. **Phytother R** 12:488-493, 1995.

Libby, P. Inflammation in atherosclerosis. **Nature** 420:868-874, 2002.

Lyons, T.J. et al. Glycosylation of low-density lipoprotein in patients with type I (insulin dependent) diabetes: correlations with other parameters of glycemic control. **Diabetologia** 29:685-691, 1986.

Mahapatra P.K. et al. Preliminary studies on glycemic effect of *Syzygium cumini* seeds. **IRCS Med Sci-Biochem** 13:129-33, 1985.

Mashima, R. et al. Oxidants and antioxidants in atherosclerosis. **Curr Oppin Lipidol** 12: 411-418, 2001.

McLellan, A.C. et al. Glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications. **Clin Sci** 87:21-29, 1994.

Montecucco, F. et al. Atherosclerosis is an inflammatory disease. **Semin Immunopathol** 31:1-3, 2009.

O'Brien, P.J. et al. Fructose-derived endogenous toxins, in: O'Brien, P.J. and Bruce, W.R. (Eds.). Endogenous Toxins: Targets for Disease Treatment and Prevention. **Wiley-VCH Verlag GmbH & Co. KGaA**, Weinheim, pp. 173-212, 2010.

Onorato, J.M. et al. Immunohistochemical and ELISA Assays for Biomarkers of Oxidative Stress in Aging and Disease. **Ann NY Acad Sci** 854:277-290, 1998.

Osterub, B. et al. Role of monocytes in atherogenesis. **Physiol Rev** 83:1069-1112, 2003.

Rabbani, N. et al. Glycation of LDL by methylglyoxal increases arterial atherogenicity. **Diabetes** 60:1973-1980, 2011.

Ramirez, R.O. et al. The gastroprotective effect of tannins extracted from duhat (*Syzygium cumini* Skeels) bark on HCl/ethanol induced gastric mucosal injury in Sprague-Dawley rats. **Cl Hemorh M** 29:253-261, 2003.

Rimm, E. B. et al. Vitamin E consumption and the risk of coronary heart disease in men. **N Engl J Med** 328:1450-1456, 1993.

Smith, S. C. et al. Atherosclerotic Vascular Disease Conference: Writing Group II: risk factors. **Circulation** 109:2613-2616, 2004.

Smith, W. C. et al. Concomitants of excess coronary deaths--major risk factor and lifestyle findings from 10,359 men and women in the Scottish Heart Health Study. **Scott Med J** 34:550-555, 1989.

Steinberg, D. et al. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. **N Engl J Med** 320:915-924, 1989.

Steinbrecher, U. et al. Decrease in reactive amino groups during oxidation or endothelial cellmodification of LDL. Correlation with changes in receptor-mediated catabolism. **Arteriosclerosis** 7:135-143, 1987.

Thornalley, P.J. et al. Suppression of the accumulation of triosephosphates and increased formation of methylglyoxal in human red blood cells during hyperglycaemia by thiamine in vitro. **Biochem J** 129:543-549, 2001.

Vikrant, V. et al. Treatment with extracts of Momordica charantia and *Eugenia jambolana* prevents hyperglycemia and hyperinsulinemia in fructose fed rats. **J Ethnopharmacol** 76:139-143, 2001.

Villa-Colinayo, V. et al. Genetics of atherosclerosis: the search for genes acting at the level of the vessel wall. **Curr Atheroscler Rep** 2:380-389, 2000.

Vlassopoulos, A. et al. Role of oxidative stress in physiological albumin glycation: A neglected interaction. **Free Radical Biol Med** 60:318-324, 2013.

Witztum, J.L. and Steinberg, D. Role of oxidized low density lipoprotein in atherogenesis. **J Clin Invest** 88:1785-1792, 1991.

Witztum, J.L. The oxidation hypothesis of atherosclerosis. **Lancet** 344:793-795, 1994.

World Health Organization. Global atlas on cardiovascular disease prevention and control.(http://whqlibdoc.who.int/publications/2011/9789241564373_eng.pdf - acessado em 19/08/2014). 2011 b.

World Health Organization. Global status report on noncommunicable diseases 2010. (http://www.who.int/nmh/publications/ncd_report_full_en.pdf - acessado em 19/09/2014), 2011 a.

Yamaguchi, Y. et al. Assay methods of modified lipoproteins in plasma. **J Chromatogr B Analyt Technol Biomed Life Sci** 781: 313-330, 2002.

Yeboah, F.K. et al. Reactivities of D-glucose and D-fructose during Glycation of Bovine Serum Albumin. **J Agric Food Chem** 47:3164-3172, 1999.