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

AVALIAÇÃO DA ATIVIDADE ANTIOXIDANTE DE DIFERENTES CLASSES DE COMPOSTOS CONTRA A OXIDAÇÃO DE LIPOPROTEÍNAS DE BAIXA DENSIDADE

TESE DE DOUTORADO

Rafael de Lima Portella

Santa Maria, RS, Brasil
2011

AVALIAÇÃO DA ATIVIDADE ANTIOXIDANTE DE DIFERENTES CLASSES DE COMPOSTOS CONTRA A OXIDAÇÃO DE LIPOPROTEÍNAS DE BAIXA DENSIDADE

por

Rafael de Lima Portella

Tese apresentada ao Programa de Pós-Graduação em Ciência Biológicas: Bioquímica Toxicológica, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM), como requisito parcial para obtenção do grau de Doutor em Bioquímica Toxicológica.

Orientador: Félix Alexandre Antunes Soares

Santa Maria, RS, Brasil
2011

P843a Portella, Rafael de Lima
Avaliação da atividade antioxidante de diferentes classes de compostos contra a oxidação de lipoproteínas de baixa densidade / por Rafael de Lima Portella. – 2011.
145 p. ; il. ; 30 cm

Orientador: Félix Alexandre Antunes Soares
Tese (doutorado) – Universidade Federal de Santa Maria, Centro de Ciências Naturais e Exatas, Programa de Pós-Graduação em Ciências Biológicas : Bioquímica Toxicológica, RS, 2011

1. Lipoproteínas de baixa densidade 2. Aterosclerose 3. Antioxidante 4. Estresse oxidativo 5. Paullinia cupana 6. Tiosemicarbazona 7. Telúrio orgânico I. Soares, Félix Alexandre Antunes II. Título.

CDU 616.13-004.6

Ficha catalográfica elaborada por Cláudia Terezinha Branco Gallotti – CRB 10/1109
Biblioteca Central UFSM

Universidade Federal de Santa Maria
Centro de Ciências Naturais e Exatas
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica

A Comissão Examinadora, abaixo assinada, aprova a Tese de Doutorado

AVALIAÇÃO DA ATIVIDADE ANTIOXIDANTE DE DIFERENTES CLASSES DE COMPOSTOS CONTRA A OXIDAÇÃO DE LIPOPROTEÍNAS DE BAIXA DENSIDADE

elaborado por
Rafael de Lima Portella

como requisito parcial para a obtenção do grau de
Doutor em Bioquímica Toxicológica

COMISSÃO EXAMINADORA:

_____
Prof. Dr. Félix Alexandre Antunes Soares (Orientador; UFSM)

_____
Profª. Drª. Caroline Wagner (UNIPAMPA)

_____
Prof. Dr. Rafael Noal Moresco (UFSM)

_____
Prof. Dr. Ricardo Brandão (UFSM)

_____
Profª. Drª. Roselei Fachinetto (UFSM)

Santa Maria, 04 de outubro de 2011.

AGRADECIMENTOS

Agradeço à Deus por me dar forças e sempre guiar meu caminho.

À minha família, pelo amor, pelo incentivo, pela dedicação, pela compreensão, pelos valores sempre transmitidos para mim e minha namorada Alessandra pela paciência, compreensão, apoio e amor.

Ao meu orientador Prof. Félix Alexandre Antunes Soares, pela orientação e ensinamentos transmitidos ao longo de minha formação acadêmica, e principalmente pela amizade e confiança na execução deste trabalho. Admiro-o pelos seus conhecimentos e dedicação ao seu trabalho.

Aos professores João Batista Teixeira da Rocha, Cristina Wayne Nogueira e Gilson Zeni, pelos seus conhecimentos e exemplos de dedicação a serem seguidos.

A todos os colegas dos laboratórios do Prof. João, da Profª Cristina e do Prof. Gilson e, especialmente, aos colegas e ex-colegas do laboratório do Prof. Félix, pela amizade, pela ajuda, pela descontração e pelos conhecimentos compartilhados.

Aos professores e aos colegas do Programa de Pós-Graduação em Bioquímica Toxicológica, que de alguma maneira contribuíram para a minha formação científica.

Às funcionárias Márcia e Elvandir pela dedicação e competência com que realizam o seu trabalho.

À Universidade Federal de Santa Maria e ao Programa de Pós-Graduação em Bioquímica Toxicológica pela possibilidade de realização deste curso.

A CAPES pela bolsa de estudos e pelos recursos financeiros concedidos.

Enfim, agradeço a todos que de alguma forma contribuíram para a realização deste trabalho.

“A imaginação é mais importante que a ciência,
porque a ciência é limitada,
ao passo que a imaginação abrange o mundo inteiro.”
(Albert Einstein)

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

Avaliação da atividade antioxidante de diferentes classes de compostos contra a oxidação de lipoproteínas de baixa densidade

AUTOR: Rafael de Lima Portella
ORIENTADOR: Félix Alexandre Antunes Soares
DATA E LOCAL DA DEFESA: Santa Maria, outubro de 2011.

Anormalidades do metabolismo lipídico muitas vezes levam ao acúmulo patológico de lipídios na parede arterial, sequelas oxidativas e inflamatórias crônicas e à formação de lesões ateroscleróticas, levando a eventos clínicos. A modificação oxidativa de lipoproteínas de baixa densidade (LDL) na parede arterial parece ser um fator importante no desenvolvimento da aterosclerose. Seguindo a hipótese oxidativa da aterosclerose, o papel dos antioxidantes tem sido investigado em grande número de estudos epidemiológicos, clínicos e experimentais. Sendo assim, neste estudo nós investigamos a atividade antioxidante de três compostos de diferentes classes na prevenção da oxidação da LDL. No estudo com guaraná, nós mostramos que indivíduos que consumiam guaraná habitualmente (GI) apresentaram menor produção de dienos conjugados que os indivíduos que nunca consumiam guaraná (NG; redução de 27%, $p < 0,0014$), independentemente de outras variáveis. No entanto, no grupo GI a produção de dienos conjugados foi positivamente associada com os níveis de glicose. O grupo GI também apresentou nível de colesterol total significativamente menor comparado ao grupo NG. Além disso, o guaraná apresentou uma grande atividade antioxidante *in vitro*, principalmente nas concentrações de 1 e 5 µg/mL, demonstrado pela supressão da produção de dienos conjugados e substâncias reativas ao ácido tiobarbitúrico, prevenção da destruição do triptofano e alta atividade *scavenger* de radicais (TRAP). O guaraná, similar a outros alimentos ricos em cafeína e catequinas como o chá verde, tem alguns efeitos na oxidação da LDL que podem explicar parcialmente os efeitos protetores deste alimento nas doenças cardiometabólicas. No estudo com a tiosemicarbazona, a salicilaldeído-4-feniltiosemicarbazona (SPTS) apresentou atividade antioxidante contra a oxidação de LDL e soro induzidas por Cu^{2+} e 2,2'-azobis(2-metilpropionamidina) dihidrocloreto (AAPH). Além disso, a SPTS foi efetiva em prevenir a destruição do triptofano. O composto também apresentou significativa atividade *scavenger* de radicais e pode prevenir a formação de substâncias reativas ao ácido tiobarbitúrico induzidas por nitroprussiato de sódio em diferentes tecidos de ratos e por Cu^{2+} em LDL e soro humano. Estes resultados indicam que o efeito antioxidante da SPTS é causado pela combinação da atividade quelante do composto e a atividade *scavenger* de radicais livres. No estudo com o composto orgânico de telúrio, o 2-fenil-2-telurofenil vinilfosfonato (DPTVP) apresentou atividade antioxidante contra a oxidação de LDL e soro humano induzida por AAPH e Cu^{2+}. Além disso, o composto preveniu a oxidação do triptofano e a formação de substâncias reativas ao ácido tiobarbitúrico e mostrou um significativo efeito *scavenger* de radicais. O DPTVP (20 µM) não apresentou toxicidade quando exposto à fatias de aorta de ratos. Estes resultados indicam que o efeito do DPTVP é resultado de uma combinação da atividade *scavenger* de radicais do composto e da possibilidade dele bloquear os sítios de ligação de cobre da LDL. Considerando todos os resultados apresentados aqui, podemos concluir que os três compostos apresentam um grande potencial antioxidante e podem prevenir as modificações oxidativas da LDL. Esses dados nos encorajam para avaliar esses compostos em estudos *in vivo* e investigar novas propriedades que possam prevenir o processo aterogênico.

Palavras-chave: Lipoproteínas de baixa densidade; Aterosclerose; Antioxidante; Estresse oxidativo; *Paullinia cupana*; Tiosemicarbazona; Telúrio orgânico.

ABSTRACT

Thesis of Doctor's Degree

Federal University of Santa Maria, RS, Brazil

Evaluation of antioxidant activity of different classes of compounds against low density lipoprotein oxidation

AUTHOR: Rafael de Lima Portella

ADVISOR: Félix Alexandre Antunes Soares

DATE AND PLACE OF THE DEFENSE: Santa Maria, October 2011.

Abnormalities of lipid metabolism often lead to pathologic lipid accumulation in the vessel wall, oxidative and chronic inflammatory sequelae and the formation of atherosclerotic lesions, ultimately leading to clinical events. Low density lipoprotein (LDL) oxidative modification in the vascular wall seems to be a key factor in atherosclerosis development. Following the oxidation hypothesis of atherosclerosis the role of antioxidants has been investigated in a large number of epidemiological, clinical and experimental studies. Therefore, we investigated the antioxidant activity of three compounds of different classes in preventing the low density lipoprotein oxidation. In the guaraná study, we showed that subjects who habitually ingested guaraná (GI) demonstrated lower conjugated dienes production than did subjects who never ingested guaraná (NG; reduction of 27%, $p < 0,0014$), independent of other variables. However, in the GI group the conjugated dienes production was positively associated with glucose levels. The GI group also showed a total cholesterol level significantly lower than NG group. Also, guaraná demonstrated a high antioxidant activity *in vitro*, mainly at concentrations of 1 and 5 µg/mL, demonstrated by suppression of CDs and TBARS productions, tryptophan destruction and high TRAP activity. Guaraná, similar to other foods rich in caffeine and catechins such as green tea, has some effect on LDL oxidation that could partially explain the protective effects of this food in cardiometabolic diseases. In the thiosemicarbazone study, salicylaldehyde-4-phenylthiosemicarbazone (SPTS) may have antioxidant activity against Cu^{2+}- and 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH)-induced LDL and serum oxidation. Additionally, SPTS was effective at preventing tryptophan destruction. SPTS also showed significant total radical-trapping antioxidant activity and could prevent thiobarbituric acid reactive substances (TBARS) formation induced by sodium nitroprusside in different rat tissues and by Cu^{2+} in human LDL and serum. These results indicate that the antioxidant effect of SPTS is caused by a combination of transition metal chelation and free-radical-scavenging activity. In the organotellurium study, the 2-phenyl-2-tellurophenyl vinylphosphonate (DPTVP) may have antioxidant activity against Cu^{2+}- and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH)-induced LDL and serum oxidation. Additionally, DPTVP was effective at preventing tryptophan oxidation. DPTVP also showed significant total radical-trapping antioxidant activity and could prevent thiobarbituric acid reactive substance (TBARS) formation induced by Cu^{2+} in human LDL and serum. Additionally, DPTVP exhibited no toxicity in rat aorta slices. The results presented here indicate that the antioxidant effect of DPTVP is caused by a combination of free-radical-scavenging activity and possible blockade of the copper binding sites of LDL. Considering these preliminary results, we can conclude that the three compounds presented a potential antioxidant activity and could prevent the oxidative modifications of LDL. These data encourage us to evaluate these compounds in *in vivo* studies and investigate additional properties in preventing the atherogenic process.

Key words: Low density lipoprotein; Atherosclerosis; Antioxidant; Oxidative stress; *Paullinia cupana*; Thiosemicarbazone; Organotellurium.

SUMÁRIO

1. INTRODUÇÃO

A doença cardiovascular é a principal causa de morbidade e mortalidade no mundo ocidental. Em 2008, as doenças cardiovasculares foram responsáveis por mais de 17 milhões de mortes no mundo, o que representa 30,5% de todas as mortes (World Health Organization, 2008). Aproximadamente 81% das mortes por doenças cardiovasculares são de pessoas acima de 60 anos. No ano de 2002 no Brasil, as doenças cardiovasculares foram as principais causas de morte no país (31% do total) (Oliveira, G.M.M. *et al.*, 2006). Além disso, um fato que agrava esse quadro é que, aproximadamente, um terço dos óbitos por doenças cardiovasculares ocorrem precocemente em adultos na faixa etária de 35 a 64 anos (Ishitani, L.H. *et al.*, 2006).

As principais manifestações das doenças cardiovasculares são o ataque do coração e o acidente vascular cerebral. Estas manifestações representam uma seqüela clinica de um processo vascular sistêmico conhecido como aterosclerose. Dados de estudos epidemiológicos dos últimos 50 anos têm demonstrado que a aterosclerose é uma doença ligada ao envelhecimento, e que a aterosclerose prematura pode ser ocasionada por várias condições clínicas, sendo as mais proeminentes o excesso de lipoproteínas de baixa densidade (LDL), diabetes mellitus, hipertensão e o tabagismo (Smith, S.C., Jr. *et al.*, 2004).

A aterosclerose é um processo insidioso que pode persistir por muitos anos antes das manifestações clínicas se tornarem evidentes. Esta observação é devido ao fato que o processo envolvido na aterogênese requer uma prolongada exposição aos fatores de predisposição. Apenas o último estágio da doença que progride rapidamente e leva às manifestações clínicas. O processo de desenvolvimento da lesão aterosclerótica e os eventos clínicos são distintos. Devido a essa distinção, as terapias, como medicamentos hipocolesterolêmicos, a prática de exercícios físicos e a dieta balanceada, que são efetivas em prevenir o desenvolvimento da lesão podem não serem eficientes em prevenir as manifestações clínicas da doença. A principal razão é que essas muitas terapias são iniciadas depois que os indivíduos já têm a lesão bem estabelecida. Assim, já que a maioria dos indivíduos inicia a aterogênese antes da adolescência, fica muito difícil determinar uma estratégia para

prevenir o desenvolvimento da aterosclerose (Keaney, J.F., Jr., 2000). Por isso a necessidade de novos fármacos, com maior eficácia para combater a aterosclerose.

2. DESENVOLVIMENTO

2.1 Aterosclerose

2.1.1 Aterogênese

2.1.1.1 Definição

A aterosclerose pode ser definida como uma doença multifatorial, inflamatória degenerativa e progressiva das artérias, caracterizada pelo acúmulo de lipídios e elementos fibrosos nos grandes vasos. Um crescente número de fatores de risco tem sido associado com a sua evolução: idade e sexo, níveis de lipoproteínas, hipertensão, diabetes, tabagismo, parâmetros inflamatórios e coagulativos são os mais estudados (Cherubini, A. *et al.*, 2005).

A aterosclerose é uma condição patológica em que as artérias sofrem um espessamento gradual da camada íntima causando um decréscimo da elasticidade, estreitamento do lúmen e redução do suprimento sanguíneo. Os vasos sanguíneos mais comumente afetados e relevantes clinicamente são a aorta, a coronária, a carótida e as artérias cerebrais, sendo classificadas coletivamente como Doenças Cardiovasculares (CVD) (Matsuura, E. *et al.*, 2006).

2.1.1.2 Características morfológicas

A aterosclerose tipicamente é manifestada em três estágios conhecidos como lesões precoces, lesões em desenvolvimento e lesões maduras (Keaney, J.F., Jr., 2000). As lesões precoces são caracterizadas por áreas nodulares de deposição de lipídios nomeadas morfologicamente como “estrias gordurosas”. Estas representam macrófagos cheios de lipídios e células musculares lisas em áreas focais da camada íntima. Estas lesões precoces podem se desenvolver em torno dos 10 anos de idade e aumentar para ocupar até 1/3 da superfície da aorta na terceira década de vida. As lesões em desenvolvimento representam o próximo estágio das estrias gordurosas. Elas são encontradas inicialmente em áreas das artérias coronárias e aorta abdominal principalmente durante a terceira e quarta década de vida. Estas placas fibrosas têm formas elipsóides, são firmes e cobertas por uma camada fibromuscular. Por último, as lesões podem progredir para formas complicadas e avançadas, que são caracterizadas por áreas fibrosas calcificadas da artéria com ulcerações visíveis. Este tipo de lesão está associado com a formação de trombos, êmbolos e consequentemente com as doenças cardiovasculares (Keaney, J.F., Jr., 2000).

A anatomia típica da artéria normal e com lesões ateroscleróticas está ilustrada na Figura 1 (Itabe, H., 2003). Na artéria normal, existe uma camada de células endoteliais revestindo a superfície do lúmen do vaso que está em contato direto com o sangue (1a). Uma camada composta de elastina chamada lâmina elástica situa-se entre as células endoteliais e a camada de células musculares lisas. No estágio precoce da lesão aterosclerótica, o qual é referido como um espessamento difuso da camada íntima (1b), ocorre um acúmulo de fibras de colágeno e lipídios, bem como a invasão de várias células incluindo macrófagos entre as células endoteliais e a lâmina elástica. As estrias gordurosas são pedaços macroscópicos sob a parede do vaso, os quais coalescem para formar grandes lesões. Nesse estágio, há um acúmulo maciço de lipídios no espaço da camada íntima e um grande número de células espumosas, que derivam de macrófagos carregados de lipídios. Algumas células espumosas são derivadas de células musculares lisas (1c). A capa fibrosa é formada com células musculares lisas e numerosas proteínas fibrosas tais como o colágeno. Nas regiões mais profundas do ateroma são encontrados um acúmulo maciço de macrófagos, células musculares lisas e proteínas de matriz extracelular (1d). Quando a superfície luminal da lesão aterosclerótica é danificada ou rompida, as plaquetas imediatamente se aderem e se

agregam no local rompido e assim formam o trombo (1e). A formação de trombos e êmbolos pode levar a uma oclusão completa do vaso sanguíneo e esta é considerada a causa primária do infarto do miocárdio e cerebral. Diversos estudos têm apontado que placas ateromatosas ricas em lipídios e células espumosas são as mais vulneráveis e propensas a sofrer uma ruptura (Itabe, H., 2003).

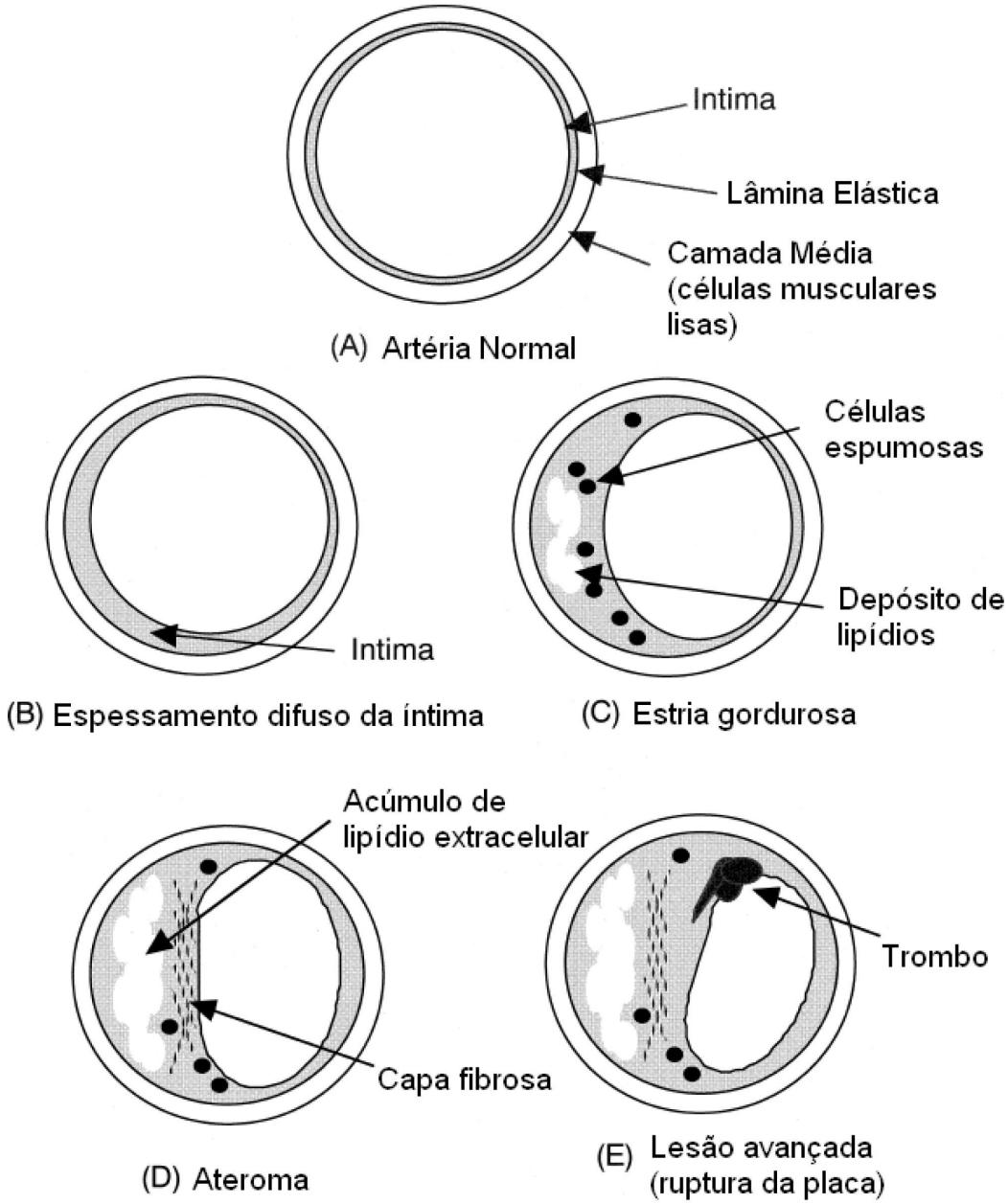


Figura 1 – Mudanças na estrutura da parede do vaso sanguíneo durante o desenvolvimento da aterosclerose (Modificado de Itabe, H., 2003).

2.1.2 Hipótese da modificação oxidativa da aterosclerose

Partículas de LDL oxidada estão presentes na lesão aterosclerótica (Ehara, S. *et al.*, 2001; Nishi, K. *et al.*, 2002) e tem sido sugerido que estas desempenham um papel significantivo na aterogênese (Steinberg, D., 1997). Uma elevação dos níveis plasmáticos de LDL leva a um aumento na velocidade de entrada das partículas de LDL dentro da parede arterial (Osterud, B. *et al.*, 2003). Enquanto as partículas de LDL estão protegidas da oxidação no plasma pelos compostos antioxidantes, as partículas presas dentro da parede arterial estão expostas ao dano oxidativo (Osterud, B. *et al.*, 2003).

Moléculas tais como radicais peroxila e alcoxila, óxido nítrico e íons de metais de transição (cobre e ferro) contêm um ou mais elétrons desemparelhados que reagem com outras moléculas de radicais ou não-radicalares resultando em novas moléculas de radicais. Se os lipídios sofrem danos por radicais livres, cria-se uma reação em cadeia que leva a peroxidação lipídica (Stocker, R. *et al.*, 2004). Antes de estarem completamente oxidada, as partículas de LDL tem que sofrer algumas modificações.

Inicialmente, as partículas de LDL nativas contêm um polipeptídeo intacto [apolipoproteína B-100 (apoB-100)], nenhum peróxido de lipídio ou aldeído, e são ricas em ácidos graxos poliinsaturados (PUFA) e antioxidantes (Parthasarathy, S. *et al.*, 1999). Partículas de LDL minimamente modificada (mm-LDL) são caracterizadas pela oxidação de fosfolipídios na superfície da LDL (Parthasarathy, S. *et al.*, 1999). Neste estágio, a estrutura da apoB-100 está intacta, porém a partícula de LDL perdeu os PUFA e os compostos antioxidantes. Como a estrutura da apoB-100 não está alterada, a LDL ainda é reconhecida pelos receptores de LDL e não é reconhecida pelos receptores *scavenger* (Mashima, R. *et al.*, 2001). A mm-LDL pode induzir a expressão de proteína quimiotática de monócitos-1 (MCP-1) e fator estimulador de colônia de macrófagos (M-CSF) pelas células endoteliais que, respectivamente, iniciam o recrutamento de monócitos para a parede arterial (Cushing, S.D. *et al.*, 1990) e promovem a diferenciação de monócitos em macrófagos (Villa-Colinayo, V. *et al.*, 2000).

Oxidações adicionais da mm-LDL levam à modificação da apoB-100 com produtos gerados do catabolismo dos peróxidos lipídicos, tais como aldeídos, que interagem com os resíduos de lisina da apoB-100, tornando a LDL carregada mais negativamente, o que resulta em um decréscimo da afinidade da LDL pelo seu receptor e um aumento da afinidade pelos receptores *scavenger* (Brown, M.S. *et al.*, 1983). Essas partículas de LDL completamente oxidadas (ox-LDL) ativam as células endoteliais que respondem expressando moléculas de adesão tais como molécula de adesão de células vasculares-1 em sua superfície (Khan, B.V. *et al.*, 1995; Takei, A. *et al.*, 2001), que, juntamente com a MCP-1, irão promover a adesão e a entrada de monócitos para dentro da parede arterial. Posteriormente, estes monócitos irão se diferenciar em macrófagos pela ação do M-CSF.

As partículas de ox-LDL são reconhecidas pelos receptores *scavenger* dos macrófagos e são internalizadas. No entanto, diferente dos receptores de LDL, que sofrem inibição por retroalimentação, os receptores *scavenger* de macrófagos não são regulados negativamente com o aumento do conteúdo de colesterol nos macrófagos. Consequentemente, os macrófagos continuam capturando ox-LDL e acumulando uma quantidade significativa de lipídios na parede arterial (Osterud, B. *et al.*, 2003), que induzirão uma liberação de citocinas pró-inflamatórias pelos macrófagos, que promoverão mais recrutamento de monócitos e acúmulo de macrófagos carregados de lipídios (células espumosas), que é o tipo de célula mais predominante na lesão aterosclerótica inicial, chamada estria gordurosa (Osterud, B. *et al.*, 2003). Assim, um ciclo vicioso de oxidação, modificação de lipoproteínas e inflamação pode se manter na artéria pela presença dessas ox-LDL. Concentrações de ox-LDL circulante tem sido relacionadas com a espessura da camada média íntima e a ocorrência de placas em carótidas e artérias femorais em homens (Hulthe, J. *et al.*, 2002; Metso, S. *et al.*, 2004), bem como com a progressão da aterosclerose nas artérias carótidas (Wallenfeldt, K. *et al.*, 2004).

2.1.3 Propriedades pró-aterogênicas da oxLDL

O interesse inicial na ox-LDL surgiu de dois conjuntos básicos de observação. A primeira observação foi que a ox-LDL é citotóxica para células endoteliais e outras células e assim, poderia causar danos diretamente para as células arteriais (Hessler, J.R. *et al.*, 1983). A segunda observação foi que a captação de LDL nativa pelo macrófago ocorre a uma velocidade suficientemente baixa para suportar a formação de células espumosas, mas a captação de ox-LDL é desregulada e leva a formação de células espumosas (Heinecke, J.W. *et al.*, 1984; Steinbrecher, U.P. *et al.*, 1984). Entretanto, agora está perfeitamente estabelecido que a ox-LDL, com seus muitos lipídios modificados oxidativamente e produtos de degradação, contribuem para a patofisiologia tanto da iniciação como da progressão da lesão aterosclerótica por muitos mecanismos, incluindo suas propriedades pró-inflamatórias, imunogênicas e citotóxicas (Tabela 1) (Navab, M. *et al.*, 1996; Steinberg, D. *et al.*, 1999). Produtos da ox-LDL contribuem para o recrutamento de monócitos e linfócitos-T, direta ou indiretamente via indução de quimioquinas e moléculas de adesão de células endoteliais. Eles alteram a expressão de genes de células vasculares levando a estimulação de fatores de crescimento e citocinas e são citotóxicas. Uma vez que a oxidação da LDL gera muitos “elementos novos” que induzem uma resposta imune ativa (Horkko, S. *et al.*, 2000), surgem respostas tanto celulares como humorais que afetam a progressão da lesão aterosclerótica de uma maneira complexa (Libby, P., 2002). De fato, o papel que a oxidação da LDL exerce na aterogênese se encaixa muito bem com a noção generalizada que a aterosclerose é um processo inflamatório crônico.

2.1.4 Evidências da oxidação da LDL *in vivo*

Mesmo no fluido extracelular, seria de supor que as concentrações de antioxidantes (proteínas, ascorbato, ácido úrico etc) seriam suficientes para inibir a modificação oxidativa induzida pelas células. Mas isso não ocorre e a oxidação acontece. Como evidências temos que: 1) As frações de lipoproteínas extraídas cuidadosamente de lesões ateroscleróticas (de coelhos e humanos) contêm ox-LDL, identificada pelas suas propriedades físicas e pelo seu reconhecimento por

receptores *scavenger* (Yla-Herttuala, S. *et al.*, 1990). 2) Imuno-histoquímica usando anticorpos gerados contra ox-LDL demonstram a presença de ox-LDL (ou antígeno muito similar à isso) na lesão arterial mas não em artérias normais (Palinski, W. *et al.*, 1989). 3) Tanto em animais como em humanos foram demonstrados anticorpos que reagem com ox-LDL no soro (Palinski, W. *et al.*, 1989). 4) A administração de antioxidantes que podem prevenir as modificações oxidativas da LDL reduzem a progressão da aterosclerose em vários modelos experimentais de animais.

Tabela 1. Potenciais mecanismos pelos quais as formas oxidadas de LDL podem influenciar na aterogênese

• Ox-LDL tem captação aumentada pelos macrófagos levando a formação de células espumosas;
• Produtos da ox-LDL são quimiotáticos para monócitos e células-T e inibem a motilidade dos macrófagos tissulares;
• Produtos da ox-LDL são citotóxicos, em parte devido aos esteróis oxidados, e podem induzir a apoptose;
• Ox-LDL, ou produtos, são mitogênicos para células musculares lisas e macrófagos;
• Ox-LDL, ou produtos, podem alterar expressão de genes de células vasculares, por exemplo, indução de MCP-1, fatores estimuladores de colônia, IL-1 e expressão de móleculas de adesão;
• Ox-LDL, ou produtos, podem aumentar a expressão de receptores *scavenger* de macrófagos, dessa maneira aumentando sua própria captação;
• Ox-LDL, ou produtos, podem induzir genes pró-inflamatórios, por exemplo, hemoxigenase e ceruloplasmina;
• Ox-LDL pode induzir a expressão e ativação do receptor ativado por proliferadores do peroxissoma (PPAR-γ), dessa maneira influenciando a expressão de muitos genes;
• Ox-LDL é imunogênico e induz a formação de anticorpos e ativação de células-T;
• Oxidação torna a LDL mais suceptível a agregação, que independentemente leva a um aumento na captação. Similarmente, ox-LDL é um substrato melhor para a esfingomielinase, que também agrega LDL;
• Ox-LDL pode aumentar as vias pró-coagulantes, por exemplo, pela indução de fatore tissular e agregação plaquetária;

Modificada de Steinberg and Witztum (1999).

2.1.5 Agentes oxidantes fisiológicos *in vivo*

É geralmente bem aceito que muito pouca oxidação da LDL ocorra na circulação sanguínea por causa da abundância de antioxidantes, como o tocoferol, ascorbato, urato, apolipoproteínas e albumina. Em vez disso, uma grande oxidação de LDL ocorre no espaço subendotelial da parede arterial, onde a LDL pode ser sequestrada pelos proteoglicanos e onde a concentração de antioxidantes é muito menor que no plasma (Levitan, I. *et al.*, 2010). Wen e Leake (Wen, Y. *et al.*, 2007) demonstraram que a LDL pode também ser oxidada intracelularmente, mais provavelmente no compartimento lisossomal de macrófagos. A oxidação da LDL pode também ocorrer nos locais de inflamação por causa da infiltração de neutrófilos e monócitos/macrófagos e por causa do aumento da permeabilidade vascular e consequente aumento na concentração de LDL nos tecidos e nos locais de inflamação. As células da parede arterial podem produzir espécies oxidantes radicalares e não-radicalares através de vários mecanismos enzimáticos. Os radicais livres produzidos pelas células incluem superóxido, radicais hidroxila, radicais com centro carbono, óxido nítrico e radicais tiil e pertiil. Embora os metais de transição livres sejam comumente usados para a oxidação *in vitro* da LDL, a sua função na oxidação fisiológica é controversa visto que quantidades significativas de ferro ou cobre livre não são encontradas *in vivo*. No entanto, proteínas contendo ferro (como ferritina, transferrina, hemoglobina, mioglobina) e proteínas contendo cobre (como ceruloplasmina) têm demonstrado oxidar LDL *in vitro* e portanto podem ser relevantes fisiologicamente na geração de ox-LDL *in vivo* (Fox, P.L. *et al.*, 2000). Além disso, ferro livre pode ser liberado da ferritina seguido de sua redução para o estado ferroso pela superóxido dismutase (SOD) (Meyers, D.G., 1996) e um dos sete átomos de cobre ligados à ceruloplasmina é permutável com quelantes (Fox, P.L. *et al.*, 2000). Os radicais livres oxidam preferencialmente os ácidos graxos poliinsaturados, cujos produtos de degradação podem derivatizar a apoB-100 e alterar seu reconhecimento pelo receptor. Os oxidantes não-radicalares que tendem a modificar as proteínas diretamente (especialmente a cisteína, metionina e tirosina) incluem H_2O_2, hipoclorito e peroxinitrito. Os oxidantes na parede arterial são gerados pela ação da NADPH oxidase, xantina oxidase, óxido nítrico sintase,

mieloperoxidase e lipoxigenase, todas as quais foram demonstradas estarem presentes na lesão aterosclerótica (Stocker, R. *et al.*, 2005). Deve ser salientado que os diversos agentes oxidantes não agem isoladamente, mas é mais provável que uma ação consecutiva de vários agentes e enzimas possam estar envolvidas na geração de uma LDL completamente oxidada *in vivo*.

2.2 Antioxidantes e aterosclerose

2.2.1 Estudos clínicos com antioxidantes

Existe uma abundância de dados científicos, provenientes de estudos *in vitro* e de modelos de diferentes animais que sustentam a validade da hipótese oxidativa da aterosclerose, isto é, que a modificação oxidativa de lipoproteínas é o evento principal na evolução das placas ateroscleróticas (Steinberg, D. *et al.*, 2002). Uma consequência dessa hipótese é que antioxidantes podem prevenir a oxidação de LDL e, portanto protege contra o desenvolvimento da aterosclerose. De acordo com esta hipótese, estudos epidemiológicos e experimentais em humanos sugerem que antioxidantes naturais podem ter um efeito protetor sobre a aterosclerose periférica e a mortalidade e morbidade das doenças coronárias (Cherubini, A. *et al.*, 2005).

Verlangieri et al. reportaram que as taxas de mortalidade das doenças coronárias e relacionadas estavam em declínio nos Estados Unidos de 1964 a 1978. Este fenômeno estava atribuído parcialmente à grande disponibilidade de frutas e vegetais, sendo que esses autores acharam uma correlação inversa entre o consumo per capita e a incidência de doenças coronárias (Verlangieri, A.J. *et al.*, 1985). Smith et al. também relataram uma taxa de mortalidade extremamente alta de doenças coronárias em escoceses (homens e mulheres) com um baixo consumo de frutas frescas e legumes (Smith, W.C. *et al.*, 1989).

Antioxidantes como α-tocoferol e β-caroteno foram determinados em tecido adiposo de pacientes com infarto do miocárdio e de controles em 12 regiões da

Europa e Israel. Os níveis de β-caroteno estavam reduzidos nos pacientes quando comparados com os controles, e a associação foi maior entre fumantes e ex-fumantes (Kardinaal, A.F. *et al.*, 1993). Entre os diferentes carotenóides, apenas os níveis de licopeno foram associados com o baixo risco de infarto do miocárdio (Kohlmeier, L. *et al.*, 1997). Um efeito protetor dos carotenóides foi relatado também por outros autores (Street, D.A. *et al.*, 1994).

WHO-MONICA STUDY encontrou que níveis sanguíneos de α-tocoferol e a razão de tocoferol/colesterol, mas não de vitamina C e β-caroteno, estavam inversamente relacionados com a taxa de mortalidade cardiovascular (Gey, K.F. *et al.*, 1987a; Gey, K.F. *et al.*, 1987b; Gey, K.F. *et al.*, 1989; Gey, K.F. *et al.*, 1991).

No Nurses' Health Study (Estudo da Saúde de Enfermeiras), que envolveu mais de 87.000 enfermeiras americanas livres de diagnósticos de câncer e doenças do coração (Stampfer, M.J. *et al.*, 1993), o risco de eventos como doenças coronárias entre as mulheres que tomavam suplementos de vitamina E, geralmente 100 UI ou mais, eram 40% menores que as mulheres que não tomavam suplemento. A associação inversa de vitamina E com o risco de doença coronária foi em grande parte atribuída à suplementação com vitamina E ao invés da ingestão dietética.

Em outro estudo (Health Professionals Follow-up Study), que considerou mais de 40.000 profissionais da saúde masculinos que estavam livres de doenças do coração e diabetes (Rimm, E.B. *et al.*, 1993), os homens no maior quintil de consumo de vitamina E de suplementos tinham um risco reduzido de infarto do miocárdio não-fatal ou eventos fatais de doenças do coração. Os efeitos estavam altamente limitados àqueles que consumiam no mínimo 100 UI de suplementação de vitamina E por pelo menos 2 anos (Rimm, E.B. *et al.*, 1993).

Os flavanóis e flavonóis encontrados no chá têm sido demonstrados apresentarem potentes efeitos antioxidantes. A capacidade antioxidante total do chá preto e do chá verde são muito similares (Wiseman, S.A. *et al.*, 1997). Acredita-se que esses efeitos antioxidantes são um mecanismo primário que medeia o efeito cardio-protetor do chá. Além disso, há algumas evidências que o aumento da biodisponibilidade de antioxidantes restaura a resposta dependente do óxido nítrico nos vasos, que podem ser perdidos pelo estresse oxidativo (Patel, R.P. *et al.*, 2000).

2.2.2 Guaraná (*Paullinia cupana*)

O gênero Paullinia é classificado como sendo da família Sapindaceae, que inclui carvalho (Acer spp.), castanhas (Aesculus spp.) e lichia (Litchi chinensis). Existem aproximadamente 195 espécies de *Paullinia* distribuídas nos trópicos e subtrópicos da América (Angelo, P.C. *et al.*, 2008). Destas, ao menos nove espécies são descritas como nativas do Brasil, incluindo a *P. cupana* (Kunth) var. *sorbilis* [(Mart.) Ducke].

O guaraná é comercialmente cultivado apenas no Brasil, onde a EMBRAPA mantém um programa de produção. A produção anual é cerca de 4.000 toneladas de sementes tostada (IBGE 2006), dos quais 70% são consumidos como refrigerante industrializado. Farmácias e lojas alternativas de produtos naturais vendem o restante, principalmente na forma de pó da semente tostada.

O transcriptoma da *P. cupana* inclui pelo menos 129 seqüências relacionadas ao metabolismo dos flavonóides, incluindo todas as principais enzimas na rota central da sua biossíntese, que indicam ser uma rota muito ativa na semente da fruta (Angelo, P.C. *et al.*, 2008).

A planta do guaraná é rica em metilxantinas tais como cafeína, teobromina e teofilina e contém taninos, saponinas, catequinas, epicatequinas, proantocianidinas, bem como traços de muitos outros compostos (Belliardo, F. *et al.*, 1985). Alguns destes compostos estão representados na Figura 2. As principais xantinas e catequinas presentes no extrato de guaraná utilizado em nosso estudo foram determinadas por cromatografia e dessa análise foi encontrado cafeína (12,240mg/g), teobromina (6,733mg/g), catequinas totais (4,336mg/g) e taninos condensados (22mg/g).

Estudos prévios em modelos experimentais e análises *in vitro* têm descrito vários efeitos biológicos que o guaraná compartilha com o chá verde, com atividade antioxidante (Mattei, R. *et al.*, 1998; Basile, A. *et al.*, 2005; Jimoh, F.O. *et al.*, 2007), efeito antimicrobiano (Pinheiro, C.E. *et al.*, 1987; da Fonseca, C.A. *et al.*, 1994; Yamaguti-Sasaki, E. *et al.*, 2007) e propriedades anticarcinogênicas e antitumorais (Fukumasu, H. *et al.*, 2006; Leite, R.P. *et al.*, 2011). Outros estudos em animais e voluntários humanos têm demonstrado que a ingestão de guaraná exibe importantes efeitos biológicos, com um melhoramento no desempenho cognitivo (Espinola, E.B.

et al., 1997; Kennedy, D.O. *et al.*, 2004) e um efeito antidepressivo (Campos, A.R. *et al.*, 2005; Otobone, F.J. *et al.*, 2007).

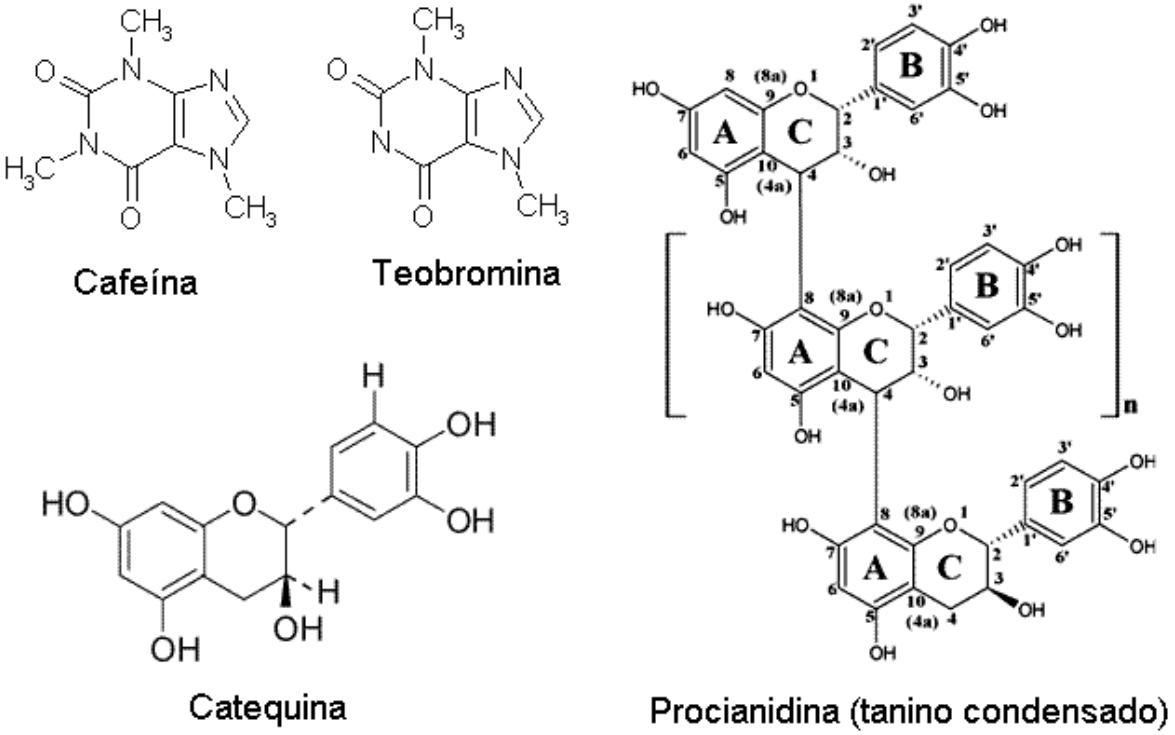


Figura 2 – Estruturas químicas de alguns representantes de metilxantinas (cafeína e teobromina) e compostos fenólicos (catequina e procianidina).

Investigações têm mostrado que o guaraná afeta positivamente o metabolismo de lipídios (Lima, W.P. *et al.*, 2005), aumenta a perda de peso (Boozer, C.N. *et al.*, 2001; Opala, T. *et al.*, 2006), e aumenta o consumo de energia basal (Berube-Parent, S. *et al.*, 2005). Portanto, esses dados sugerem que o guaraná tem um potencial anti-obesogênico. Além disso, o guaraná tem efeito cardioprotetor por evitar a agregação plaquetária (Bydlowski, S.P. *et al.*, 1988; Bydlowski, S.P. *et al.*, 1991).

Um estudo controlado foi realizado para analisar a associação entre o consumo habitual de guaraná com a prevalência de doenças metabólicas (obesidade, hipertensão, diabetes tipo 2 e dislipidemia) em uma população idosa que vive em Maués (AM). O estudo observou uma menor prevalência de

hipertensão, obesidade e síndrome metabólica nos sujeitos que relataram consumir guaraná habitualmente comparado com os sujeitos que não ingeriam guaraná. Além disso, foi encontrada uma associação entre o consumo de guaraná e menores níveis de colesterol (total e LDL) e produtos avançados de proteínas oxidadas (AOPP) (Costa Krewer, C. *et al.*, 2011).

2.2.3 Salicilaldeído-4-feniltiosemicarbazona

Os efeitos químicos e biológicos das tiosemicarbazonas têm recebido um considerável interesse pela química medicinal por muitos anos. Isto está atribuído, em primeiro lugar, à sua ampla utilidade farmacológica que inclui as atividades antineoplásica, antibacteriana, antiviral e antifúngica (Beraldo, H. *et al.*, 2004), e em segundo lugar, pela sua versatilidade como ligante, que lhes permitem dar origem a uma grande variedade de modos de coordenação (Pedrido, R. *et al.*, 2008). É também conhecido que as tiosemicarbazonas são excelentes quelantes de metais de transição tais como o ferro, o cobre e o zinco (French, F.A. *et al.*, 1966; DeConti, R.C. *et al.*, 1972; Pandeya, S.N. *et al.*, 1999). Sua habilidade de quelar íons metálicos depende da presença do sistema de coordenação tridentado N, N, S (doador brando) (Yu, Y. *et al.*, 2009). O grupo das salicilaldeídos tiosemicarbazonas e seus análogos têm cerca de 300 estruturas caracterizadas, onde um doador O-fenolato liga com um doador N- e um S- (Yu, Y. *et al.*, 2009). Tal habilidade para quelar metais é também uma estratégia atrativa no desenvolvimento de drogas anticâncer, devido à alta exigência das células neoplásicas por metais essenciais necessários para o crescimento e a proliferação (Kalinowski, D.S. *et al.*, 2005). De fato, a classe das tiosemicarbazonas foi a primeira classe de quelantes a ser amplamente avaliada como agente antineoplásico contra células cancerígenas (Sartorelli, A.C. *et al.*, 1967) *in vitro* e em ensaios clínicos (Krakoff, I.H. *et al.*, 1974).

Recentemente, nosso grupo mostrou o efeito da isatin-3-N^4-benziltiosemicarbazona (IBTC) na proteção da oxidação de lipoproteínas de baixa densidade (LDL) induzida por sulfato de cobre e AAPH (Barcelos, R.P. *et al.*, 2011). Zhong et al. mostraram que dois compostos derivados de tiosemicarbazonas

mostraram uma forte atividade *scavenger* de radicais superóxido e hidroxil além da atividade quelante (Zhong, Z. *et al.*, 2010). Prathima et al. (Prathima, B. *et al.*, 2010) mostraram que o composto benziloxibenzaldeido-4-fenil-3-tiosemicarbazona apresentou atividade *scavenger* de DPPH (radical 1,1-difenil-2-picrilhidrazila) e inibiu a peroxidação lipídica induzida por ferro. No entanto os complexos de cobre (II) e níquel (II) do composto não apresentaram efeito nestes ensaios.

O composto sallicilaldeído-4-feniltiosemicarbazona (SPTS; figura 3) é inédito, sintetizado recentemente e consequentemente pouco se sabe sobre suas propriedades farmacológicas e/ou toxicológicas.

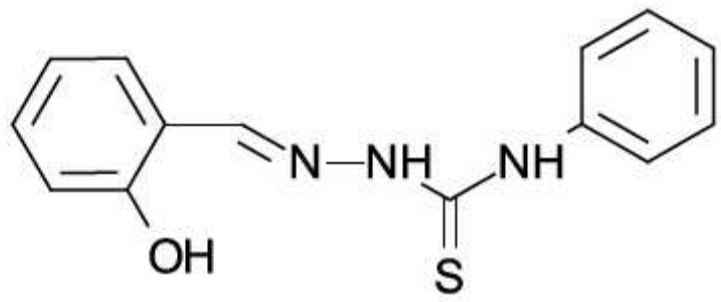


Figura 3 – Estrutura química da salicilaldeído-4-feniltiosemicarbazona.

2.2.4 Dietil 2-fenil-2-telurofenil vinilfosfonato

O dietil-2 fenil-2-telurofenil vinilfosfonato (DPTVP; figura 4) é um composto de telúrio orgânico de estrutura bastante distinta de outros já descritos na literatura. É um β-organocalcogenil vinilfosfonato de grande potencial sintético, visto que combina a conhecida reatividade química dos fosfonatos vinílicos e a capacidade dos calcogenetos vinílicos de facilmente se transformarem em outros compostos orgânicos com retenção 10 da configuração (Minami e Motoyoshiya, 1992; Comasseto et al., 1997; Zeni et al., 2006). O DPTVP tem sido descrito como um composto com baixa toxicidade em roedores. Em camundongos, administrações

diárias por até 12 dias de 250 μmol/Kg de DPTVP pela via subcutânea ocasionaram um índice de apenas 20% de mortalidade (De Avila et al., 2006). Nos animais sobreviventes, não foram observadas alterações na atividade da enzima δ-ALA-D em fígado, rins e cérebro (De Avila et al., 2006). Além disso, administrações diárias por doze dias de até 500 μmol/Kg pela via intraperitoneal em camundongos não causaram mortalidade nos animais nem alterações em diversos parâmetros analisados, tais como níveis de TBARS, atividade de enzimas antioxidantes como SOD e catalase, atividades séricas da AST e ALT e da δ-ALA-D (Avila et al.,2007). *In vitro*, este telureto vinílico mostrou-se capaz de reagir com grupos sulfidrílicos, aumentando a oxidação do DTT e inibindo a δ-ALA-D (De Avila et al., 2006), porém em concentrações bastante elevadas quando comparadas às concentrações utilizadas de ditelureto de difenila, um composto de telúrio extremamente tóxico (Nogueira, Zeni et al., 2004). Aliado a isso, os autores reportaram a potente atividade antioxidante deste composto em fígado, rim e cérebro, uma vez que o DPTVP reduziu a peroxidação lipídica induzida por Fe (II) (De Avila et al., 2006).

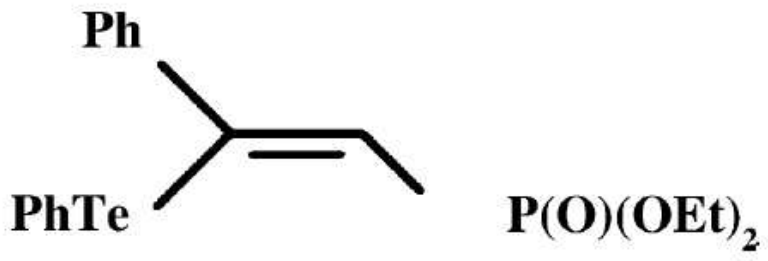


Figura 4 – Estrutura química do dietil-2 fenil-2-telurofenil vinilfosfonato.

3. OBJETIVOS

3.1 Objetivo Geral

Considerando os seguintes fatos:

- A evidência do papel da oxidação das lipoproteínas de baixa densidade na aterogênese;
- Diversos estudos sobre o papel protetor de agentes antioxidantes na prevenção de doenças relacionadas a esse processo;
- Doença com maior número de mortalidade e morbidade do mundo;
- Necessidade de um fármaco com maior eficácia que os existentes no mercado;

O objetivo deste estudo foi avaliar a capacidade de três compostos de classes diferentes em prevenir modificações oxidativas nas porções lipídicas e proteicas das lipoproteínas de baixa densidade, mediadas por diferentes oxidantes, procurando encontrar os possíveis mecanismos de ação destes.

3.2 Objetivos Específicos

- Avaliar o efeito da ingestão de guaraná (*Paullinia cupana* Kunth) quanto à resistência da LDL à oxidação em indivíduos idosos;
- Avaliar o efeito do guaraná, do DPTVP e do SPTS na prevenção da oxidação lipídica e proteica de LDL isolada e de soro de humanos;
- Avaliar o efeito do SPTS na prevenção de lipoperoxidação em tecidos de ratos induzidas por nitroprussiato de sódio;
- Determinar a capacidade antioxidante total (TRAP) do guaraná, do DPTVP e do SPTS;

4. ARTIGOS CIENTÍFICOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos, os quais se encontram aqui organizados. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos. Os artigos estão dispostos na forma em que foram submetidos para as revistas.

4.1 Artigo 1

4.1.1 Efeitos do guaraná (*Paullinia cupana* Kunth) na oxidação de LDL humana

Guaraná (*Paullinia cupana* Kunth) effects on LDL oxidation in elderly people: an *in vitro* and *in vivo* study

Rafael de Lima Portella, Rômulo Pillon Barcelos, Edovando José Flores da Rosa, Euler Esteves Ribeiro, Ivana Beatrice Mânica da Cruz, Leila Suleiman, Felix Alexandre Antunes Soares

Guaraná (*Paullinia cupana* Kunth) effects on LDL oxidation in elderly people: an *in vitro* and *in vivo* study

Rafael de Lima Portella[1], Rômulo Pillon Barcelos[1], Edovando José Flores da Rosa[1], Euler Esteves Ribeiro[2], Ivana Beatrice Mânica da Cruz[1,2,3], Leila Suleiman[1], Felix Alexandre Antunes Soares[1]*

[1]Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Campus UFSM, Santa Maria, RS, Brazil.

[2]Universidade Aberta da Terceira Idade, Universidade do Estado do Amazonas, AM, Brazil.

[3]Departamento de Morfologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Campus UFSM, Santa Maria, RS, Brazil.

*CORRESPONDING AUTHOR:

Félix Alexandre Antunes Soares

Departamento de Química - CCNE - Universidade Federal de Santa Maria

97105-900 - Santa Maria - RS - Brazil

Phone: +55-55-3220-9522

Fax: +55-55-3220-8978

E-mail: felix_antunes_soares@yahoo.com.br

Running title: Guaraná effects on human LDL oxidation

Key words: Low density lipoprotein; Lipoperoxidation; Humans; Antioxidant; Plant extract.

Abstract

Previous experimental investigations have suggested that guaraná (*Paullinia cupana* Kunth, supplied by EMBRAPA Oriental) consumption is associated with a lower prevalence of cardiovascular metabolic diseases and has positive effects on lipid metabolism, mainly related to LDL-cholesterol levels. As LDL-cholesterol oxidation is an important initial event in the development of atherosclerosis, we performed *in vitro* and *in vivo* studies to observe the potential effects of guaraná on LDL-cholesterol and serum oxidation. The *in vivo* protocol was performed using blood samples from 42 healthy elderly subjects who habitually ingested guaraná (GI) or never ingested guaraná (NG). The formation of conjugated dienes (CDs) was analyzed from serum samples. The *in vitro* protocols were performed using LDL obtained from 3 healthy, non-fasted, normolipidemic voluntary donors who did not habitually ingest guaraná in their diets. The LDL samples were exposed to 5 different guaraná concentrations (0.05, 0.1, 0.5, 1, and 5 μg/mL). GI subjects demonstrated lower LDL oxidation than did NG subjects (reduction of 27%, $p < 0.0014$), independent of other variables. However, in the GI group the LDL oxidation was positively associated with glucose levels. Also, guaraná demonstrated a high antioxidant activity *in vitro*, mainly at concentrations of 1 and 5 μg/mL, demonstrated by suppression of CDs and TBARS productions, tryptophan destruction and high TRAP activity. Guaraná, similar to other foods rich in caffeine and catechins such as green tea, has some effect on LDL oxidation that could partially explain the protective effects of this food in cardiometabolic diseases.

1. Introduction

Foods rich in catechins and caffeine, such as tea and coffee, are the most popular beverages in the world, and have been consumed for thousands of years because of their alluring flavors and health benefits. Several epidemiological and experimental investigations have described an inverse association between tea consumption and cardiovascular diseases (Hodgson, J.M. *et al.*, 2010). A recent study described an association between coffee and tea consumption and a low morbidity and mortality risk from stroke, coronary heart disease (CHD), and all causes of mortality in 37,514 subjects followed for 13 years (de Koning Gans, J.M. *et al.*, 2010). A possible causal factor associated with tea and coffee consumption is the role of bioactive compounds present in these foods in metabolic pathways related to body weight loss, and a consequent reduction of the overall risk for developing metabolic syndrome (Heckman, M.A. *et al.*, 2010).

However, it is important to analyze whether other foods rich in these compounds show biological properties similar to those of the guaraná used in energy drinks (*Paullinia cupana* H.B.K., Sapindaceae) (Angelo, P.C. *et al.*, 2008). Guaraná is a rainforest vine that was domesticated in the Amazon for its caffeine-rich fruits. During the last two decades, guaraná has emerged as a key ingredient in various 'sports' and energy drinks (Smith, N. *et al.*, 2007). Energy drinks have increased in popularity with adolescents and young adults. Caffeine, the most physiologically active ingredient in energy drinks, is generally considered safe by the US Food and Drug Administration (FDA), although adverse effects can occur at varying amounts. Guaraná, which contains caffeine in addition to small amounts of theobromine, theophylline, and tannins, is also recognized as safe by the FDA (Duchan, E. *et al.*, 2010). Because the consumption of guaraná is growing in many countries, studies on its functional properties are needed.

Previous experimental investigations have suggested that guaraná has positive effects on lipid metabolism (Lima, W.P. *et al.*, 2005), in body weight loss (Boozer, C.N. *et al.*, 2001), and increases basal energy expenditure (Berube-Parent, S. *et al.*, 2005). Furthermore, studies suggested that guaraná exhibits a cardioprotective effect by inhibiting platelet aggregation (Bydlowski, S.P. *et al.*, 1988). These all positive effects contributed to reduce the risk factors for cardiovascular

diseases. However, in contrast to green tea and coffee, on which many epidemiological studies have been performed, investigations involving guaraná consumption are difficult to perform because guaraná originates in a specific Brazilian Amazonian region (Maués-AM) (Smith, N. *et al.*, 2007). Besides, there are no informations whether guaraná consumption might increase the resistance to LDL oxidation.

For this reason, a controlled study was recently performed to analyze the association between habitual guaraná consumption and the prevalence of metabolic disease (obesity, hypertension, type 2 diabetes, and dyslipidemia) in an elderly population living in the Amazon's Riverine region (Maués-AM). The study observed a lower prevalence of hypertension, obesity, and metabolic syndrome in the subjects which self-reported habitual guaraná consumption (GI) than in subjects who reported never ingesting guaraná (NG). Additionally an association was found between habitual guaraná consumption and lower cholesterol (total and LDL-c) and advanced oxidative protein product (AOPP) levels (Costa Krewer, C. *et al.*, 2011). The potential effect of guaraná on LDL-cholesterol levels as well as oxidative biomarkers (AOPP) could provide a possible causal explanation for the lower prevalence of some cardiovascular metabolic diseases observed in Maués's study.

These results lead us to verify whether guaraná may have a possible antiatherogenic activity. Lipid peroxidation induced by free radicals has been implicated in the pathogenesis of various diseases. Numerous *in vitro* and animal studies have shown that oxidative modification of low density lipoprotein (LDL) is an important initial event for the development of atherosclerosis (Niki, E., 2011). Moreover, it is known that age is one of the major risk factor for atherosclerotic vascular disease (Bruckdorfer, K.R., 2008). Plasma cholesterol, triglyceride, LDL-cholesterol, polyunsaturated fatty acid (PUFA), total fatty acid and malondialdehyde levels, were found to increase in aged humans compared with young groups (Mehmetcik, G. *et al.*, 1997). In addition, VLDL+LDL oxidizability increased and total thiol content levels in plasma decreased in aged humans and rats compared with young groups (Mehmetcik, G. *et al.*, 1997).

The experimental studies described earlier and the established inverse relationship between the consumption of fruit and vegetables and cardiovascular diseases have led to a number of new studies on patients and populations that, for the most part, seem to reinforce the central role of antioxidants as protective

nutrients (Bruckdorfer, K.R., 2008). Considering these facts, we performed an *in vivo* study to investigate the potential effects of guaraná in elderly people on LDL-cholesterol and serum oxidation and an *in vitro* study in order to investigate the possible mechanism involved in these effects.

2. Materials and methods

2.1. *In vivo* assay: effect of habitual guaraná consumption on serum oxidation

From a previous data and biological bank of the study performed by Krewer et al (2011) (Costa Krewer, C. *et al.*, 2011) that investigated elderly included in GI (n=421) and NG groups (NG, n=239) we select 42 samples from 22 males and 20 females (≥ 60 years of age, 23 GI and 19 NG) to perform a serum oxidation analysis. We selected subjects without previous life style as smoking habit and higher alcoholic beverage consumption and morbidities that could to influence the analysis of serum oxidation as: diabetes mellitus 2, obesity, dyslipidemia, severe hypertension, metabolic syndrome, cardiovascular diseases, neoplasias and other metabolic diseases. The methodology used to determine biochemical parameters from elderly Riverine inhabitants who habitually ingest guaraná (GI) and those who never ingest guaraná (NG) are described in materials and methods from Krewer et al (2011) (Costa Krewer, C. *et al.*, 2011).

The Maués elderly population study was approved by the Ethical Committee of the Universidade do Estado do Amazonas, Brasil (nº 807/04). Since the vast majority of the elderly included in this study were illiterate, oral consent or fingerprint in Term was obtained to indicate their voluntary participation in the study after the researchers read the consent form to the patients.

Venous blood was drawn from these 42 previous selected elderly subjects into tubes containing no anticoagulant and centrifuged at 1000g for 15 min and the serum was stored at -20°C until to be analyzed. Serum diluted 100-fold was incubated at 37°C in a medium containing 10 mM phosphate buffer (pH 7.4). The oxidation was initiated by the addition of $CuSO_4$ (30μM) and conjugated dienes (CD) formation was monitored at 245 nm as previously described (Schnitzer, E. *et al.*, 1998).

2.2. Guaraná extract

Powdered *Paullinia cupana* Kunth seed produced and supplied by EMBRAPA Oriental (Agropecuary Research Brazillian Enterprise) located Western Amazon in Maués, Amazonas-Brazil was used in all experiments. The guaraná powder was conserved in dry conditions at ± 4°C, protected against light action until the extracts preparations.

We used a hydro-alcoholic extract of *Paullinia cupana* Kunth using alcohol and water (70:30) to 100mL of extraction fluid prepared at a concentration of 300mg/mL. The detailed description and determination of mainly bioactive compounds presents guaraná extract used in this study is presented in Bittencourt et al. (Bittencourt LS, Machado DC, Machado MM, Santos GFF, Algarve TD, Marinowic DR, Ribeiro EE, Soares FAA, Athayde ML, Cruz IBM, unpublished results). Briefly, after 21 days of guaraná extraction the extract was centrifuged for 1000g during 10 min and the supernatant was isolated and lyophilized. The mainly xanthines and catechins presented in guaraná extract was analyzed by chromatography and from this analysis was found caffeine = 12.240mg/g, theobromine = 6.733mg/g, total catechins = 4.336mg/g, and condensed tannins = 22mg/g.

The guaraná solution used in the study was prepared based in Santa Maria et al. (1998) (Santa Maria, A. *et al.*, 1998) protocol. The extract obtained and lyophilized was diluted in distillated water prepared at a concentration of 200mg/mL. The mixture was infused for 7 min in boiling, and centrifuged (600g, 15 min) and filtered. Five guaraná concentrations were tested here: 0.05, 0.1, 0.5, 1 and 5µg/mL. In TRAP assay were used different guaraná concentrations (0.01-10µg/mL).

2.3. LDL isolation to *in vitro* protocol tests

To perform *in vitro* LDL-oxidation assays, firstly the LDL was isolated from fresh human plasma by discontinuous density-gradient ultracentrifugation as described by Silva et al., 1998 (Luiz da Silva, E. *et al.*, 1998), with few modifications. Briefly, plasma of non-fasted three healthy normolipidemic voluntary donors that did not ingest guaraná in your habitual diet collected with EDTA (1 mg/mL) was pooled and sucrose (final concentration, 0.5%) was added to prevent LDL aggregation. Five

milliliters of EDTA-plasma adjusted to a density of 1.22g/mL with solid KBr (0.326g/mL) was layered on the bottom of a centrifuge tube. Then, 5 mL EDTA-containing sodium chloride solution (density 1.006g/mL) was overlaid on the top of the plasma. Ultracentrifugation was run at 350,000g for 2h at 4ºC, in a Himac CP80MX ultracentrifuge. LDL particles were collected by the aspiration of the yellow/orange band at the middle of the saline layer and dialyzed exhaustively overnight at 4ºC with 10mM phosphate buffer (pH 7.4). Protein concentration in LDL solution was determined by Lowry's method (Lowry, O.H. *et al.*, 1951). The purity of LDL preparation was verified by agarose gel electrophoresis. Isolated LDL was stored at -20ºC for no longer than 2 weeks.

2.4. In vitro LDL oxidation analysis

2.4.1. Conjugated dienes and TBARS formation

LDL samples (50µg protein/mL) were pre-incubated at 37°C in a medium containing 10mM phosphate buffer (pH 7.4) and different guaraná concentrations (0.05 - 5µg/mL). After 5 minutes, the oxidation was initiated by the addition of either $CuSO_4$ (5µM). The oxidation was monitored by measuring the increase in absorbance at 234nm due to conjugated diene (CD) formation as previously described (Gieseg, S.P. *et al.*, 1994). Aliquots were also removed at different time points for evaluating thiobarbituric acid reactive substances (TBARS) production as previously described (Ohkawa, H. *et al.*, 1979).

2.4.2. Measurement of LDL- Tryptophan fluorescence

The fluorescence spectra of native LDL display a single band centered at approximately 332nm, which is assigned to the tryptophan (Trp) residues in apolipoprotein B-100 (apoB-100) (Giessauf, A. *et al.*, 1995). Loss of Trp fluorescence is a marker for oxidations at the protein core of LDL (Giessauf, A. *et al.*, 1995). Trp fluorescence was measured in a solution of LDL (50µg protein/mL) in PBS (10mM) pH 7.4 at 37°C, using a Shimatzo espectrofluorometer (excitation at 282nm and emission at 331nm) (Giessauf, A. *et al.*, 1995). The kinetics of LDL oxidation was followed by measuring the decrease of Trp-fluorescence, corresponding to the

decomposition of this amino acid, after the addition of 5μM $CuSO_4$, in absence or presence of guaraná (0.05 – 5μg/mL). The cuvettes had to be removed from the excitation light between the single measurements to avoid photooxidation of the Trp residues; fluorescence was measured every 20 min. Data are shown as the percent decrease of Trp fluorescence in each sample. The time required for reaching half Trp fluorescence ($t_{1/2}$) was calculated.

2.5. In vitro serum oxidation

Venous blood was drawn from nonfasted healthy normolipidemic voluntary donors into tubes containing no anticoagulant and centrifuged at 1000g for 15 min. Serum diluted 100-fold was incubated at 37°C in a medium containing 10mM phosphate buffer (pH 7.4) and different guaraná concentrations (0.05 – 5μg/mL). The oxidation was initiated by the addition of $CuSO_4$ (30μM) and CD formation was monitored at 245nm as previously described (Schnitzer, E. *et al.*, 1998).

2.6. Determination of lag phase and maximum oxidation rate

In the studies of CD formation, there are several parameters which can be obtained from diene vs. time profiles. The value of the lag phase is commonly determined graphically by the intercept of the tangents to the slow and fast increase of the diene absorption. Another parameter is the maximum oxidation rate, given by the peak of the first derivative, i.e. change of A_{234} as a function of time (Gieseg, S.P. *et al.*, 1994).

2.7. Total radical-trapping antioxidant potential (TRAP)

TRAP was determined by measuring the chemiluminescence intensity of luminol induced by [2,20-azo-bis(2-amidinopropane)-dihydrochloride] (AAPH) thermolysis in a luminometer BioTek Synergy 2 (Dresch, M.T. *et al.*, 2009). The reaction mixture contained AAPH (10mM) and luminol (35μM) dissolved in 0.1M glycine buffer (pH 8.6). Incubation of this mixture generates an almost constant light intensity at room temperature after stabilization. Guaraná was added in different concentrations to determine the TRAP activity. At this point, the luminescence

intensity is practically abolished. In the course of time, with the loss of antioxidant capacity of guaraná, the luminescence intensity returns to the initial values. The area under curve (AUC) was evaluated for each guaraná concentration and compared to vehicle AUC (Dresch, M.T. *et al.*, 2009).

2.8. Statistical analysis

Statistical analysis was performed using the SPSS/PC statistical package, version 17.0 (SPSS, Inc., IL). Data are expressed as means ± SD. Comparison between characteristics baselines and serum oxidation of GI and NG elderly subjects was performed by Student T test. Multivariate logistic regression (Backward Wald method) and Pearson correlation tests were performed to observe possible intervenient factors. The follow variables considered in these analysis: sex, age, BMI, blood pressure, glucose, cholesterol total, HDL-cholesterol,, triglycerides, TBARS, total polyphenols and protein carbonilation. *In vitro* LDL-oxidation assays were statistical analyzed using a one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls test when appropriate. In addition, linear regression was performed to identify a possible dose dependent effect. Values of $p<0.05$ were considered significant.

3. Results

3.1. Baseline characteristics of subjects

The baseline characteristics of GI and NG elderly subjects included in the *in vivo* serum oxidation assay are presented in Table 1. In general, the two elderly groups were similar in body mass index (BMI), blood pressure, glucose levels, and certain lipid parameters. However, the levels of total cholesterol were higher in the NG subjects than in the GI subjects. Additionally, the oxidative biomarker parameters such as TBARS, total polyphenols, and protein carbonylation were similar for the two groups.

3.2. Conjugated diene levels in Maués's population

The serum samples from elderly GI and NG subjects were evaluated for maximum CD production. The diene levels were significantly different between the two groups analyzed here. The GI group showed lower diene formation (reduction of 27%, $p < 0.0014$) than the NG group (Figure 1A). The potential gender effect on diene formation in GI and NG groups was also analyzed. Figure 1B shows that both men (reduction of 21%, $p < 0.0168$) and women (reduction of 33%, $p < 0.0311$) who drank guaraná showed a significant decrease in maximum CD production compared to their respective gender subjects that did not drink guaraná.

Additionally, a correlation analysis of diene levels and age, blood pressure, BMI, glucose, lipid, and oxidative biomarkers was performed in the GI and NG groups. The GI group showed a positive and significant correlation between diene levels and plasma glucose concentrations ($r^2=0.494$, $p=0.016$). In the NG group, we could not observe any significant correlation with the variables analyzed in this study.

3.3. *In vitro* guaraná effect on LDL oxidation

3.3.1. Conjugated diene (CD) production:

Isolated LDL samples incubated with different concentrations of guaraná showed a concentration-dependent increase in the lag phase of LDL oxidation ($r^2=0.88$, $p<0.001$). Guaraná concentrations in the range of 0.5 – 5µg/mL significantly inhibited $CuSO_4$-induced LDL oxidation, increasing the lag phase (Figure 2A). At concentrations of 1 and 5µg/mL, guaraná totally prevented LDL oxidation during an assay time of 180 min (Figure 2B). However, guaraná had no effect on the maximum rate of oxidation (data not shown).

3.3.2. TBARS production

LDL oxidation was also evaluated by TBARS formation. Table 2 shows a significant effect of guaraná in preventing TBARS formation induced by $CuSO_4$. The protective effect of guaraná was similar to that demonstrated in the CD experiments. Guaraná at 1µg/mL showed a significant effect during an assay time of 60 – 180

minutes. At a concentration of 5µg/mL, guaraná prevented TBARS formation during an assay time of 60 – 360 min.

3.3.3. LDL tryptophan fluorescence

Figure 3 shows the time required to achieve 50% Trp fluorescence ($t_{1/2}$) during $CuSO_4$-induced LDL oxidation. When compared to the control group, the presence of guaraná at all concentrations tested significantly increased the $t_{1/2}$ of Trp. Guaraná at 1 and 5µg/mL showed a total protection during 180 minutes. This result was similar that for the conjugated dienes and TBARS formation.

3.4. CD production in serum

Serum oxidation was determined by CD formation at 245nm. No oxidation occurred in serum when the medium did not contain $CuSO_4$. Figure 4 shows that guaraná at concentrations of 1 and 5µg/mL was able to cause a significant increase in the lag phase of oxidation. At the highest concentration, guaraná totally inhibited the serum oxidation during the assay time. Similar to its effect on LDL oxidation, guaraná demonstrated no effect on the maximum oxidation rate in serum oxidation (data not shown).

3.5. Total radical-trapping antioxidant potential of guaraná

Figure 5 shows the antioxidant potential of guaraná when AAPH was used as pro-oxidant. This method is based on the use of a water-soluble azo compound, AAPH [2,20-azo-bis(2-amidinopropane)-dihydrochloride], as a reliable and quantifiable source of alkyl peroxyl radicals. The thermal decomposition of these compounds in the presence of luminol produces luminescence, which is quenched by the addition of peroxyl radical scavengers (Dresch, M.T. *et al.*, 2009). Guaraná concentrations ranging from 0.01 to 10µg/mL were used in this experiment. We observed that all concentrations tested were able to reduce the AAPH-induced luminol oxidation, and that guaraná concentrations of 0.5 – 10µg/mL demonstrated very strong inhibition.

4. Discussion

To our knowledge, this is the first study conducted to investigate the potential association between guaraná's effect on LDL and serum oxidation. The results showed that guaraná ingestion (GI) resulted in lower maximum conjugated diene production than that found in an elderly NG group (Figure 1). Furthermore, *in vitro* assays showed that guaraná increased the lag phase in the oxidation of LDL and serum *in vitro* (Figures 2 and 4), and also prevented TBARS production (Table 2) and Trp destruction (Figure 3) in LDL oxidation. Moreover, we observed that guaraná extract demonstrates a peroxyl radical scavenger activity (Figure 5).

The effects of guaraná on LDL and serum oxidation are probably associated with some bioactive compounds (catechins and xantines) that are similar to those found in other foods, such as green tea (Angelo, P.C. *et al.*, 2008). Tea polyphenols are well studied and there are numerous studies consistently describing these compounds as having antioxidative, antithrombogenic, antiinflammatory, hypotensive, hypocholesterolemic, antihypertensive, and antiobesogenic effects (Yung, L.M. *et al.*, 2008). The potential beneficial effects of daily guaraná consumption described by Krewer et al. (2011) (Costa Krewer, C. *et al.*, 2011) include a lower prevalence of hypertension, obesity, and metabolic syndrome, and lower cholesterol (total and LDL-c) and AOPP levels in GI subjects. These effects are similar to those described in epidemiological studies involving green tea (Chacko, S.M. *et al.*, 2010). We believe that our results contribute in the elucidation of potential causal factors related to these associations.

First, it is important to consider the bioactive effects of guaraná related to the main chemical compounds. The antioxidant effects of guaraná extract might be due to methylxanthines, such as caffeine, theobromine, and theophylline, and also to tannins, saponins, catechins, epicatechins, proanthocyanidols, as well as trace concentrations of many other compounds (Belliardo, F. *et al.*, 1985). Effect of caffeine on LDL resistance to oxidative modification has been excluded by several *in vitro* (Krisko, A. *et al.*, 2005) and *ex vivo* (Hodgson, J.M. *et al.*, 2000) studies. On the other hand, caffeine has been linked to increased thermogenesis and decreased body weight in some clinical studies (Westerterp-Plantenga, M.S., 2010). These effects of caffeine may contribute to prevent the other risk factors related to

atherosclerotic vascular disease such as waist circumference and blood pressure (Costa Krewer, C. *et al.*, 2011).

It has been shown that guaraná inhibits the lipid peroxidation process, an effect apparently associated with the high tannin content of the seeds, which reach concentrations between 16% and 31% (Mattei, R. *et al.*, 1998; Yamaguti-Sasaki, E. *et al.*, 2007). Fresh tea leaf is unusually rich in the flavanol group of polyphenols known as catechins which may constitute up to 30% of the dry leaf weight and there is no tannic acid in tea (Graham, H.N., 1992). Tannins are polyphenolic compounds having molecular masses between 500 and 3000 Da and a sufficiently large number of phenolic groups (Quideau, S. *et al.*, 2011). Yamaguti-Sasaki et al. 2007 (Yamaguti-Sasaki, E. *et al.*, 2007) identified some procyanidins (condensed tannins) as epicatechin-(4β→8)-epicatechin (procyanidin B2), catechin-(4α→8)-catechin (procyanidin B3), and catechin-(4α→8)-epicatechin (procyanidin B4). It has been demonstrated that green tea catechins demonstrate antioxidant activity by scavenging free radicals and chelating redox active transition-metal ions (Babu, P.V. *et al.*, 2008). Catechins have many phenolic hydroxyl groups in their structures and have been shown to inhibit oxidative modification of LDL when added before initiation of oxidation (Miura, S. *et al.*, 1995). However, the mechanisms by which these flavonoids inhibit LDL oxidation have not been clarified. Because of their amphipathic nature, flavonoids may act within the LDL particle in a manner similar to that of vitamin E, or may act in a manner comparable with that of ascorbic acid in the extraparticle environment of LDL. Considering our *in vivo* results (Figure 1), we might suppose that polyphenols from guaraná could incorporate into LDL, turning the serum from GI subjects less susceptible to oxidation *in vitro*.

Guaraná's effect on lipid peroxidation *in vitro* is in agreement with Mattei et al. (1998) (Mattei, R. *et al.*, 1998), who showed that guaraná extract inhibited lipoperoxidation even at low concentrations (1.2µg/mL). Besides their *in vitro* effects, catechins demonstrate additional effects *in vivo* by inhibiting redox active transcriptional factors, inhibiting pro-oxidant enzymes, and inducing antioxidant enzymes, which could explain their beneficial effects *in vivo* (Babu, P.V. *et al.*, 2008). These effects make the potential use of guaraná extract even most promising to promote protection against atherogenesis.

In our *in vivo* study, the diene levels were significantly lower in GI than in NG subjects. It may indicate that guaraná intake is able to provide an additional

antioxidant protection to serum and, mainly, to LDL. It was in agree with our *in vitro* data, which could increase the lag phase of serum and LDL oxidation, prevent TBARS production and tryptophan destruction (Figures 2, 3 and 4 and Table 2). In the actual *in vivo* condition, the polyphenols and methylxanthines in plasma may work together to prevent LDL oxidation. These polyphenols may be more easily incorporated into LDL *in vivo* than *in vitro*. Although guaraná polyphenols may be metabolized quickly after entering the circulation, it is possible that these metabolites also exert preventive effects on LDL oxidation. Repeated exposure of LDL particles to guaraná polyphenols over a long period of time may enrich the LDL particles sufficiently to make them less susceptible to oxidative stress.

Certain physiological aspects must be considered with respect to guaraná's effect on LDL oxidation. The atherogenesis process related to cardiovascular diseases initially involves the oxidative modification of LDL. This is the first step in foam cell formation. Oxidized LDL (oxLDL) is produced in the subendothelial space and taken up by resident macrophages via scavenger receptors, leading to their transformation into foam cells (Thomson, M.J. *et al.*, 2007). Additionally, oxLDL can induce the expression of adhesion molecules, plus the synthesis of cytokines, oxygen radical species, and certain growth factors in endothelial cells, macrophages, and smooth muscle cells (Thomson, M.J. *et al.*, 2007). Therefore, the potential effect of guaraná on LDL-cholesterol oxidation described here is biologically plausible because we found that blood samples from elderly GI subjects showed a lower maximum production of conjugated dienes than samples from NG subjects. Additionally, our data corroborate with previous studies that described an association between green tea (Gomikawa, S. *et al.*, 2008) and cocoa powder (Baba, S. *et al.*, 2007) consumption and a decrease in LDL oxidation.

Multivariate and correlation analyses conducted in this study found just one significant association between glucose and serum oxidation. Interestingly, glucose and LDL-c levels were also positively correlated only in the GI group. This finding could indicate that conjugated diene production is associated with both glucose and LDL-c levels together. Evidence concerning the role of oxidative and glycative/glycoxidative modifications of low-density lipoprotein (LDL) induced by hyperglycemia has been published in the literature (Knott, H.M. *et al.*, 2003). Previous studies have shown that high glucose (HG) increases the susceptibility of LDL to oxidation (Otero, P. *et al.*, 2002). On the other hand, the investigation

performed by Wu et al (Wu, C.H. *et al.*, 2009) observed that the introduction of flavonoids into LDL particles protects the lipoprotein against glycotoxin-mediated adverse effects. Our results suggest that even within the glucose range expected (≤100 mg/dL), higher glucose levels directly impact the effect of guaraná on LDL oxidation. However, studies investigating whether the presence of different glucose and insulin concentrations affects guaraná's action on LDL oxidation and other biological variables need to be conducted.

It is important to ponder some considerations associated with the methodological design of our *in vivo* protocol. Since the *in vivo* study was performed using a group of elderly subjects, and without controlling for the amounts of guaraná ingested or the ingestion of other foods rich in antioxidants or bioactive compounds, it is important to conduct additional controlled studies on the possible effect of guaraná ingestion on LDL oxidation levels to confirm the results described here.

In summary, *in vivo* results showed that guaraná intake could reduce the diene levels in serum from elderly subject. This positive effect of guaraná could be confirmed by *in vitro* results which showed an increased resistance to LDL oxidation. It was due to high content of polyphenolic compounds, which may act to prevent atherosclerosis through a combination of effects, including the other positive effects of guaraná on lipid metabolism (Lima, W.P. *et al.*, 2005), in body weight loss (Boozer, C.N. *et al.*, 2001), and increases basal energy expenditure (Berube-Parent, S. *et al.*, 2005), besides the lower prevalence of hypertension, metabolic syndrome, and lower cholesterol and AOPP levels in GI subjects (Costa Krewer, C. *et al.*, 2011). Considering these results, our study indicates that consumption of guaraná regularly or its possible inclusion in diet-based therapies could yield certain health benefits and potential defense against atherogenesis and metabolic disorders.

5. Acknowledgments

We are grateful to the Maués governmental team and to Amazonas Governmental Health Service (ESF-SUS) and the all research team that assisted in data and blood sample collection. We are grateful to BioMed Proofreading. This work was supported by the FINEP research grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)" # 01.06.0842-00 and INCT for Excitotoxicity and

Neuroprotection- MCT/CNPq, CAPES, FAPEAM and CNPq. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Table 1. Baseline characteristics from elderly Riverine inhabitants who habitually ingest guaraná (GI) and those who never ingest guaraná (NG).

Variables	GI Means±SD	NG Means±SD	*p*
Age (years)	73.6±6.5	75.2±9.4	0.557
BMI (Kg/m^2)	27.4±3.3	27.0±4.2	0.580
Waist circumference (cm)	87.6±11.1	82.5±10.9	0.140
SBP (mmHg)	126.1±15.9	130.0±15.8	0.445
BSP (mmHg)	73.51±8.3	76.51±4.9	0.195
Glucose (mg/dL)	103.7±12.8	108.6±14.1	0.215
Cholesterol total (mg/dL)	189.7±30.8	230.2±61.6	0.009
Triglycerides (mg/dL)	128.7±47.7	150.9±51.7	0.57
LDL-chol (mg/dL)	132.5±45.8	161.1±52.6	0.08
HDL-chol (mg/dL)	42.6±24.0	42.2±19.1	0.958
Uric acid (mg/dL)	4.2±1.9	5.4±2.9	0.164
TBARS	21.7±7.9	19.9±7.0	0.441
Protein carbonilation	0.17±0.09	0.14±0.07	0.248
Total polyphenols	2.7±0.8	2.7±0.5	0.977

SD=standard deviation; BMI= Body mass index (Kg/m^2); SBP= systolic blood pressure; DBP= diastolic blood pressure. Comparison between GI and NG elderly subjects performed by *T Student* test. *p* value= statistical significance.

Table 2. Effects of guaraná concentrations on time-dependent changes in the thiobarbituric acid reactive substances (TBARS) production during LDL oxidation.

		Time (min)						
$CuSO_4$	**Guaraná**	**30**	**60**	**120**	**180**	**240**	**300**	**360**
-	**-**	0,28 ± 0,1	0,34 ± 0,22	0,35 ± 0,25	0,41 ± 0,16	0,54 ± 0,22	0,55 ± 0,26	0,71 ± 0,26
+	**-**	0,73 ± 0,15	1,73 ± 0,31**§**	3,34 ± 0,42**§**	3,53 ± 0,52**§**	3,87 ± 0,2**§**	3,6 ± 0,21**§**	3,31 ± 0,26**§**
+	**0.05µg/mL**	0,83 ± 0,15	2,09 ± 0,57	3,41 ± 0,43	3,73 ± 0,6	4,1 ± 0,1	3,71 ± 0,17	3,39 ± 0,33
+	**0.1µg/mL**	0,88 ± 0,22	1,89 ± 0,19	3,52 ± 0,34	3,75 ± 0,43	4,18 ± 0,16	3,73 ± 0,25	3,64 ± 0,24
+	**0.5µg/mL**	0,44 ± 0,15	0,9 ± 0,59	2,94 ± 0,84	3,84 ± 0,46	3,98 ± 0,13	3,82 ± 0,17	3,5 ± 0,02
+	**1µg/mL**	0,32 ± 0,11	0,46 ± 0,25#	0,61 ± 0,49*	1,12 ± 0,72*	3,3 ± 0,62	3,8 ± 0,2	3,69 ± 0,16
+	**5µg/mL**	0,37 ± 0,2	0,45 ± 0,31#	0,44 ± 0,28*	0,66 ± 0,33*	0,92 ± 0,16*	1,01 ± 0,21*	1,08 ± 0,19#

LDL (50 µg protein/mL) was incubated at 37°C in the presence of 5µM $CuSO_4$. Data are expressed as mean ± SD of three experiments. The values were expressed by nmol MDA/ 20µg protein LDL.

* $p < 0.01$ compared to $CuSO_4$ without guaraná at same time assay.

# $p < 0.001$ compared to $CuSO_4$ without guaraná at same time assay.

§ $p<0.001$ compared to group without $CuSO_4$ and guaraná at same time assay.

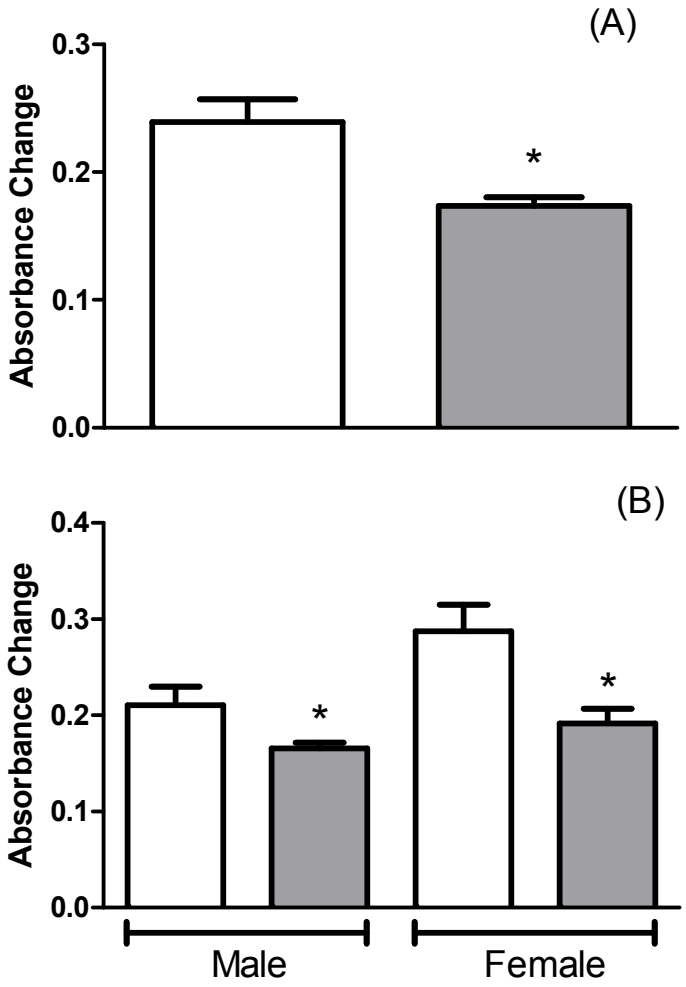


Figure 1. Maximum conjugated dienes production in serum from Maués inhabitants. Serum (diluted 100-fold) in PBS 10mM (pH7.4) was incubated at 37°C for 180min in the presence of 30 μM $CuSO_4$. Conjugated diene formation was measured by determining the absorbance at 245 nm every 20 min. In panel A, maximum CD production in serum of GI and NG subjects. In panel B, gender effect on maximum CD production between GI and NG groups. NG (white bars) and GI (gray bars). * $p<0.05$ compared to white bar.

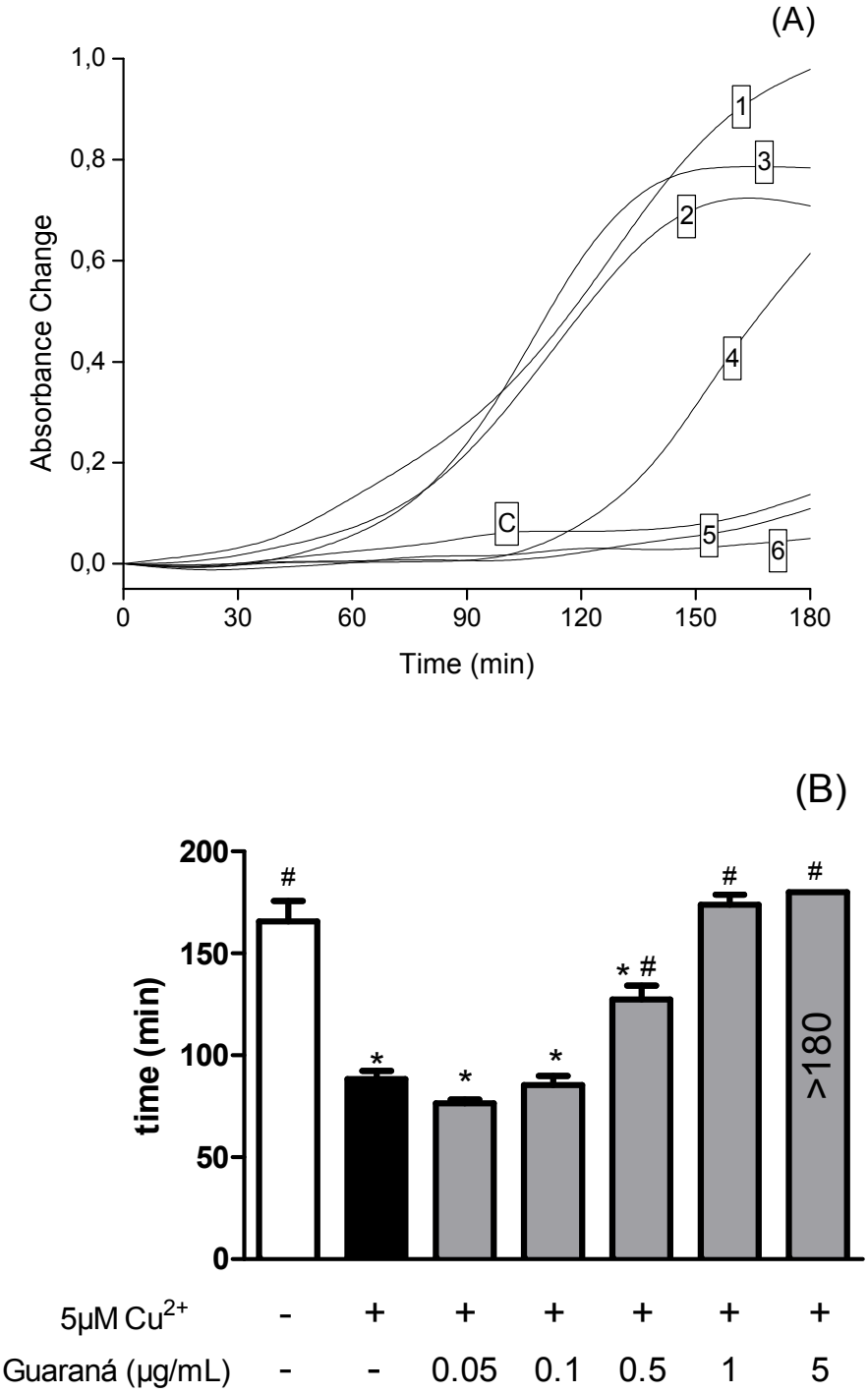


Figure 2. Effects of guaraná on LDL oxidation. LDL (50 μg protein/mL) was incubated in PBS 10mM (pH 7.4) at 37°C in the presence of $CuSO_4$ 5 μM. Conjugated diene formation was measured by determining the absorbance at 234 nm every 20 min. In panel A, incubation medium did not contain guaraná (1) or

contained 0.05 µg/mL (2), 0.1 µg/mL (3), 0.5 µg/mL (4), 1 µg/mL (5) or 5 µg/mL (6) of guaraná. (C) Control without $CuSO_4$ and guaraná. In panel B, the value of the lag phase determined graphically by the intercept of the tangents to the slow and fast increase of the diene absorption. Experiments were repeated six times, showing similar results. * $p < 0.05$ compared to group without $CuSO_4$ and guaraná. # $p < 0.05$ compared to group with $CuSO_4$ and without guaraná.

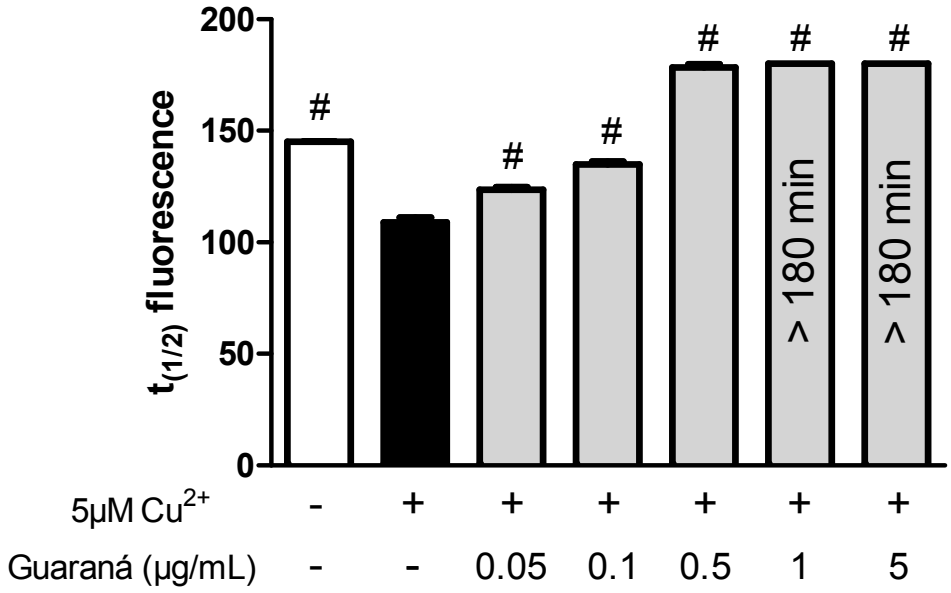


Figure 3. Time required for reaching half Trp fluorescence ($t_{1/2}$) during $CuSO_4$-mediated oxidation of LDL with different concentrations of guaraná. LDL (50 μg protein/mL) was incubated in PBS with 5 μM $CuSO_4$. Fluorescence (Ex/Em = 282/331 nm) was measured at intervals of 20 min at 37°C. Experiments were repeated three times, showing similar results. # $p<0.05$ compared to black bar.

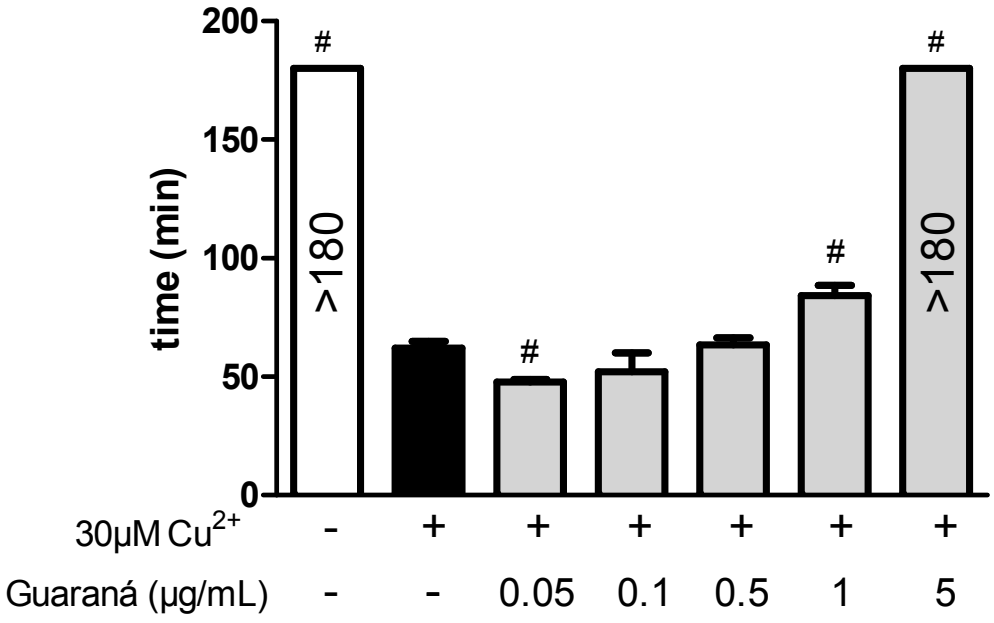


Figure 4. Effects of guaraná concentrations on phase lag of conjugated dienes formation during serum oxidation. Serum (diluted 100-fold) in PBS 10mM (pH7.4) was incubated at 37°C for 180min in the presence of 30 μM $CuSO_4$. Conjugated diene formation was measured by determining the absorbance at 245 nm every 20 min. Experiments were repeated three times, showing similar results. # $p<0.05$ compared to black bar.

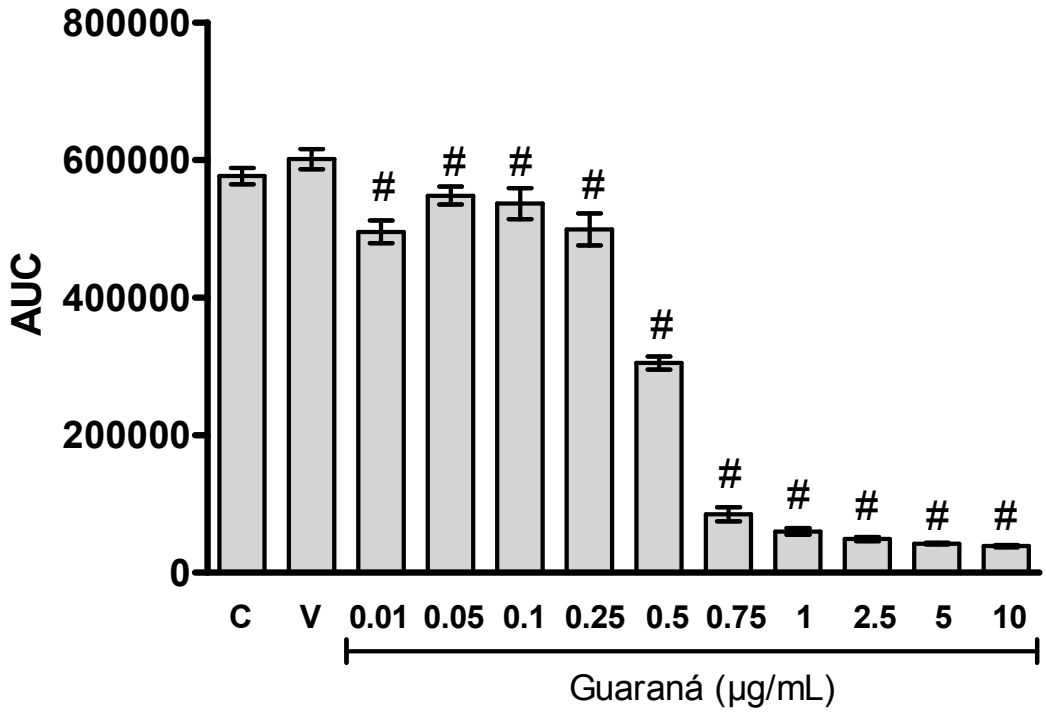


Figure 5. *In vitro* effect of guaraná on total radical-trapping antioxidant potential (TRAP). The reaction mixture contained 10 mM AAPH, 35 µM luminol and guaraná at different concentrations dissolved in 0.1M glycine buffer (pH 8.6). The luminescence was measured every 5 minutes during 5 hours. The area under curve (AUC) was evaluated. (V) Vehicle and (C) Control. The bars represent mean ± S.D. of five different experiments. # $p<0.001$ compared to control and vehicle group. There is no difference between vehicle and control group.

6. References

Angelo, P.C., et al. Guarana (Paullinia cupana var. sorbilis), an anciently consumed stimulant from the Amazon rain forest: the seeded-fruit transcriptome. **Plant Cell Rep,** V. 27, n. 1, p. 117-124, 2008.

Baba, S., et al. Continuous intake of polyphenolic compounds containing cocoa powder reduces LDL oxidative susceptibility and has beneficial effects on plasma HDL-cholesterol concentrations in humans. **Am J Clin Nutr,** V. 85, n. 3, p. 709-717, 2007.

Babu, P.V., Liu, D. Green tea catechins and cardiovascular health: an update. **Curr Med Chem,** V. 15, n. 18, p. 1840-1850, 2008.

Belliardo, F., Martelli, A., Valle, M.G. HPLC determination of caffeine and theophylline in Paullinia cupana Kunth (guarana) and Cola spp. samples. **Z Lebensm Unters Forsch,** V. 180, n. 5, p. 398-401, 1985.

Berube-Parent, S., et al. Effects of encapsulated green tea and Guarana extracts containing a mixture of epigallocatechin-3-gallate and caffeine on 24 h energy expenditure and fat oxidation in men. **Br J Nutr,** V. 94, n. 3, p. 432-436, 2005.

Boozer, C.N., et al. An herbal supplement containing Ma Huang-Guarana for weight loss: a randomized, double-blind trial. **Int J Obes Relat Metab Disord,** V. 25, n. 3, p. 316-324, 2001.

Bruckdorfer, K.R. Antioxidants and CVD. **Proc Nutr Soc,** V. 67, n. 2, p. 214-222, 2008.

Bydlowski, S.P., Yunker, R.L., Subbiah, M.T. A novel property of an aqueous guarana extract (Paullinia cupana): inhibition of platelet aggregation in vitro and in vivo. **Braz J Med Biol Res,** V. 21, n. 3, p. 535-538, 1988.

Chacko, S.M., et al. Beneficial effects of green tea: a literature review. **Chin Med,** V. 5, n. p. 13, 2010.

Costa Krewer, C., et al. Habitual Intake of Guarana and Metabolic Morbidities: An Epidemiological Study of an Elderly Amazonian Population. **Phytother Res,** V. n. 2011.

de Koning Gans, J.M., et al. Tea and coffee consumption and cardiovascular morbidity and mortality. **Arterioscler Thromb Vasc Biol,** V. 30, n. 8, p. 1665-1671, 2010.

Dresch, M.T., et al. Optimization and validation of an alternative method to evaluate total reactive antioxidant potential. **Anal Biochem,** V. 385, n. 1, p. 107-114, 2009.

Duchan, E., Patel, N.D., Feucht, C. Energy drinks: a review of use and safety for athletes. **Phys Sportsmed,** V. 38, n. 2, p. 171-179, 2010.

Gieseg, S.P., Esterbauer, H. Low density lipoprotein is saturable by pro-oxidant copper. **FEBS Lett,** V. 343, n. 3, p. 188-194, 1994.

Giessauf, A., Steiner, E., Esterbauer, H. Early destruction of tryptophan residues of apolipoprotein B is a vitamin E-independent process during copper-mediated oxidation of LDL. **Biochim Biophys Acta,** V. 1256, n. 2, p. 221-232, 1995.

Gomikawa, S., et al. Effect of ground green tea drinking for 2 weeks on the susceptibility of plasma and LDL to the oxidation ex vivo in healthy volunteers. **Kobe J Med Sci,** V. 54, n. 1, p. E62-72, 2008.

Graham, H.N. Green tea composition, consumption, and polyphenol chemistry. **Prev Med,** V. 21, n. 3, p. 334-350, 1992.

Heckman, M.A., Weil, J., Gonzalez de Mejia, E. Caffeine (1, 3, 7-trimethylxanthine) in foods: a comprehensive review on consumption, functionality, safety, and regulatory matters. **J Food Sci,** V. 75, n. 3, p. R77-87, 2010.

Hodgson, J.M., Croft, K.D. Tea flavonoids and cardiovascular health. **Mol Aspects Med,** V. 31, n. 6, p. 495-502, 2010.

Hodgson, J.M., et al. Acute effects of ingestion of black and green tea on lipoprotein oxidation. **Am J Clin Nutr,** V. 71, n. 5, p. 1103-1107, 2000.

Knott, H.M., et al. Glycation and glycoxidation of low-density lipoproteins by glucose and low-molecular mass aldehydes. Formation of modified and oxidized particles. **Eur J Biochem,** V. 270, n. 17, p. 3572-3582, 2003.

Krisko, A., Kveder, M., Pifat, G. Effect of caffeine on oxidation susceptibility of human plasma low density lipoproteins. **Clin Chim Acta,** V. 355, n. 1-2, p. 47-53, 2005.

Lima, W.P., et al. Lipid metabolism in trained rats: effect of guarana (Paullinia cupana Mart.) supplementation. **Clin Nutr,** V. 24, n. 6, p. 1019-1028, 2005.

Lowry, O.H., et al. Protein measurement with the Folin phenol reagent. **J Biol Chem,** V. 193, n. 1, p. 265-275, 1951.

Luiz da Silva, E., Tsushida, T., Terao, J. Inhibition of mammalian 15-lipoxygenase-dependent lipid peroxidation in low-density lipoprotein by quercetin and quercetin monoglucosides. **Arch Biochem Biophys,** V. 349, n. 2, p. 313-320, 1998.

Mattei, R., et al. Guarana (Paullinia cupana): toxic behavioral effects in laboratory animals and antioxidants activity in vitro. **J Ethnopharmacol,** V. 60, n. 2, p. 111-116, 1998.

Mehmetcik, G., et al. Age-related changes in plasma lipid peroxidation and antioxidant system in humans and rats. **Arch Gerontol Geriatr,** V. 25, n. 3, p. 305-310, 1997.

Miura, S., et al. Effects of various natural antioxidants on the Cu(2+)-mediated oxidative modification of low density lipoprotein. **Biol Pharm Bull,** V. 18, n. 1, p. 1-4, 1995.

Niki, E. Do free radicals play causal role in atherosclerosis? Low density lipoprotein oxidation and vitamin E revisited. **J Clin Biochem Nutr,** V. 48, n. 1, p. 3-7, 2011.

Ohkawa, H., Ohishi, N., Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. **Anal Biochem,** V. 95, n. 2, p. 351-358, 1979.

Otero, P., Herrera, E., Bonet, B. Dual effect of glucose on LDL oxidation: dependence on vitamin E. **Free Radic Biol Med,** V. 33, n. 8, p. 1133-1140, 2002.

Quideau, S., et al. Plant Polyphenols: Chemical Properties, Biological Activities, and Synthesis. **Angew Chem Int Ed Engl,** V. n. 2011.

Santa Maria, A., et al. Evaluation of the toxicity of guarana with in vitro bioassays. **Ecotoxicol Environ Saf,** V. 39, n. 3, p. 164-167, 1998.

Schnitzer, E., et al. Lipid oxidation in unfractionated serum and plasma. **Chem Phys Lipids,** V. 92, n. 2, p. 151-170, 1998.

Smith, N., Atroch, A.L. Guarana's Journey from Regional Tonic to Aphrodisiac and Global Energy Drink. **Evid Based Complement Alternat Med,** V. n. 2007.

Thomson, M.J., Puntmann, V., Kaski, J.C. Atherosclerosis and oxidant stress: the end of the road for antioxidant vitamin treatment? **Cardiovasc Drugs Ther,** V. 21, n. 3, p. 195-210, 2007.

Westerterp-Plantenga, M.S. Green tea catechins, caffeine and body-weight regulation. **Physiol Behav,** V. 100, n. 1, p. 42-46, 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,** V. 57, n. 11, p. 5058-5064, 2009.

Yamaguti-Sasaki, E., et al. Antioxidant capacity and in vitro prevention of dental plaque formation by extracts and condensed tannins of Paullinia cupana. **Molecules,** V. 12, n. 8, p. 1950-1963, 2007.

Yung, L.M., et al. Tea polyphenols benefit vascular function. **Inflammopharmacology,** V. 16, n. 5, p. 230-234, 2008.

4.2 Artigo 2

4.2.1 Efeitos do salicilaldeído-4-feniltiosemicarbazona na oxidação de LDL humana

The Potential Antioxidant Activity of a Thiosemicarbazone Against LDL and Serum Oxidation

Rafael de Lima Portella, Rômulo Pillon Barcelos, Edovando José Flores da Rosa, Vanessa Santana Carratu, Leandro Bresolin, João Batista Teixeira da Rocha, Félix Alexandre Antunes Soares.

The Potential Antioxidant Activity of a Thiosemicarbazone Against LDL and Serum Oxidation

Rafael de Lima Portella[1], Rômulo Pillon Barcelos[1], Edovando José Flores da Rosa[1], Vanessa Santana Carratu[2], Leandro Bresolin[2], João Batista Teixeira da Rocha[1], Félix Alexandre Antunes Soares[1]*.

[1]Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Campus UFSM, Santa Maria, RS, Brasil.

[2]Departamento de Química, Fundação Universidade de Rio Grande, Campus Carreiros, Rio Grande, RS, Brasil.

*CORRESPONDING AUTHOR:

Félix Alexandre Antunes Soares

Departamento de Química - CCNE - Universidade Federal de Santa Maria

97105-900 - Santa Maria - RS - Brazil

Phone: +55-55-3220-9522

Fax: +55-55-3220-8978

E-mail: felix_antunes_soares@yahoo.com.br

Abstract

The oxidation of lipoproteins is hypothesized to be an important early step in atherogenesis. Various studies have shown a positive effect of antioxidant compounds on oxidative modification of LDL and atherogenesis. We investigated the possible antioxidant effect of a thiosemicarbazone, salicylaldehyde-4-phenylthiosemicarbazone (SPTS), on the prevention of LDL and serum oxidation and tissue lipoperoxidation. Our results demonstrated that SPTS may have antioxidant activity against Cu^{2+}- and 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH)-induced LDL and serum oxidation. Additionally, SPTS was effective at preventing tryptophan destruction. SPTS also showed significant total radical-trapping antioxidant activity and could prevent thiobarbituric acid reactive substances (TBARS) formation induced by sodium nitroprusside in different rat tissues and by Cu^{2+} in human LDL and serum. These results indicate that the antioxidant effect of SPTS is caused by a combination of transition metal chelation and free-radical-scavenging activity. We suggest that SPTS may be studied *in vivo* as a potential antioxidant compound against several diseases in which free radicals are involved.

Keywords: Low density lipoprotein, Copper, Thiosemicarbazone, Metals, Atherosclerosis, Antioxidant.

1. Introduction

Atherosclerosis is a pathophysiological condition in which arteries undergo a gradual increase in intima thickness, causing decreased elasticity, narrowing, and reduced blood supply. This affects the arterial wall and leads to angina pectoris, myocardial infarction, and cerebral infarction (Matsuura, E. *et al.*, 2008).

The hypothesis that the oxidative modification of low-density lipoprotein (LDL) plays a pivotal role in the progression of atherosclerosis has been widely accepted (Niki, E., 2004). Native LDL accumulates in the extracellular subendothelial space of arteries (Madamanchi, N.R. *et al.*, 2005) and can be oxidatively modified by all major arterial wall cell types (Ting, H.H. *et al.*, 1997). Both the lipid and protein moieties of lipid particles can be oxidized, yielding a broad spectrum of oxidized LDL species that differ structurally and functionally, depending on the degree of oxidative modification (Frei, B., 1999; Steinberg, D. *et al.*, 2002).

Oxidized LDL (ox-LDL) promotes the initiation of monocyte invasion (Maier, J.A. *et al.*, 1994; Hashimoto, K. *et al.*, 2007). Ox-LDL is not recognized by the LDL receptor apo (B/E) but is taken up in a non-regulated manner by scavenger receptors in monocytes-macrophages and endothelial cells. This process leads to the accumulation of cholesterol in the macrophages and the formation of foam cells, a hallmark of atherosclerotic lesion (Ross, R., 1999). Ox-LDL has been shown to be present in atherosclerotic lesions in laboratory animals and humans (Witztum, J.L. *et al.*, 2001). Additionally, human and animal studies have demonstrated the presence of autoantibodies that react with ox-LDL, suggesting the presence of ox-LDL or a similar epitope *in vivo* (Steinberg, D., 1997).

One possible method of preventing atherosclerotic disease may be the administration of antioxidative substances that render LDL less sensitive to oxidation. Numerous animal experiments and *in vitro* studies have demonstrated the beneficial effects of antioxidants in the prevention of atherosclerosis (Bocan, T.M. *et al.*, 1992; Fruebis, J. *et al.*, 1994; Shaish, A. *et al.*, 1995; de Lima Portella, R. *et al.*, 2008; de Bem, A.F. *et al.*, 2009).

Recent studies have investigated the free-radical scavenger activity of thiosemicarbazones and related compounds (Shih, M.H. *et al.*, 2004; Karatas, F. *et al.*, 2006; Ghosh, S. *et al.*, 2009). The chemical and biological effects of

thiosemicarbazones have received considerable attention from medicinal chemists for many years, which is attributable to their wide pharmacological utility. Both thiosemicarbazones and their metal complexes have been studied as potential antiviral, antibacterial, antimycobacterial, antiprotozoal, antifungal, and antineoplastic agents (Pandeya, S.N. *et al.*, 1999; Smee, D.F. *et al.*, 2003; Beraldo, H. *et al.*, 2004; Chohan, Z.H. *et al.*, 2004; Kalinowski, D.S. *et al.*, 2005, 2007). Furthermore, their anticonvulsant and neurotropic effects have been reported (Beraldo, H. *et al.*, 2004). Thiosemicarbazones are typically excellent chelators of transition metals, such as iron (Fe), copper (Cu), and zinc (Zn) (French, F.A. *et al.*, 1966; DeConti, R.C. *et al.*, 1972; Pandeya, S.N. *et al.*, 1999).

The present study investigated the possible *in vitro* antioxidant effects of a thiosemicarbazone, salicylaldehyde-4-phenylthiosemicarbazone (SPTS; Fig. 1), on LDL and serum oxidation and lipoperoxidation.

2. Materials and methods

2.1. Synthesis of salicylaldehyde-4-phenylthiosemicarbazone

The compound was prepared by the mixture of equimolar quantities of 4-phenylthiosemicarbazide and salicylaldehyde as described by (Klayman, D.L. *et al.*, 1979). The spectral analysis of salicylaldehyde 4-phenylthiosemicarbazone have been discussed in a previous paper (Seena, E.B. *et al.*, 2008). SPTS was solubilized in dimethyl sulfoxide (DMSO).

2.2. Animals

Adult Wistar rats (200-250g) were obtained from University of Santa Maria and maintained in an air conditioned room (20–25ºC) under natural lighting conditions with water and food (Guabi-RS, Brazil) ad libitum. All the experimental procedures performed were conducted according to the guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil.

2.3. LDL isolation

LDL was isolated from fresh human plasma by discontinuous density-gradient ultracentrifugation as described by Silva et al., 1998 (Luiz da Silva, E. *et al.*, 1998), with few modifications. Briefly, plasma of non-fasted healthy normolipidemic voluntary donors collected with EDTA (1 mg/mL) was pooled and sucrose (final concentration, 0.5%) was added to prevent LDL aggregation. Five milliliters of EDTA-plasma adjusted to a density of 1.22 g/mL with solid KBr (0.326 g/mL) was layered on the bottom of a centrifuge tube. Then, 5 mL EDTA-containing sodium chloride solution (density 1.006 g/mL) was overlaid on the top of the plasma. Ultracentrifugation was run at 350.000xg for 2 h at 4º C, in a Himac CP80MX ultracentrifuge. LDL particles were collected by the aspiration of the yellow/orange band at the middle of the saline layer and dialyzed exhaustively overnight at 4º C with 10 mM phosphate buffer (pH 7.4). Protein concentration in LDL solution was determined by Lowry's method (Lowry, O.H. *et al.*, 1951). The purity of LDL preparation was verified by agarose gel electrophoresis. Isolated LDL was stored at -20º C for no longer than 2 weeks.

2.4. LDL oxidation

2.4.1. Conjugated dienes and TBARS formation

LDL samples (50 µg protein/mL) were pre-incubated at 37 °C in a medium containing 10 mM phosphate buffer (pH 7.4) and different SPTS concentrations (0-5 µM). After 5 minutes, the oxidation was initiated by the addition of either $CuSO_4$ (5 µM) or 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH; 1 mM). The oxidation was monitored by measuring the increase in absorbance at 234 nm due to conjugated diene (CD) formation as previously described (Esterbauer, H. *et al.*, 1989). Aliquots were also removed at different time points for evaluating thiobarbituric acid reactive substances (TBARS) production as previously described (Ohkawa, H. *et al.*, 1979).

2.4.2. Measurement of LDL- Tryptophan fluorescence

The fluorescence spectra of native LDL display a single band centered at approximately 332 nm, which is assigned to the tryptophan (Trp) residues in apolipoprotein B-100 (apoB-100) (Giessauf, A. *et al.*, 1995). Loss of Trp fluorescence is a marker for oxidations at the protein core of LDL (Reyftmann, J.P. *et al.*, 1990; Giessauf, A. *et al.*, 1995). Trp fluorescence was measured in a solution of LDL (50 µg protein/ml) in PBS (10 mM) pH 7.4 at 37°C, using a Shimatzo espectrofluorometer (excitation at 282 nm and emission at 331 nm) (Reyftmann, J.P. *et al.*, 1990; Giessauf, A. *et al.*, 1995). The kinetics of LDL oxidation was followed by measuring the decrease of Trp-fluorescence, corresponding to the decomposition of this amino acid, after the addition of 5 µM $CuSO_4$, in absence or presence of SPTS. The cuvettes had to be removed from the excitation light between the single measurements to avoid photooxidation of the Trp residues; fluorescence was measured every 20 min. Data are shown as the percent decrease of Trp fluorescence in each sample. The time required for reaching half Trp fluorescence ($t_{1/2}$) was calculated.

2.4.3. Serum oxidation

Venous blood was drawn from non-fasted healthy normolipidemic voluntary donors into tubes containing no anticoagulant and centrifuged at 1000 g for 15 min. Serum diluted 100-fold was incubated at 37°C in a medium containing 10 mM phosphate buffer (pH 7.4) and different SPTS concentrations (0-5 µM). The oxidation was initiated by the addition of $CuSO_4$ (30 µM) and CD formation was monitored at 245 nm as previously described (Schnitzer, E. *et al.*, 1998).

TBARS formation was also evaluated in serum oxidation. Briefly, serum samples were diluted 1:4 in 10 mM potassium phosphate buffer, pH 7.4 and incubated at 37°C with $CuSO_4$ (100 µM) and/or DPTVP (0–10 µM). The total volume was 9 mL. At different time points, aliquots (200 µL) were removed for evaluating thiobarbituric acid reactive substances (TBARS) levels as previously described (Ohkawa, H. *et al.*, 1979).

2.4.4. Determination of lag phase and maximum oxidation rate

In the studies of CD formation, there are several parameters which can be obtained from diene vs. time profiles. The value of the lag phase is commonly determined graphically by the intercept of the tangents to the slow and fast increase of the diene absorption. Another parameter is the maximum oxidation rate, given by the peak of the first derivative, i.e. change of A_{234} as a function of time (Gieseg, S.P. *et al.*, 1994).

2.5. Lipid peroxidation assay

Lipid peroxidation was determined by measuring TBARS as described by Ohkawa et al. (1979) from liver, kidney and brain rats homogenates. Aliquots of 200 µL of liver, kidney and brain homogenates were mixed to incubating medium containing Tris–HCl (0.01 mM), SPTS at indicated concentrations (see figure legends), and sodium nitroprusside (SNP) 5 µM and then incubated at 37°C for 60 min. The reaction was stopped by adding 0.5 mL of acetic acid buffer, and lipid peroxidation products were measured by the addition of 0.5 mL of TBA 0.6 %. Tubes were then incubated at 90ºC for 60 min before spectrophotometric analysis. TBARS levels were measured at 532 nm using a standard curve of malondialdehyde (MDA). The pro-oxidant SNP was added as positive control for lipid peroxidation.

2.6. Total radical-trapping antioxidant potential (TRAP)

TRAP was determined by measuring the chemiluminescence intensity of luminol induced by AAPH thermolysis in a luminometer BioTek Synergy 2 (Lissi, E. *et al.*, 1992). The reaction mixture contained 300 µL of 10 mM AAPH and 10 µL of 1 mM luminol dissolved in 0.1M glycine buffer (pH 8.6). Incubation of this mixture generates an almost constant light intensity at room temperature after stabilization. SPTS was added in different concentrations to determine the TRAP activity. At this point, the luminescence intensity is practically abolished. In the course of time, with the loss of antioxidant capacity of SPTS, the luminescence intensity returns to the initial values. The area under curve (AUC) was evaluated for each SPTS concentration and compared to vehicle AUC (Dresch, M.T. *et al.*, 2009).

2.7. Statistical analysis

Data are expressed as means ± SD. Statistical analysis was performed using a one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls test when appropriate. In addition, linear regression was performed to identify a possible dose dependent effect. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Effects of SPTS on the LDL oxidation

3.1.1. Conjugated Diene (CD) production:

Isolated LDL samples incubated with different concentrations of SPTS showed a concentration-dependent increase in the lag phase of LDL oxidation ($r^2 = 0.88$, $p < 0.001$). Significant increase was showed at lowest SPTS concentration (Table 1, Fig. 2A). At highest concentration, SPTS totally prevents the LDL oxidation during assay time. However, SPTS caused a decrease in maximum oxidation rate only at 0.75 and 1 μM (Table 1).

On the other hand, when AAPH was used to oxidize LDL, SPTS showed a lowest antioxidant capacity when compared the results obtained in the Cu^{2+} oxidation assay (Fig. 2B). With AAPH as pro-oxidant the SPTS showed a significant effect only at 5 and 10 μM.

3.1.2. TBARS Production

The LDL oxidation was also evaluated by TBARS formation. Figure 2C showed a significant effect of SPTS in preventing TBARS formation induced by Cu^{2+}. The protective effect of SPTS was similar to the ones presented in the CD experiments. SPTS at 0.75 and 1μM totally prevented the TBARS formation during the assay time.

3.1.3. Effects of SPTS on the LDL tryptophan fluorescence

Figure 2D reports the time course of Trp fluorescence decrease during Cu^{2+}-induced LDL oxidation. When compared to the control group, the presence of SPTS at concentrations higher than 0.5 μM significantly increased $t_{1/2}$ of Trp. SPTS at 1 μM showed a total protection during 180 minutes. This result was similar to the conjugated dienes and TBARS formation.

3.2. The effect of SPTS on serum oxidation

3.2.1. Conjugated Diene (CD) production

Serum oxidation was determined by CD formation at 245 nm (Fig. 3). No oxidation occurred in serum when the medium did not contain Cu^{2+} ions. Table 1 shows that SPTS was able to cause a significant increase in the lag phase at concentrations ranging from 0.25 to 1 μM. It was in a concentration dependent manner (r^2 = 0.878, P<0.001, linear regression). Interestingly, SPTS significantly decreased the maximum oxidation rate at all concentrations tested here (Table 1).

3.2.2. TBARS Production

Effect of SPTS on TBARS production was evaluated on Cu^{2+}-induced serum oxidation (Fig. 4). SPTS showed a significant serum oxidation protection at concentrations from 5 to 20 μM. This protection was observed at 90 and 180 minutes.

3.3. Effect of SPTS added at different times on LDL and serum oxidation

In figure 5 (A and B), we could observe that SPTS prevented the LDL or serum oxidation only when it was added before the rapid increase in conjugated dienes production. In LDL oxidation, when SPTS was added after the end of the lag phase, the protective effect was totally abolished. On the other hand, in serum oxidation, SPTS showed a slight effect when added in the end of lag phase.

3.4. The effect of SPTS on lipid peroxidation

In order to determine if SPTS presents antioxidant effect in other assays, we evaluated its effect on SNP-induced lipoperoxidation in different rat tissues (Fig 6). SPTS showed a significant protective effect at 1, 5 and 10 μM in all tissues tested here. SPTS presented significant effect at 0.5 μM only in liver lipoperoxidation assay (Fig.6A).

3.5. Total radical-trapping antioxidant potential of SPTS

In Figure 7 we demonstrated the antioxidant potential of SPTS when AAPH was used as pro-oxidant. Concentrations of SPTS range from 0.5 to 5 μM showed a significant effect on total radical-trapping antioxidant potential.

4. Discussion

The inhibition of copper-induced lipid peroxidation in human LDL is considered to be a biologically relevant model for evaluating the antioxidant effects of new compounds (Miranda, C.L. *et al.*, 2000; Lapeyre, C. *et al.*, 2005). The present study demonstrated that SPTS may have antioxidant effects on LDL and serum oxidation. SPTS increased the lag phase and reduced the maximum oxidation rate in both LDL and serum oxidation induced by Cu^{2+}. Trp destruction was also prevented by SPTS. Moreover, SPTS significantly protected against AAPH-induced LDL oxidation and in the TRAP assay. It was also effective at protecting the rat liver, kidney, and brain *in vitro* from lipoperoxidation induced by SNP.

Lipid peroxidation in LDL performed under the present conditions usually exhibits three phases. The lag phase occurs first, with very slow formation of conjugated dienes, during which endogenous lipophilic antioxidants prevent radical chain propagation. After consumption of these endogenous antioxidants, chain propagation begins, which is accompanied by a rapid increase in CD formation. Later in the process, CD can decompose to other radicals and eventually to aldehydes, such as malondialdehyde and 4-hydroxynonenal (Ramos, P. *et al.*, 1995).

Antioxidant compounds have been found to interfere not only with the propagation reactions of free radicals by hydrogen atom donation, but also with the formation of radicals by chelating the transition metal involved in the initiation and

propagation reactions. Therefore, most lipid peroxidation processes feature a combination of transition metal (usually Cu^{2+}) chelation and radical scavenging activities (Turchi, G. *et al.*, 2009). Thiosemicarbazone compounds are typically excellent chelators of transition metals, such as iron (Fe), copper (Cu), and zinc (Zn) where a phenolate O-donor binds in addition to N1- and the S-donor (French, F.A. *et al.*, 1966; DeConti, R.C. *et al.*, 1972; Pandeya, S.N. *et al.*, 1999; Yu, Y. *et al.*, 2009). Our results corroborate the chelating activity of SPTS. In both LDL and serum oxidation, SPTS was able to decrease the maximum oxidation rate, suggesting that its protection could be attributable to complex formation (Ziouzenkova, O. *et al.*, 1998; Lopez-Alarcon, C. *et al.*, 2007).

The fact that SPTS inhibited LDL oxidation at lower concentrations than Cu^{2+} could indicate that the effects of SPTS on the chelating activity of Cu^{2+} was not the only mechanism of action. We found that SPTS could prevent AAPH-induced LDL oxidation but at concentrations higher than those used in the Cu^{2+} oxidation assay. Moreover, determining whether antioxidation involves direct trapping of azo compound-derived peroxyl radicals or, more importantly, lipoperoxides from the oxidizable substrate is difficult (Halliwell, B., 1990). SPTS might scavenge AAPH-derived peroxyl radicals directly in the aqueous medium, thus reducing the rate of initiation. The TRAP assay confirmed that SPTS had a significant effect at concentrations similar to the AAPH-induced LDL oxidation assay. Interestingly, SPTS exhibited a significant effect on AAPH-induced LDL oxidation only at concentrations at which TRAP activity was maximal. Raja et al. (Raja, D.S. *et al.*, 2011) analyzed antioxidant activity of some thiosemicarbazones compounds and demonstrated that the Cu^{2+} chelation is responsible for their antioxidant activity. It could explain the highest activity presented by SPTS in the Cu^{2+}-induced LDL oxidation. Additionally, the highest activity among all the compounds analyzed by Raja et al. may be due to the phenyl substitution in the terminal nitrogen of the compound, a similar structure to SPTS.

The polyunsaturated fatty acids in cholesteryl esters, phospholipids, and triglycerides are also subjected to free-radical-initiated oxidation to yield a wide array of smaller fragments, including aldehydes and ketones, that can become conjugated to amino lipids or apolipoprotein B-100 (apoB-100) (Esterbauer, H. *et al.*, 1987). In the present study, SPTS totally inhibited TBARS formation at concentrations of 0.75 and 1 μM for 240 min. The prevention of TBARS formation can avoid the interaction

between aldehydes and apoB-100, which facilitates the recognition of oxidized LDL by scavenger receptors and deregulates the uptake of LDL-cholesterol into cells (Kawamura, M. *et al.*, 1994).

LDL oxidation *in vivo*, however, is thought to occur in the interstitial space of the arterial wall where LDL may be shielded from the various antioxidants present in plasma (Schwartz, C.J. *et al.*, 1991; Witztum, J.L. *et al.*, 1991). Our results indicate that SPTS could prevent the effect of TBARS formation on serum oxidation at low concentrations (5-20 μM) compared with Cu^{2+} concentration (100 μM).

ApoB-100 has been postulated to be essential for copper-catalyzed LDL oxidation by providing the ligand binding sites for the formation of LDL-Cu complexes (Kuzuya, M. *et al.*, 1992). The oxidative modification of apoB-100 may be more important than lipid oxidation for scavenger receptor recognition in macrophages. ApoB-100 may also be oxidized by both radical and non-radical mechanisms (Uchida, K., 2000). In an LDL tryptophan fluorescence assay, SPTS significantly increased $t_{1/2}$ at concentrations higher than 0.5 μM. This effect may be a combination of Cu^{2+} chelation and radical scavenger activity of SPTS.

Trp residues play a key role in the Cu^{2+}-dependent initiation of lipid peroxidation in the LDL particle (Giessauf, A. *et al.*, 1995). Supporting this hypothesis are the observations that Cu^{2+} ions bind in close vicinity to the Trp residues of apo B-100 and Trp destruction is an early event that begins immediately after the addition of Cu^{2+} to LDL and proceeds to the onset of the lipid peroxidation propagation phase (Giessauf, A. *et al.*, 1996). When we added SPTS at different times after the addition of Cu^{2+}, the compound exhibited a significant effect only when added before the initiation of the propagation phase. After consumption of the endogenous antioxidants (i.e., the end of the lag-phase), SPTS could not prevent LDL or serum oxidation. This might indicate that the mechanism by which SPTS protects against LDL or serum oxidation involves the prevention of endogenous antioxidant consumption. Such a process may occur because of the radical scavenger activity of SPTS.

To evaluate whether SPTS could prevent oxidation in other systems, we assessed the antioxidant activity of SPTS on SNP-induced lipoperoxidation in rat tissue. We found that SPTS was effective in all tissues tested here and against another oxidant agent. Additionally, our results demonstrated that tissue components could not inhibit or impair the effects of SPTS. SNP is well recognized as a classic

nitric oxide (NO) donor in the presence of reducing agents (Bates, J.N. *et al.*, 1991). The NO released from SNP molecule decomposition could react with intracellular superoxide anions ($O2^{-}$) and form peroxynitrite ($ONOO^{-}$), which is a damaging oxidant involved in tissue damage (Darley-Usmar, V. *et al.*, 1995). However, the decomposition of SNP molecules also releases a pentacyanoferrate complex (Bates, J.N. *et al.*, 1991; Rao, D.N. *et al.*, 1991; Roncaroli, F. *et al.*, 2005), which could lead to oxidative reactions involved in the generation of radical species (Welch, K.D. *et al.*, 2002). SPTS might contribute to the prevention of tissue damage by scavenging NO and $ONOO^{-}$ directly in an SNP-induced lipoperoxidation assay, which is consistent with the TRAP and AAPH oxidation experiments.

Our results demonstrated that SPTS has potential antioxidant effects against a wide range of oxidant agents, including Cu^{2+}, AAPH, and SNP. SPTS was effective against AAPH- and Cu^{2+}-induced LDL oxidation. This antioxidant activity may contribute to the prevention of LDL oxidation *in vivo* and thus atherogenesis. SPTS might present a dual mechanism that includes chelating activity and peroxyl scavenger activity. Considering the substantial involvement of free radicals in many diseases, we speculate that SPTS may be studied *in vivo* as a potential treatment for various diseases, including atherosclerosis, Alzheimer's disease, Huntington's disease, diabetes, and poison toxicity.

5. Acknowledgement

This work was supported by the FINEP research grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)" # 01.06.0842-00 and INCT for Excitotoxicity and Neuroprotection- MCT/CNPq. J.B.T.R and F.A.A.S. receive a fellowship by CNPq. R.L.P. receive a fellowship by CAPES. Additional support giving by FAPERGS and CAPES. This manuscript has been edited by native English-speaking experts of BioMed Proofreading.

6. Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Table 1. Lag phase and maximum oxidation rate for conjugated diene formation in LDL and serum and oxidation $t_{1/2}$ values for Trp fluorescence.

	LDL		Serum		Trp fluorescence
	lag phase	**oxidation rate**	**lag phase**	**oxidation rate**	**$t_{1/2}$**
$CuSO_4$	50,1 ± 8,6 a	0,155 ± 0,033	27,3 ± 6 a	0,079 ± 0,01	111 ± 6,904
0.1µM	71,6 ± 13 * b	0,144 ± 0,009	34,5 ± 5,6 a	0,061 ± 0,013 *	116,8 ± 7,969
0.25µM	83,7 ± 12,2 * b	0,143 ± 0,011	59,1 ± 13,4 * b	0,056 ± 0,017 *	123,9 ± 7,512
0.5µM	105 ± 16 ** c	0,128 ± 0,007	105,1 ± 22,7 ** c	0,057 ± 0,009 *	170,4 ± 2,086 **
0.75µM	145 ± 4,7 ** d	0,073 ± 0,014 **	124,2 ± 5,1 ** c	ND	>180 **
1µM	>200 ** e	0,041 ± 0,002 **	>180 d	ND	>180 **

LDL (50 µg/mL) was oxidized in the presence of 5 µM Cu^{2+} and serum was oxidized in the presence of 30 µM Cu^{2+}. Different concentrations of SPTS were used. Different letters indicate statistically significant differences among different mean ($P < 0.05$). ND: Not determined.

* $p < 0.05$ compared to $CuSO_4$ group

** $p < 0.001$ compared to $CuSO_4$ group

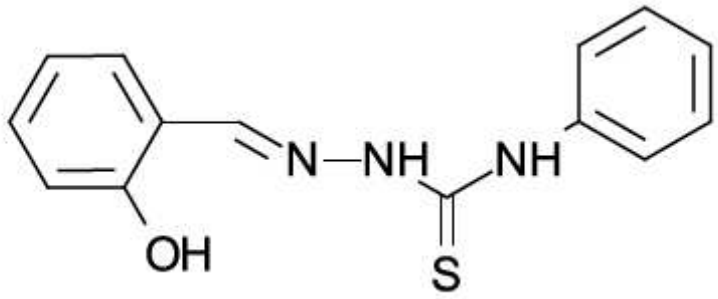
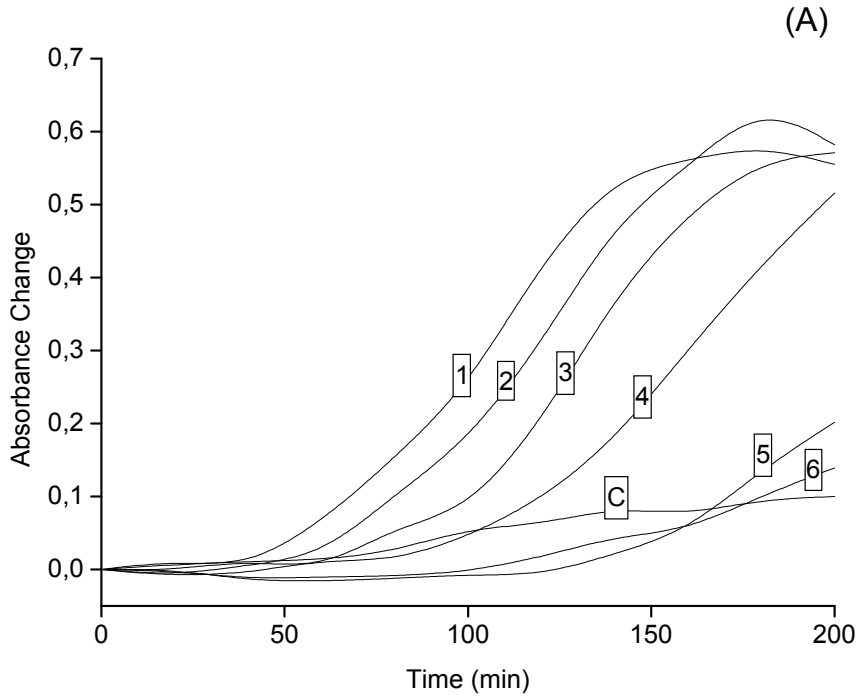
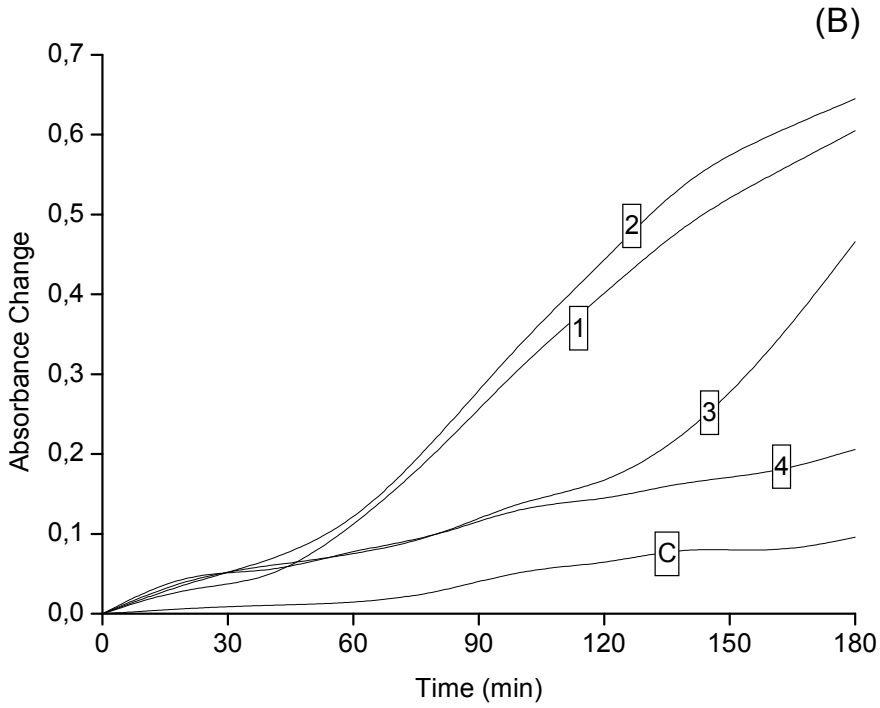
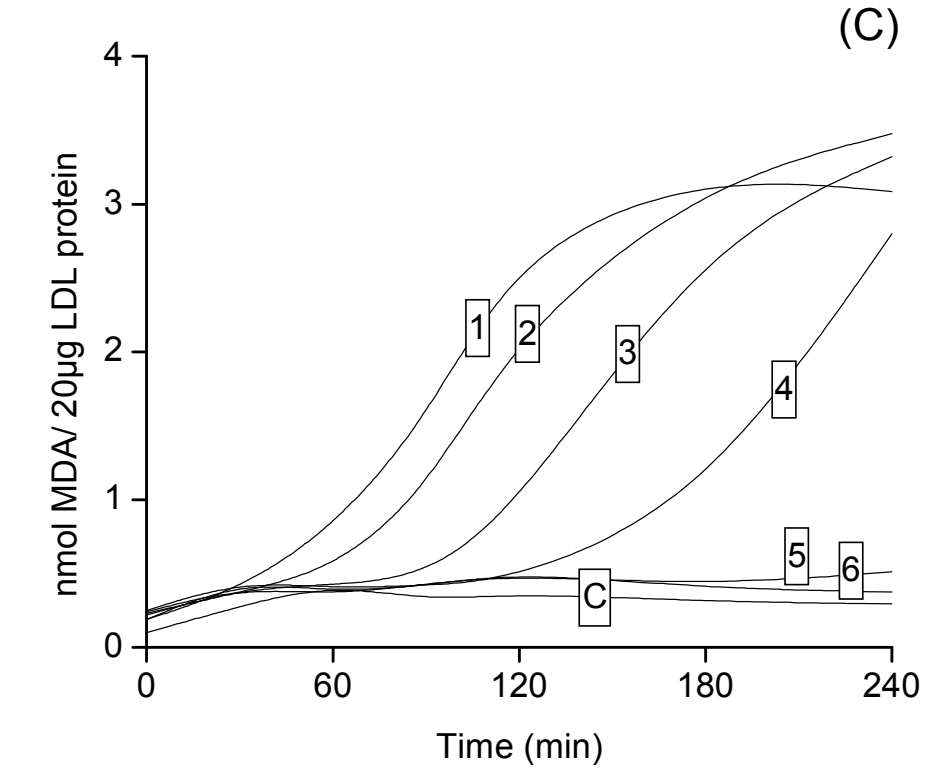


Figure 1. Chemical structures of salicylaldehyde-4-phenylthiosemicarbazone.


(A)
Absorbance Change
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0
50
100
150
200
Time (min)
1
2
3
4
5
6
C


(B)
Absorbance Change
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0
30
60
90
120
150
180
Time (min)
1
2
3
4
C


(C)
nmol MDA/ 20µg LDL protein
Time (min)
0
1
2
3
4
0
60
120
180
240
1
2
3
4
5
6
C

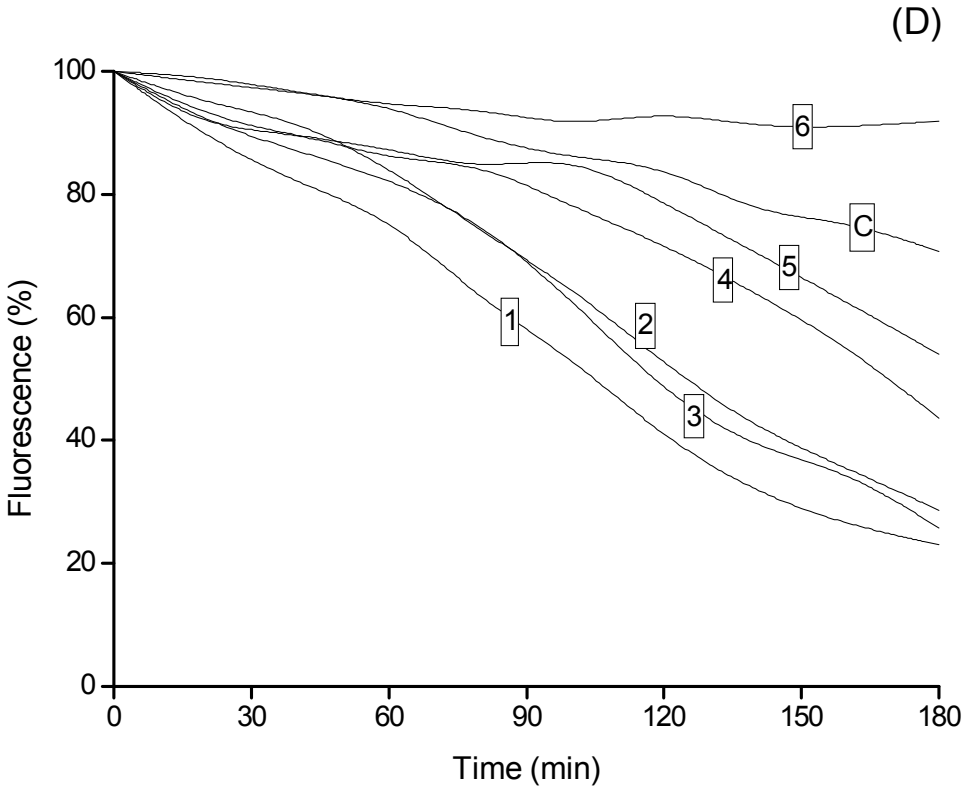

(D)
Fluorescence (%)
Time (min)
0
20
40
60
80
100
0
30
60
90
120
150
180
1
2
3
4
5
6
C

Figure 2. Effects of SPTS on LDL oxidation. LDL (50 µg protein/mL) was incubated in PBS 10mM (pH 7.4) at 37°C in the presence of Cu^{2+} 5 µM (panel A) or AAPH 1mM (panel B). Conjugated diene formation was measured by determining the absorbance at 234 nm every 20 min. Effects of SPTS concentrations on time-dependent changes in the thiobarbituric acid reactive substances (TBARS) production during Cu^{2+}-induced LDL oxidation (panel C). Decrease of Trp fluorescence during Cu^{2+}-mediated oxidation of LDL with different concentrations of SPTS (panel D). LDL (50 µg protein/mL) was incubated in PBS with 5 µM Cu^{2+}. Fluorescence (Ex/Em = 282/331 nm) was measured at intervals of 20 min at 37°C. In panel A, C and D, incubation medium did not contain SPTS (1) or contained 0.1 µM (2), 0.25 µM (3), 0.5 µM (4), 0.75 µM (5) or 1 µM (6) of SPTS. In panel B, incubation medium did not contain SPTS (1) or contained 1 µM (2), 5 µM (3) or 10 µM (4) of SPTS. (C) Control without oxidant agent and SPTS in all panels. Experiments were repeated at least three times, showing similar results.

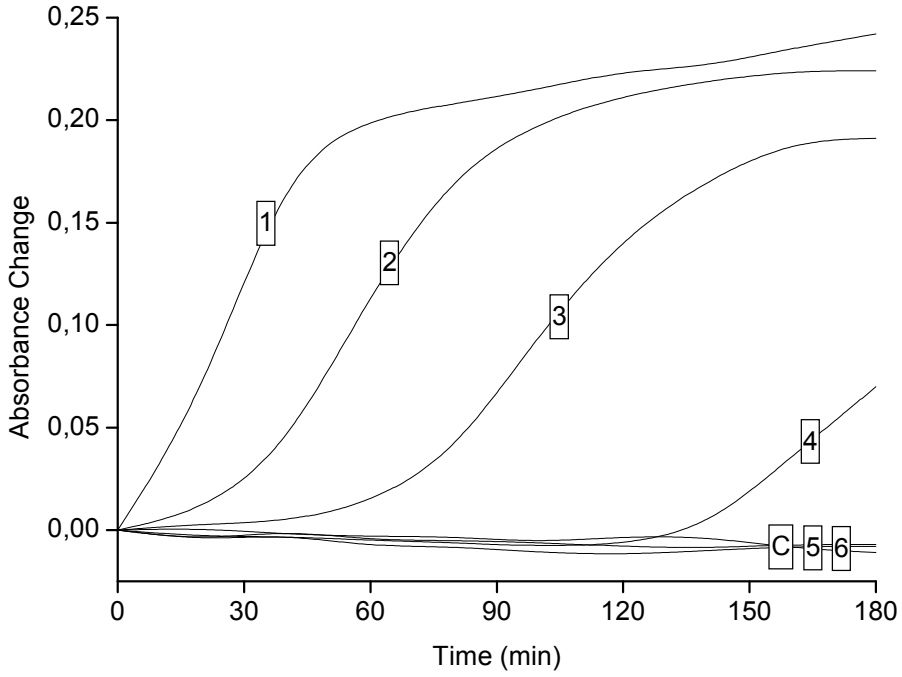


Figure 3. Effects of SPTS concentrations on conjugated dienes formation during serum oxidation. Serum (diluted 100-fold) in PBS 10mM (pH7.4) was incubated at 37°C for 180min in the presence of 30 µM Cu^{2+}. Incubation medium did not contain SPTS (1) or contained 0.1 µM (2), 0.25 µM (3), 0.5 µM (4), 0.75 µM (5) or 1 µM (6) of SPTS. Conjugated diene formation was measured by determining the absorbance at 245 nm every 20 min. (C) Control without $CuSO_4$ and SPTS. Experiments were repeated at least three times, showing similar results.

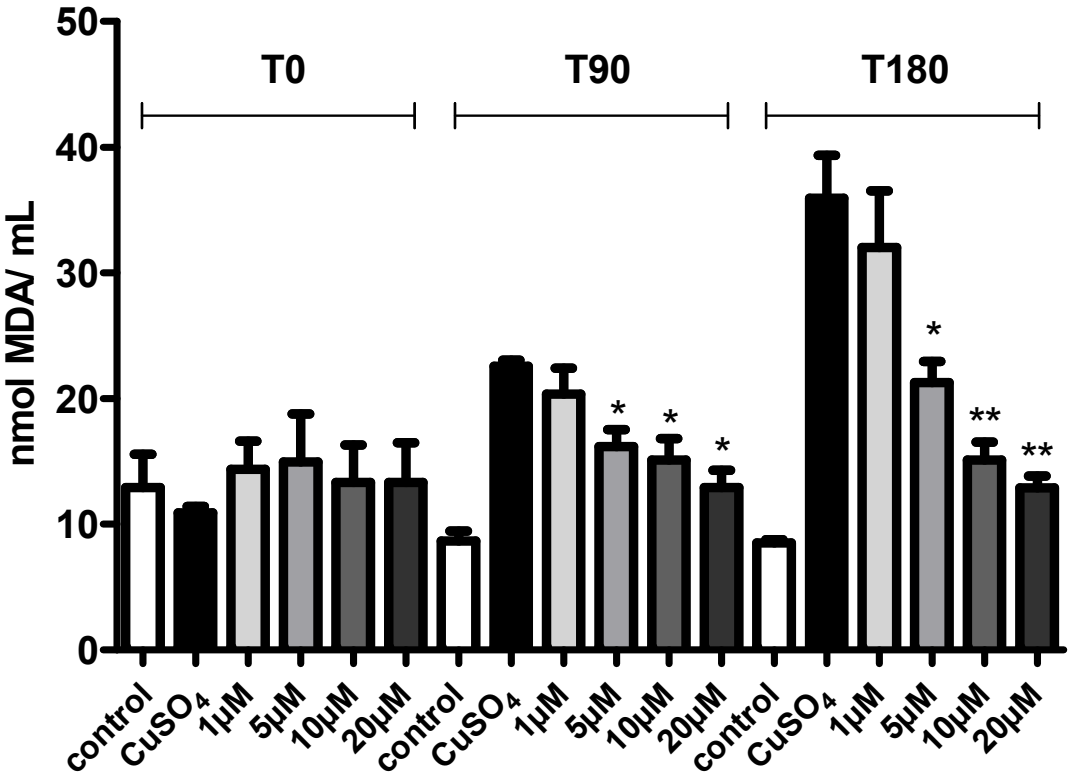
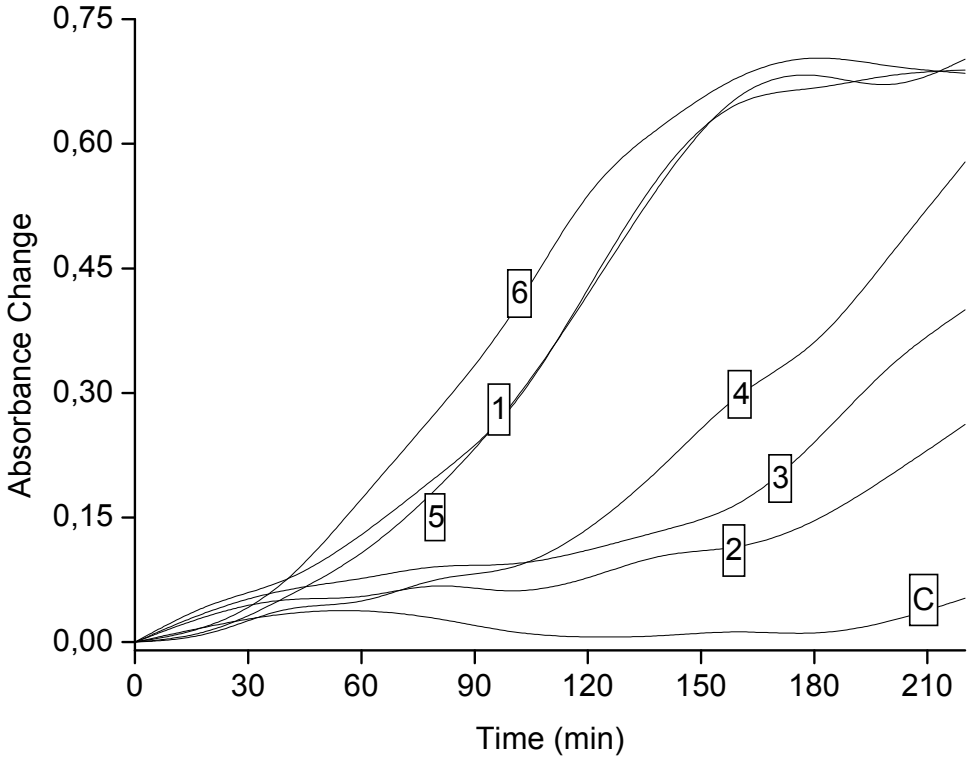


Figure 4. Effect of SPTS on serum oxidation. Serum samples were diluted 1:4 in 10mM potassium phosphate buffer, pH 7.4 and incubated at 37°C with $CuSO_4$ (100μM) and/or SPTS (0–20μM). At different time points, aliquots (200μL) were removed for evaluating thiobarbituric acid reactive substances (TBARS) levels. The bars represent mean ± S.E.M. of three different experiments. Statistically significant differences between the $CuSO_4$ and SPTS groups within the same time are denoted by * $P < 0.05$ and ** $P < 0.001$.

(A)


0,75
0,60
0,45
0,30
0,15
0,00
Absorbance Change
0
30
60
90
120
150
180
210
Time (min)
6
1
5
4
3
2
C

(B)

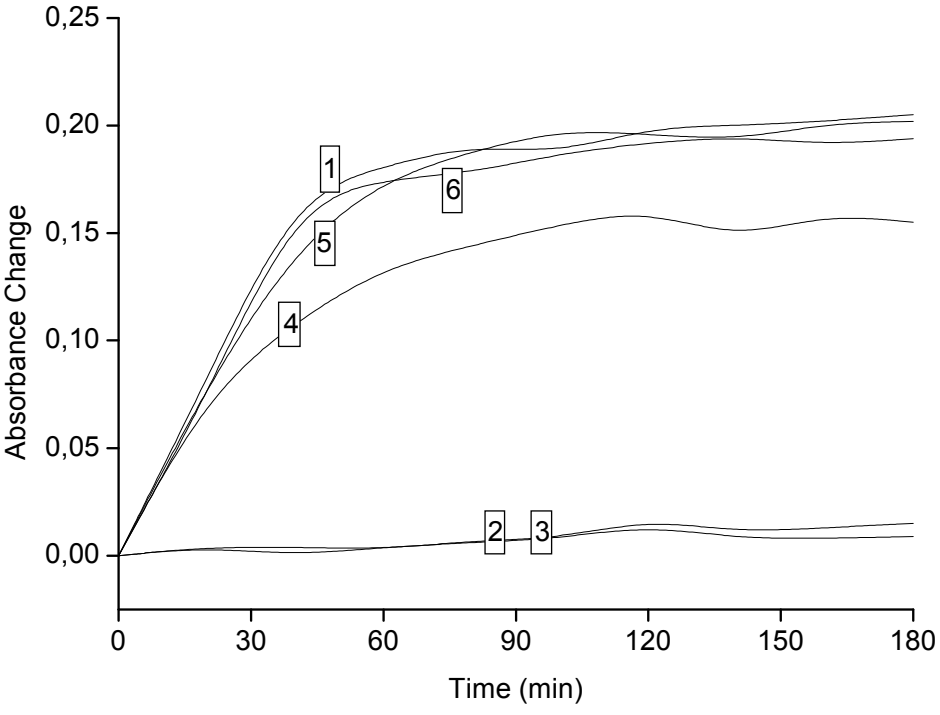
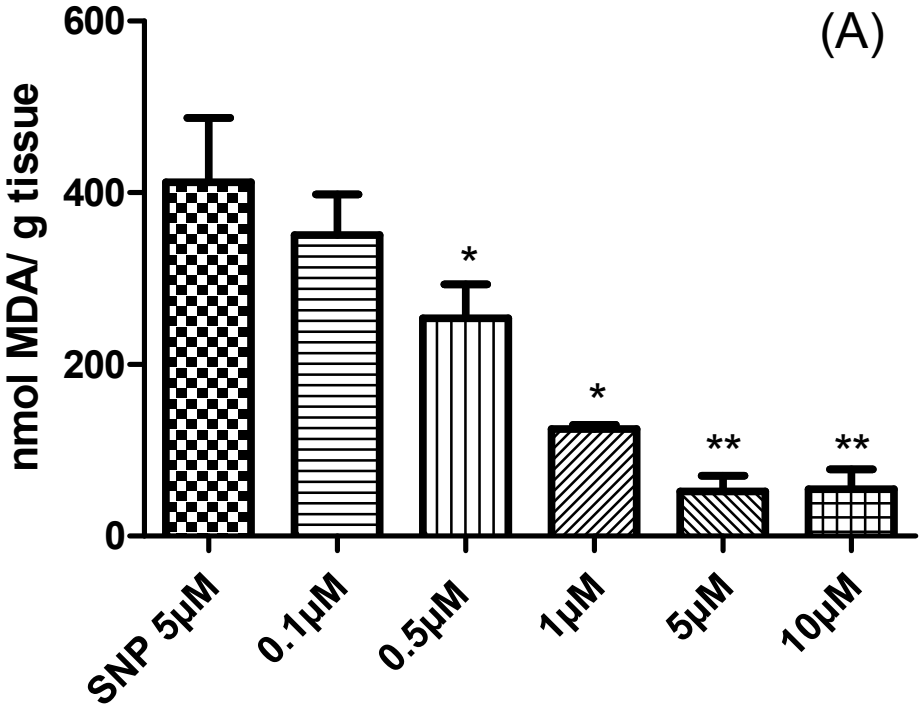
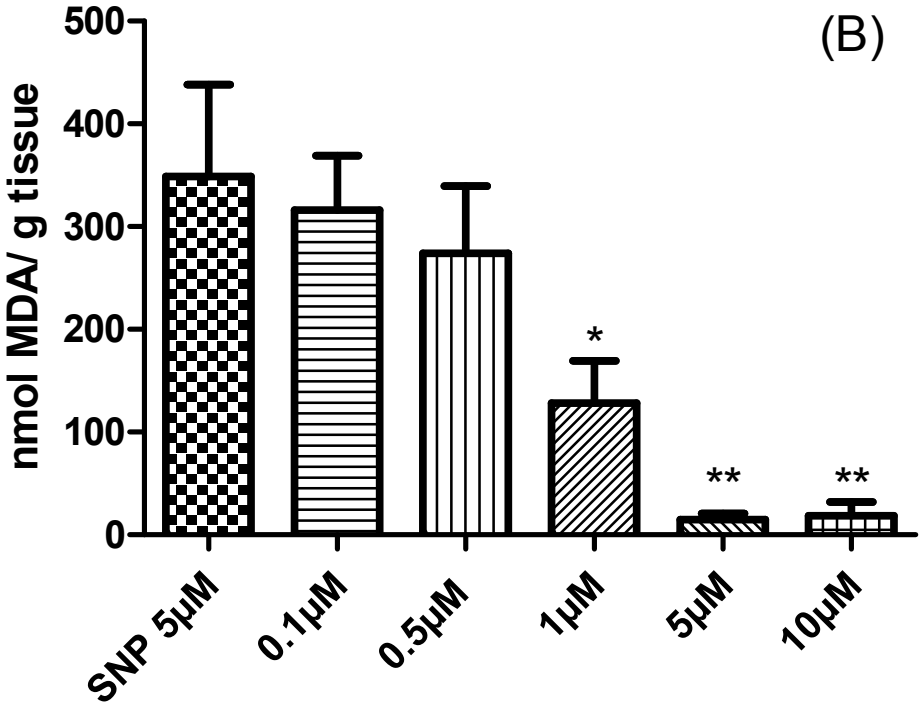

0,25
0,20
0,15
0,10
0,05
0,00
Absorbance Change
0
30
60
90
120
150
180
Time (min)
1
6
5
4
2
3

Figure 5. Effect of SPTS when added at different times of LDL (panel A) or serum (panel B) oxidation. LDL (50 µg protein/mL) was incubated in PBS 10mM (pH 7.4) at 37°C in the presence of Cu^{2+} 5 µM and SPTS 1 µM. Serum (diluted 100-fold) in PBS 10mM (pH7.4) was incubated at 37°C for 180min in the presence of 30 µM Cu^{2+} and SPTS 1µM. In panel A, incubation medium did not contain SPTS (1) or SPTS was added at zero (2), 20 (3), 40 (4), 50 (5) or 60 (6) minutes. In panel B, incubation medium did not contain SPTS (1) or SPTS was added at zero (2), 10 (3), 20 (4), 30 (5) or 40 (6) minutes. (C) Control without CuSO4 and SPTS in all panels. Experiments were repeated at least three times, showing similar results.


(A)
600
400
200
0
nmol MDA/ g tissue
*
*
**
**
SNP 5μM
0.1μM
0.5μM
1μM
5μM
10μM


(B)
500
400
300
200
100
0
nmol MDA/ g tissue
*
**
**
SNP 5μM
0.1μM
0.5μM
1μM
5μM
10μM

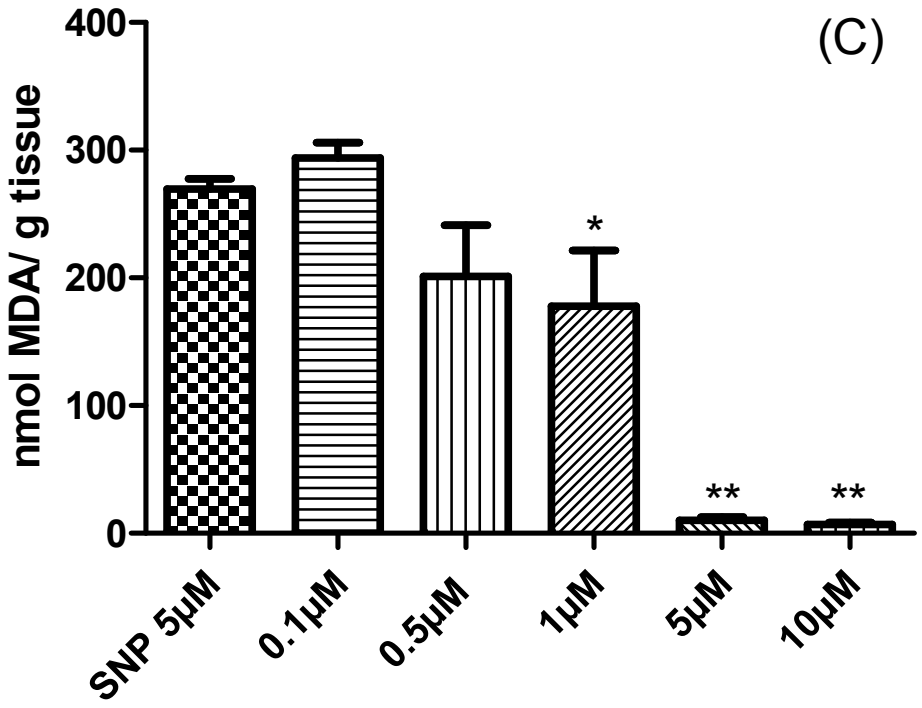


Figure 6. Effect of SPTS on liver (A) kidney (B) and brain (C) homogenates TBARS production. Sodium nitroprusside (SNP) at 5 μM was used as pro-oxidants to induce TBARS production. The bars represent mean ± S.E.M. of three different experiments. Statistically significant differences between the SNP and SPTS groups are denoted by * $P < 0.05$ and ** $P < 0.001$.

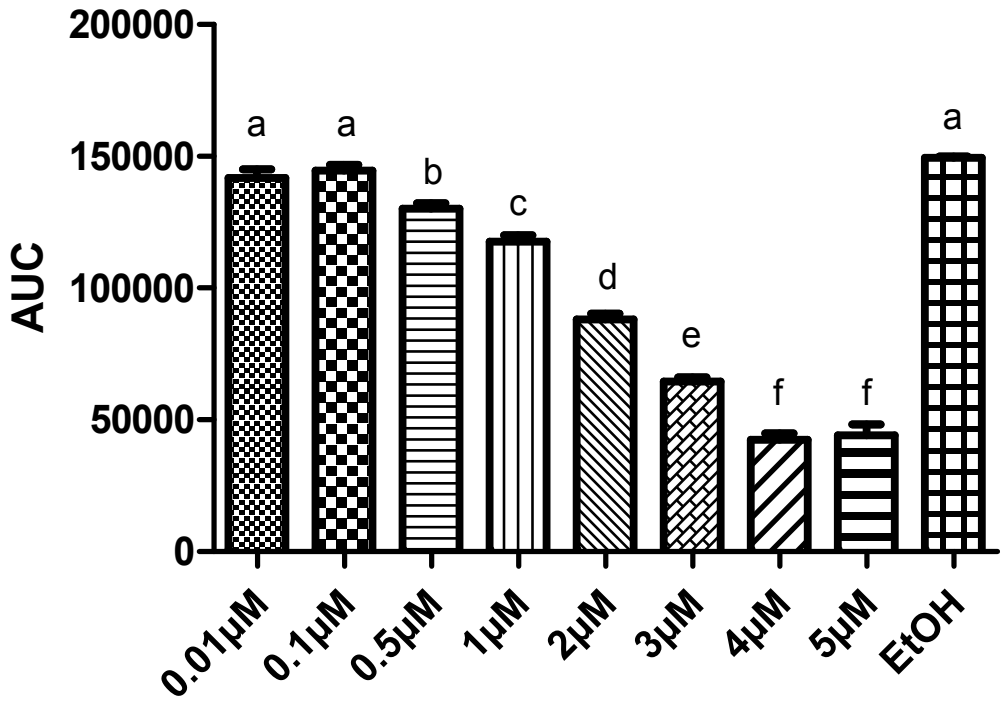


Figure 7. *In vitro* effect of SPTS on total radical-trapping antioxidant potential (TRAP). The reaction mixture contained 10 mM AAPH, 1 mM luminol and SPTS at different concentrations dissolved in 0.1M glycine buffer (pH 8.6). The luminescence was measured every 5 minutes during 5 hours. The area under curve (AUC) was evaluated. The bars represent mean ± S.E.M. of five different experiments. Different letters indicate statistically significant differences among different mean ($P < 0.05$).

7. References

Bates, J.N., et al. Nitric oxide generation from nitroprusside by vascular tissue. Evidence that reduction of the nitroprusside anion and cyanide loss are required. **Biochem Pharmacol,** V. 42 Suppl, n. p. S157-165, 1991.

Beraldo, H., Gambino, D. The wide pharmacological versatility of semicarbazones, thiosemicarba-zones and their metal complexes. **Mini Rev Med Chem,** V. 4, n. 1, p. 31-39, 2004.

Bocan, T.M., et al. Antiatherosclerotic effects of antioxidants are lesion-specific when evaluated in hypercholesterolemic New Zealand white rabbits. **Exp Mol Pathol,** V. 57, n. 1, p. 70-83, 1992.

Chohan, Z.H., et al. Isatin-derived antibacterial and antifungal compounds and their transition metal complexes. **J Enzyme Inhib Med Chem,** V. 19, n. 5, p. 417-423, 2004.

Darley-Usmar, V., Wiseman, H., Halliwell, B. Nitric oxide and oxygen radicals: a question of balance. **FEBS Lett,** V. 369, n. 2-3, p. 131-135, 1995.

de Bem, A.F., et al. Diphenyl diselenide decreases serum levels of total cholesterol and tissue oxidative stress in cholesterol-fed rabbits. **Basic Clin Pharmacol Toxicol,** V. 105, n. 1, p. 17-23, 2009.

de Lima Portella, R., et al. Oximes as inhibitors of low density lipoprotein oxidation. **Life Sci,** V. 83, n. 25-26, p. 878-885, 2008.

DeConti, R.C., et al. Clinical and pharmacological studies with 5-hydroxy-2-formylpyridine thiosemicarbazone. **Cancer Res,** V. 32, n. 7, p. 1455-1462, 1972.

Dresch, M.T., et al. Optimization and validation of an alternative method to evaluate total reactive antioxidant potential. **Anal Biochem,** V. 385, n. 1, p. 107-114, 2009.

Esterbauer, H., et al. Autoxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes. **J Lipid Res,** V. 28, n. 5, p. 495-509, 1987.

Esterbauer, H., et al. Continuous monitoring of in vitro oxidation of human low density lipoprotein. **Free Radic Res Commun,** V. 6, n. 1, p. 67-75, 1989.

Frei, B. On the role of vitamin C and other antioxidants in atherogenesis and vascular dysfunction. **Proc Soc Exp Biol Med,** V. 222, n. 3, p. 196-204, 1999.

French, F.A., Blanz, E.J., Jr. The carcinostatic activity of alpha-(N)-heterocyclic carboxaldehyde thiosemicarbazones. II. 3-Hydroxypyridine-2-carboxaldehyde thiosemicarbazone. **Cancer Res,** V. 26, n. 8, p. 1638-1640, 1966.

Fruebis, J., et al. A comparison of the antiatherogenic effects of probucol and of a structural analogue of probucol in low density lipoprotein receptor-deficient rabbits. **J Clin Invest,** V. 94, n. 1, p. 392-398, 1994.

Ghosh, S., et al. Syntheses and evaluation of glucosyl aryl thiosemicarbazide and glucosyl thiosemicarbazone derivatives as antioxidant and anti-dyslipidemic agents. **Bioorg Med Chem Lett,** V. 19, n. 2, p. 386-389, 2009.

Gieseg, S.P., Esterbauer, H. Low density lipoprotein is saturable by pro-oxidant copper. **FEBS Lett,** V. 343, n. 3, p. 188-194, 1994.

Giessauf, A., Steiner, E., Esterbauer, H. Early destruction of tryptophan residues of apolipoprotein B is a vitamin E-independent process during copper-mediated oxidation of LDL. **Biochim Biophys Acta,** V. 1256, n. 2, p. 221-232, 1995.

Giessauf, A., et al. Formation of N-formylkynurenine suggests the involvement of apolipoprotein B-100 centered tryptophan radicals in the initiation of LDL lipid peroxidation. **FEBS Lett,** V. 389, n. 2, p. 136-140, 1996.

Halliwell, B. How to characterize a biological antioxidant. **Free Radic Res Commun,** V. 9, n. 1, p. 1-32, 1990.

Hashimoto, K., et al. Oxidized LDL specifically promotes the initiation of monocyte invasion during transendothelial migration with upregulated PECAM-1 and downregulated VE-cadherin on endothelial junctions. **Atherosclerosis,** V. 194, n. 2, p. e9-17, 2007.

Kalinowski, D.S., Richardson, D.R. The evolution of iron chelators for the treatment of iron overload disease and cancer. **Pharmacol Rev,** V. 57, n. 4, p. 547-583, 2005.

Kalinowski, D.S., Richardson, D.R. Future of toxicology--iron chelators and differing modes of action and toxicity: the changing face of iron chelation therapy. **Chem Res Toxicol,** V. 20, n. 5, p. 715-720, 2007.

Karatas, F., et al. Synthesis and oxidant properties of novel (5-bromobenzofuran-2-yl)(3-methyl-3-mesitylcyclobutyl)ketonethiosemicarbaz one. **Eur J Med Chem,** V. 41, n. 5, p. 664-669, 2006.

Kawamura, M., Heinecke, J.W., Chait, A. Pathophysiological concentrations of glucose promote oxidative modification of low density lipoprotein by a superoxide-dependent pathway. **J Clin Invest,** V. 94, n. 2, p. 771-778, 1994.

Klayman, D.L., et al. 2-Acetylpyridine thiosemicarbazones. 1. A new class of potential antimalarial agents. **J Med Chem,** V. 22, n. 7, p. 855-862, 1979.

Kuzuya, M., et al. Role of lipoprotein-copper complex in copper catalyzed-peroxidation of low-density lipoprotein. **Biochim Biophys Acta,** V. 1123, n. 3, p. 334-341, 1992.

Lapeyre, C., et al. Design, synthesis, and evaluation of pharmacological properties of cinnamic derivatives as antiatherogenic agents. **J Med Chem,** V. 48, n. 26, p. 8115-8124, 2005.

Lissi, E., Pascual, C., Del Castillo, M.D. Luminol luminescence induced by 2,2'-Azo-bis(2-amidinopropane) thermolysis. **Free Radic Res Commun,** V. 17, n. 5, p. 299-311, 1992.

Lopez-Alarcon, C., Speisky, H., Lissi, E. Antioxidant effect of 5-amino salicylic acid on copper-mediated LDL oxidation. **Biol Res,** V. 40, n. 2, p. 155-162, 2007.

Lowry, O.H., et al. Protein measurement with the Folin phenol reagent. **J Biol Chem,** V. 193, n. 1, p. 265-275, 1951.

Luiz da Silva, E., Tsushida, T., Terao, J. Inhibition of mammalian 15-lipoxygenase-dependent lipid peroxidation in low-density lipoprotein by quercetin and quercetin monoglucosides. **Arch Biochem Biophys,** V. 349, n. 2, p. 313-320, 1998.

Madamanchi, N.R., Vendrov, A., Runge, M.S. Oxidative stress and vascular disease. **Arterioscler Thromb Vasc Biol,** V. 25, n. 1, p. 29-38, 2005.

Maier, J.A., et al. The protective role of high-density lipoprotein on oxidized-low-density-lipoprotein-induced U937/endothelial cell interactions. **Eur J Biochem,** V. 221, n. 1, p. 35-41, 1994.

Matsuura, E., Hughes, G.R., Khamashta, M.A. Oxidation of LDL and its clinical implication. **Autoimmun Rev,** V. 7, n. 7, p. 558-566, 2008.

Miranda, C.L., et al. Antioxidant and prooxidant actions of prenylated and nonprenylated chalcones and flavanones in vitro. **J Agric Food Chem,** V. 48, n. 9, p. 3876-3884, 2000.

Niki, E. Antioxidants and atherosclerosis. **Biochem Soc Trans,** V. 32, n. Pt 1, p. 156-159, 2004.

Ohkawa, H., Ohishi, N., Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. **Anal Biochem,** V. 95, n. 2, p. 351-358, 1979.

Pandeya, S.N., et al. Synthesis, antibacterial, antifungal and anti-HIV activities of Schiff and Mannich bases derived from isatin derivatives and N-[4-(4'-chlorophenyl)thiazol-2-yl] thiosemicarbazide. **Eur J Pharm Sci,** V. 9, n. 1, p. 25-31, 1999.

Raja, D.S., et al. Effect of terminal N-substitution in 2-oxo-1,2-dihydroquinoline-3-carbaldehyde thiosemicarbazones on the mode of coordination, structure, interaction with protein, radical scavenging and cytotoxic activity of copper(II) complexes. **Dalton Trans,** V. 40, n. 17, p. 4548-4559, 2011.

Ramos, P., et al. Effect of temperature and phase transition on oxidation resistance of low density lipoprotein. **J Lipid Res,** V. 36, n. 10, p. 2113-2128, 1995.

Rao, D.N., Elguindi, S., O'Brien, P.J. Reductive metabolism of nitroprusside in rat hepatocytes and human erythrocytes. **Arch Biochem Biophys,** V. 286, n. 1, p. 30-37, 1991.

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,** V. 1042, n. 2, p. 159-167, 1990.

Roncaroli, F., van Eldik, R., Olabe, J.A. Release of NO from reduced nitroprusside ion. Iron-dinitrosyl formation and NO-disproportionation reactions. **Inorg Chem,** V. 44, n. 8, p. 2781-2790, 2005.

Ross, R. Atherosclerosis--an inflammatory disease. **N Engl J Med,** V. 340, n. 2, p. 115-126, 1999.

Schnitzer, E., et al. Lipid oxidation in unfractionated serum and plasma. **Chem Phys Lipids,** V. 92, n. 2, p. 151-170, 1998.

Schwartz, C.J., et al. The pathogenesis of atherosclerosis: an overview. **Clin Cardiol,** V. 14, n. 2 Suppl 1, p. I1-16, 1991.

Seena, E.B., Prathapachandra Kurup, M.R., Suresh, E. Crystal Study of Salicylaldehyde N(4)-Phenylthiosemicarbazone. **J Chem Crystallogr,** V. 38, n. 2, p. 93-96, 2008.

Shaish, A., et al. Beta-carotene inhibits atherosclerosis in hypercholesterolemic rabbits. **J Clin Invest,** V. 96, n. 4, p. 2075-2082, 1995.

Shih, M.H., Ke, F.Y. Syntheses and evaluation of antioxidant activity of sydnonyl substituted thiazolidinone and thiazoline derivatives. **Bioorg Med Chem,** V. 12, n. 17, p. 4633-4643, 2004.

Smee, D.F., Sidwell, R.W. A review of compounds exhibiting anti-orthopoxvirus activity in animal models. **Antiviral Res,** V. 57, n. 1-2, p. 41-52, 2003.

Steinberg, D. Low density lipoprotein oxidation and its pathobiological significance. **J Biol Chem,** V. 272, n. 34, p. 20963-20966, 1997.

Steinberg, D., Witztum, J.L. Is the oxidative modification hypothesis relevant to human atherosclerosis? Do the antioxidant trials conducted to date refute the hypothesis? **Circulation,** V. 105, n. 17, p. 2107-2111, 2002.

Ting, H.H., et al. Vitamin C improves endothelium-dependent vasodilation in forearm resistance vessels of humans with hypercholesterolemia. **Circulation,** V. 95, n. 12, p. 2617-2622, 1997.

Turchi, G., Alagona, G., Lubrano, V. Protective activity of plicatin B against human LDL oxidation induced in metal ion-dependent and -independent processes. Experimental and theoretical studies. **Phytomedicine,** V. n. 2009.

Uchida, K. Role of reactive aldehyde in cardiovascular diseases. **Free Radic Biol Med,** V. 28, n. 12, p. 1685-1696, 2000.

Welch, K.D., et al. Deleterious iron-mediated oxidation of biomolecules. **Free Radic Biol Med,** V. 32, n. 7, p. 577-583, 2002.

Witztum, J.L., Steinberg, D. Role of oxidized low density lipoprotein in atherogenesis. **J Clin Invest,** V. 88, n. 6, p. 1785-1792, 1991.

Witztum, J.L., Steinberg, D. The oxidative modification hypothesis of atherosclerosis: does it hold for humans? **Trends Cardiovasc Med,** V. 11, n. 3-4, p. 93-102, 2001.

Yu, Y., et al. Thiosemicarbazones from the old to new: iron chelators that are more than just ribonucleotide reductase inhibitors. **J Med Chem,** V. 52, n. 17, p. 5271-5294, 2009.

Ziouzenkova, O., et al. Copper can promote oxidation of LDL by markedly different mechanisms. **Free Radic Biol Med,** V. 24, n. 4, p. 607-623, 1998.

4.3 Artigo 3

4.3.1 Efeitos do dietil 2-fenil-2-telurofenil vinilfosfonado na oxidação de LDL humana

Diethyl 2-phenyl-2-tellurophenyl vinylphosphonate prevents LDL and serum oxidation

Rafael de Lima Portella, Rômulo Pillon Barcelos, Edovando José Flores da Rosa, João Batista Teixeira da Rocha, Félix Alexandre Antunes Soares.

Diethyl 2-phenyl-2-tellurophenyl vinylphosphonate prevents LDL and serum oxidation

Rafael de Lima Portella[1], Rômulo Pillon Barcelos[1], Edovando José Flores da Rosa[1], João Batista Teixeira da Rocha[1], Félix Alexandre Antunes Soares[1]*.

[1] Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Campus UFSM, Santa Maria, RS, Brasil.

*CORRESPONDING AUTHOR:

Félix Alexandre Antunes Soares

Departamento de Química - CCNE - Universidade Federal de Santa Maria

97105-900 - Santa Maria - RS - Brazil

Phone: +55-55-3220-9522

Fax: +55-55-3220-8978

E-mail: felix@ufsm.br

Abstract

Many lines of evidence suggest that the oxidation of low-density lipoprotein (LDL) plays an important role in the pathogenesis of atherosclerosis. The LDL oxidation hypothesis of atherosclerosis predicts that LDL oxidation is an early event in atherosclerosis and that oxidized LDL contributes to atherogenesis. Various studies have shown a positive effect of antioxidant compounds on the oxidative modification of LDL and atherogenesis. We investigated the possible antioxidant effect of an organotellurium compound, 2-phenyl-2-tellurophenyl vinylphosphonate (DPTVP), on the prevention of LDL and serum oxidation. Our results demonstrated that DPTVP may have antioxidant activity against Cu^{2+}- and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH)-induced LDL and serum oxidation. Additionally, DPTVP was effective at preventing tryptophan oxidation. DPTVP also showed significant total radical-trapping antioxidant activity and could prevent thiobarbituric acid reactive substance (TBARS) formation induced by Cu^{2+} in human LDL and serum. Additionally, DPTVP exhibited no toxicity in rat aorta slices. The results presented here indicate that the antioxidant effect of DPTVP is caused by a combination of free-radical-scavenging activity and blockade of the copper binding sites of LDL. We suggest that DPTVP may be a potential molecule to be studied *in vivo* against atherosclerosis.

Keywords: Low density lipoprotein; Antioxidant; Copper; Atherosclerosis; Tellurium.

1. Introduciton

Atherosclerosis and cardiovascular disease are known to be pivotal causes of morbidity and mortality worldwide, especially in Western populations (Verhoye, E. *et al.*, 2009). Oxidized low-density lipoprotein (oxLDL) is involved in the early yet critical steps of atherogenesis, such as endothelial injury, the expression of adhesion molecules, leukocyte recruitment and retention, and foam cell and thrombus formation (Steinberg, D. *et al.*, 1989; Berliner, J.A. *et al.*, 1995; Steinberg, D., 1997).

The oxidation of LDL is a complex process. Both the protein and lipid moieties can be oxidatively attacked. Moreover, each of the lipid classes, including sterols, fatty acids in phospholipids, cholesterol esters, and triglycerides, and most of the major and minor components, including the many antioxidants in LDL, can be attacked (Steinberg, D. *et al.*, 1990; Parthasarathy, S. *et al.*, 1992).

Although unclear is how LDL is oxidized *in vivo*, several mechanisms have been discussed in the literature, involving metal ions (Swain, J. *et al.*, 1995), lipoxygenase (Parthasarathy, S. *et al.*, 1989), myeloperoxidase (Daugherty, A. *et al.*, 1994), reactive nitrogen species, superoxide radicals, and reduced thiols (Halliwell, B., 1995). Generally, the loss of antioxidants is followed by lipid peroxidation, in which conjugated dienes develop through the oxidation of polyunsaturated fatty acids with localized double bonds (PUFA). The process is terminated with lipid peroxide decomposition to aldehydes and other products (Esterbauer, H. *et al.*, 1992). The adduction of aldehydes to apolipoprotein B (apo B) in LDL has been strongly implicated in the mechanism by which LDL is converted to an atherogenic form that is taken up by macrophages, culminating in the formation of foam cells (Steinberg, D. *et al.*, 1989; Steinberg, D., 1995).

This prevalent oxidation hypothesis implies that the antioxidants that inhibit the oxidation of LDL should effectively suppress atherosclerosis. In fact, the results of numerous *in vitro* and *in vivo* studies support the involvement of LDL oxidation in the pathogenesis of atherosclerosis and the beneficial effects of antioxidants. Another issue and challenge is the development of anti-atherogenic drugs that surpass natural antioxidants. Probucol is one example, but it has the unfortunate side-effect of lowering high-density lipoprotein (HDL). Its butyric acid mono-ester, AGI-1067, has been tested as a multifunctional anti-atherogenic drug (Tardif, J.C., 2003). Another

candidate is BO-653 (2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-*di*-t-butylbenzofuran), which was designed based on *in vitro* studies (Noguchi, N. *et al.*, 1997) and has shown potent anti-atherogenic effects in all animal models studied thus far (Cynshi, O. *et al.*, 1998). We recently showed that the oximes butane-2,3-dionethiosemicarbazone and 3-(phenylhydrazono)butan-2-one prevented LDL oxidation at low concentrations (de Lima Portella, R. *et al.*, 2008). Diphenyl diselenide, an organoselenium compound, has also shown a protective effect against LDL oxidation (Bem, A.F. *et al.*, 2008), in addition to significantly reducing hypercholesterolemia in cholesterol-fed rabbits (de Bem, A.F. *et al.*, 2009).

Organotellurium compounds have been reported to be excellent antioxidants in several models of oxidative stress (Engman, L. *et al.*, 1995; Briviba, K. *et al.*, 1998; Jacob, C. *et al.*, 2000). Diethyl 2-phenyl-2-tellurophenyl vinylphosphonate (DPTVP) is an asymmetric telluride used as an intermediate with great synthetic potential because it combines the chemical reactivity of vinylic tellurides and vinylic phosphonates (Zeni, G. *et al.*, 2003). In a previous study, this compound showed potent antioxidant activity *in vitro* and unexpectedly low toxicity *in vivo* when injected subcutaneously or intraperitoneally in mice (de Avila, D.S. *et al.*, 2006; Avila, D.S. *et al.*, 2007). Additionally, telluro vinylphosphonate showed a potent antioxidant effect against iron-induced lipid peroxidation *in vitro* (de Avila, D.S. *et al.*, 2006) and is a potent antioxidant at low concentrations against two mimetic agents of glutamatergic excitotoxicity (Avila, D.S. *et al.*, 2008).

The most compelling study that supports the oxidation hypothesis showed that the treatment of hypercholesterolemic animals with various antioxidants led to the suppression of atherogenesis (Witztum, J.L. *et al.*, 2001). Although not all such studies have shown beneficial protective effects, the vast majority have. As reviewed in the first AHA Science Advisory (Tribble, D.L., 1999) on antioxidant vitamins, epidemiological and population studies reported that some micronutrients may beneficially affect cardiovascular disease risk (e.g., the antioxidants vitamin E, vitamin C, and β-carotene). These findings have been supported by *in vitro* studies that established a role of oxidative processes in the development of atherosclerotic plaque. Therefore, we investigated the possible antioxidant effect of DPTVP on LDL and serum oxidation.

2. Materials and methods

2.1. Synthesis of diethyl 2-phenyl-2-tellurophenyl vinylphosphonate

DPTVP (Fig. 1) synthesis was performed by addition of alkynylphosphonates to a solution of sodium organyl tellurolate, prepared by the reduction of diorganyl ditellurides with sodium borohydride in ethanol at room temperature (Braga, A.L. *et al.*, 2000).

2.2. LDL isolation

LDL was isolated from fresh human plasma by discontinuous density-gradient ultracentrifugation as described by Silva et al., 1998 (Luiz da Silva, E. *et al.*, 1998), with few modifications. Briefly, plasma of non-fasted healthy normolipidemic voluntary donors collected with EDTA (1 mg/mL) was pooled and sucrose (final concentration, 0.5%) was added to prevent LDL aggregation. Five milliliters of EDTA-plasma adjusted to a density of 1.22 g/mL with solid KBr (0.326 g/mL) was layered on the bottom of a centrifuge tube. Then, 5 mL EDTA-containing sodium chloride solution (density 1.006 g/mL) was overlaid on the top of the plasma. Ultracentrifugation was run at 350,000 g for 2 h at 4º C, in a Himac CP80MX ultracentrifuge. LDL particles were collected by the aspiration of the yellow/orange band at the middle of the saline layer and dialyzed exhaustively overnight at 4º C with 10 mM phosphate buffer (pH 7.4). Protein concentration in LDL solution was determined by Lowry's method (Lowry, O.H. *et al.*, 1951). The purity of LDL preparation was verified by agarose gel electrophoresis. Isolated LDL was stored at -20º C for no longer than 2 weeks.

2.3. LDL oxidation

2.3.1. Conjugated dienes and TBARS formation

LDL samples (50 µg protein/mL) were pre-incubated at 37 °C in a medium containing 10 mM phosphate buffer (pH 7.4) and different concentrations of DPTVP (0 - 5 µM). After 5 minutes, the oxidation was initiated by the addition of either $CuSO_4$ (1.6, 5 or 10µM) or 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH; 1mM). In another set of experiments, DPTVP (5 µM) was added at different time

points in an attempt to evaluate whether DPTVP could inhibit Cu^{2+}-induced LDL oxidation once the process was started. The oxidation was monitored by measuring the increase in absorbance at 234 nm due to conjugated diene (CD) formation as previously described (Esterbauer, H. *et al.*, 1989). Aliquots were also removed at different time points for evaluating thiobarbituric acid reactive substances (TBARS) production as previously described (Ohkawa, H. *et al.*, 1979).

2.3.2. Measurement of LDL- Tryptophan fluorescence

The fluorescence spectra of native LDL display a single band centered at approximately 332 nm, which is assigned to the tryptophan (Trp) residues in ApoB-100 (Giessauf, A. *et al.*, 1995). Loss of tryptophan fluorescence is a marker for oxidations at the protein core of LDL (Reyftmann, J.P. *et al.*, 1990; Giessauf, A. *et al.*, 1995). Tryptophan (Trp) fluorescence was measured in a solution of LDL (50µg protein/ml) in PBS (10 mM) pH 7.4 at 37°C, using a Shimatzo espectrofluorometer (excitation at 282 nm and emission at 331 nm) (Reyftmann, J.P. *et al.*, 1990; Giessauf, A. *et al.*, 1995). The kinetics of LDL oxidation was followed by measuring the decrease of Trp-fluorescence, corresponding to the decomposition of this amino acid, after the addition of 5µM $CuSO_4$, in absence or presence of DPTVP. The cuvettes had to be removed from the excitation light between the single measurements to avoid photooxidation of the tryptophan residues; fluorescence was measured every 20 minutes. Data are shown as the percent decrease of Trp fluorescence in each sample. The time required for reaching half Trp fluorescence ($t_{1/2}$) was calculated.

2.4. Serum oxidation

Venous blood was drawn from nonfasted healthy normolipidemic voluntary donors into tubes containing no anticoagulant and centrifuged at 1000 g for 15 minutes. Serum diluted 100-fold was incubated at 37°C in a medium containing 10 mM phosphate buffer (pH 7.4) and different DPTVP concentrations (0 - 2 µM). The oxidation was initiated by the addition of $CuSO_4$ (30 µM) and CD formation was monitored at 245nm as previously described (Schnitzer, E. *et al.*, 1998). In another set of experiments, DPTVP (2 µM) was added at different time points in an attempt to

evaluate whether DPTVP could inhibit Cu^{2+}-induced serum oxidation once the process was started.

TBARS formation was also evaluated in serum oxidation. Briefly, serum samples were diluted 1:4 in 10mM potassium phosphate buffer, pH 7.4 and incubated at 37°C with $CuSO_4$ (100μM) and/or DPTVP (0 – 10μM). At different time points, aliquots (200μL) were removed for evaluating thiobarbituric acid reactive substances (TBARS) levels as previously described (Ohkawa, H. *et al.*, 1979).

2.5. Determination of lag phase and maximum oxidation rate

In the studies of CD formation, there are several parameters which can be obtained from diene vs. time profiles. The value of the lag phase is commonly determined graphically by the intercept of the tangents to the slow and fast increase of the diene absorption. Another parameter is the maximum oxidation rate, given by the peak of the first derivative, i.e. change of A_{234} as a function of time (Gieseg, S.P. *et al.*, 1994).

2.6. Chelating activity of DPTVP

The copper chelating capacity of DPTVP was evaluated for spectral shifts produced when copper ions were incubated with the test compound as described by Turchi et al. (Turchi, G. *et al.*, 2009b). The reaction mixture consisted of 2mL of phosphate buffer saline (PBS, 10mM, pH 7.4), 20μL of DPTVP solution (in ethanol, final concentration of 37.5, 75, 150 and 300μM) and 50μL of $CuSO_4$ solution (in distilled water, final concentration 150μM). Spectra (200-600nm) were recorded immediately after preparing the mixtures and again after 15 minutes, in the presence and absence of copper ions.

2.7. Total radical-trapping antioxidant potential (TRAP)

TRAP was determined by measuring the chemiluminescence intensity of luminol induced by AAPH thermolysis in a luminometer BioTek Synergy 2 (Lissi, E. *et al.*, 1992). The reaction mixture contained 300 μL of 10 mM AAPH and 10 μL of 1 mM luminol dissolved in 0.1M glycine buffer (pH 8.6). Incubation of this mixture

generates an almost constant light intensity at room temperature after stabilization. DPTVP was added in different concentrations to determine the TRAP activity. At this point, the luminescence intensity is practically abolished. In the course of time, with the loss of antioxidant capacity of DPTVP, the luminescence intensity returns to the initial values. The area under curve (AUC) was evaluated for each DPTVP concentration and compared to vehicle AUC (Dresch, M.T. *et al.*, 2009).

2.8. Aortic slices viability

Adult Wistar rats were decapitated, thoracic aorta were quickly dissected and then removed, rinsed and submerged in a petri dish filled with ice-cold isosmotic phosphate buffer (NaCl 124 mM, Na_2HPO_4 10 mM, NaH_2PO_4 5 mM, KH_2PO_4 5 mM, glucose 10 mM, pH 7.4) and cleaned of adherent fat and connective tissue in an ice-bath (Bem, A.F. *et al.*, 2008). Slices were normalized by weight and incubated with DPTVP concentrations (1 – 20 μM) at 37 °C. After 60 minutes slices were washed three times with isosmotic phosphate buffer. Immediately after incubation with treatments, slices were assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test for 60 minutes (37 °C) (Cordova, F.M. *et al.*, 2004). After incubation, aorta slices were washed with isosmotic phosphate buffer three times (pH 7.4).

MTT is converted into a purple formazan after cleavage of the tetrazolium ring by mitochondrial dehydrogenases. Formazan was dissolved by the addition of DMSO, resulting in a colored compound whose optical density, following by a centrifugation for 1000 g for 5 minutes, the supernatants were read at 570 nm with spectrophotometer (Liu, Y. *et al.*, 1997). This data are calculated as ABS/mg of tissue and expressed as percentage of control.

2.9. Statistical analysis

Data are expressed as means ± SD. Statistical analysis was performed using a one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls test when appropriate and the Student´s “t” test when proper. In addition, linear regression was performed to identify a possible dose dependent effect. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Effects of DPTVP on LDL and serum oxidation

DPTVP concentration-dependently increased the lag phase in both Cu^{2+} and AAPH-induced LDL oxidation (Fig. 2A and B; r^2 = 0.867 and r^2 = 0.927, respectively; $p < 0.001$). Fig. 2 shows the CD production *vs*. time profiles of DPTVP.

DPTVP significantly increased the lag phase of Cu^{2+}-induced LDL oxidation at all concentrations and completely prevented oxidation at 5 μM during the assay time (Table 1). In AAPH-induced LDL oxidation, DPTVP significantly increased the lag phase only at 2, 3, and 5 μM (Table 1). However, DPTVP was unable to prevent the total oxidation of LDL when AAPH was used as an oxidant (Fig. 2B). Additionally, organotellurium (0.5-5 μM) significantly decreased the LDL oxidation rate induced by Cu^{2+} but not AAPH (Table 1). When LDL was oxidized with different Cu^{2+} concentrations, DPTVP (2 μM) presented the same lag phase time as in controls without DPTVP (data not shown) and the same oxidation rate at 1.6, 5, and 10 μM Cu^{2+}, unlike the control group (Fig. 3).

Serum oxidation was determined by CD formation at 245 nm (Fig. 4). No oxidation occurred in serum when the medium did not contain Cu^{2+} ions. Table 2 shows that DPTVP significantly and concentration-dependently increased the lag phase at concentrations from 0.75 to 2 μM (r^2 = 0.828, $p < 0.001$). Furthermore, DPTVP reduced the oxidation rate at concentrations of 1 and 1.5 μM (Table 2), similar to the LDL experiments.

3.2. Effects of DPTVP on LDL tryptophan fluorescence

Fig. 2C shows that the time course of Trp fluorescence decreased during Cu^{2+}-induced LDL oxidation. LDL incubated with different concentrations of DPTVP showed a concentration-dependent increase in the $t_{1/2}$ of Trp (r^2 = 0.943, $p < 0.001$; Fig. 2C). Compared with the control group, the presence of DPTVP at the lowest concentrations significantly increased the $t_{1/2}$ of Trp (Table 2). At concentrations of 2, 3, and 5 μM, the $t_{1/2}$ of Trp was prolonged to more than 180 min (Table 2).

3.3. TBARS production

LDL oxidation was also evaluated by TBARS formation. Table 3 shows a significant effect of DPTVP in preventing TBARS formation induced by Cu^{2+}. The protective effects of DPTVP were similar to the effects found in the CD experiments. DPTVP at the lowest concentration already protected against TBARS formation induced by 5 μM Cu^{2+} in LDL, reflected by significantly low TBARS levels recorded at 60 and 90 min compared with control levels (Table 3). Moreover, DPTVP at 3 and 5 μM continued to maintain low TBARS formation in LDL induced by 5 μM Cu^{2+} during the assay time (240 min) compared with 60-90 min in controls (Table 3).

When TBARS formation was measured in Cu^{+2}-induced serum oxidation, DPTVP at concentrations of 5 and 10 μM completely inhibited the increase in TBARS levels during the assay time (Table 4). At a concentration of 2.5 μM, little effect was observed.

3.4. Effect of DPTVP added at different times on LDL and serum oxidation

In another set of experiments, DPTVP was added at different time points to evaluate whether it could inhibit Cu^{2+}-induced lipid oxidation once the process began. Fig. 5A and B show that DPTVP was able to protect against LDL and serum oxidation only when it was added during the lag phase. When DPTVP was added either at the beginning or during the high conjugated diene production phase, no effect was observed on LDL or serum oxidation.

3.5. Total radical-trapping antioxidant potential of DPTVP

Fig. 6 shows the antioxidant potential of DPTVP when AAPH was used as a pro-oxidant. The concentrations of DPTVP ranged from 2 to 20 μM, showing a significant effect on total radical-trapping antioxidant potential. However, DPTVP had a prominent effect only at 10 and 20 μM.

3.6. Chelating activity of DPTVP

In the present study, few changes in the spectra were observed for DPTVP when it was incubated with copper ions (data not shown). These data may indicate that DPTVP was unable to chelate copper ions under the present experimental conditions.

3.7. Aortic slice viability

Aortic slices in the presence of DPTVP at concentrations of 1 to 20 μM did not show any alterations in viability, reflected by MTT assays, compared with the control group (Fig. 7).

4. Discussion

The inhibition of copper-induced lipid peroxidation in human LDL is considered a biologically relevant model for evaluating the antioxidant effects of new compounds (Miranda, C.L. *et al.*, 2000; Lapeyre, C. *et al.*, 2005). The present study demonstrated that DPTVP may have antioxidant effects on LDL and serum oxidation. DPTVP increased the lag phase and reduced the maximum oxidation rate in both LDL and serum oxidation induced by Cu^{2+}. Trp destruction was also prevented by the organotellurium compound. TBARS levels were significantly reduced in Cu^{2+}-induced serum and LDL oxidation. Moreover, DPTVP significantly protected against AAPH-induced LDL oxidation and showed protective effects in the TRAP assay.

Potential antioxidant compounds have been found to interfere not only with the propagation reactions of free radicals by hydrogen atom donation, but also with the formation of radicals by chelating the transition metal involved in the initiation and propagation reactions. Therefore, most lipid peroxidation processes could feature a combination of transition metal (usually Cu^{2+}) chelation and radical scavenging activities (Turchi, G. *et al.*, 2009a). Organotellurium compounds are readily oxidized from the divalent to the tetravalent state. Consequently, this property makes tellurides attractive as scavengers of reactive oxidizing agents, such as hydrogen peroxide, hypochloride, and peroxyl radicals (Nogueira, C.W. *et al.*, 2004). We found that DPTVP increased the lag phase in both serum and LDL oxidation (Table 1 and 2). Radical scavenger activity appears to be present in this process. However, in the

TRAP assay, we found that DPTVP actually exerted a significant effect at 10 and 20 μM (Fig. 6). These results are consistent with Ávila et al. (Avila, D.S. *et al.*, 2011) who showed that DPTVP, in addition to its ability to scavenge H_2O_2, interacts with the Fenton reaction, decreasing OH• production. They observed an inhibition of the DPPH• reduction, indicating scavenging activity at 50 μM. Furthermore, vinylic telluride was also able to scavenge NO• at 50 μM (Avila, D.S. *et al.*, 2011). Although the peroxyl radical scavenging ability of the organotellurium compound alone was insufficient to explain why DPTVP is more efficient in inhibiting Cu^{2+}-mediated than AAPH-mediated LDL lipid oxidation, the results might indicate that the radical scavenger property is not a unique mechanism involved in the effects on LDL and serum oxidation presented here.

Our results could indicate that the mechanism of action of DPTVP may involve either the binding of copper ions or the blockade of the copper-binding sites of LDL (Pinchuk, I. *et al.*, 1998). However, in the chelating activity assay, we did not observe any significant changes in the DPTVP spectra with or without Cu^{2+}. Although in both LDL and serum oxidation DPTVP was able to decrease the maximum oxidation rate (Table 1 and 2), possibly suggesting that its protection could be attributable to complex formation (Ziouzenkova, O. *et al.*, 1998; Lopez-Alarcon, C. *et al.*, 2007), we can reasonably exclude this interpretation. To verify whether DPTVP could block the copper-binding sites of LDL, we measured the oxidation rate of LDL oxidation with different Cu^{2+} concentrations. An increase in Cu^{2+} concentration increases the LDL oxidation rate (Ramos, P. *et al.*, 1995). Our results showed that DPTVP at 2 μM presented the same oxidation rate at 1.6, 5, and 10 μM of Cu^{2+}, which was different from controls without DPTVP (Fig. 3). DPTVP could interact with copper-binding sites instead of forming a Cu^{2+} complex because the highest Cu^{2+} concentrations are five-fold higher than the DPTVP concentration. Additionally, when DPTVP was added either at the beginning or during the high conjugated diene production phase, little or no effect was observed on LDL or serum oxidation. Moreover, the loss of Trp fluorescence is an oxidation marker at the protein core of LDL (Reyftmann, J.P. *et al.*, 1990), and DPTVP significantly prevented the loss of Trp fluorescence at the lowest concentration used here (Table 1, Fig. 2C). These data may indicate that DPTVP protects against LDL oxidation by blocking the copper-binding sites of LDL.

Several lines of evidence suggest that histidine is the amino acid residue that partially defines the Cu^{2+}-binding sites on apolipoprotein B100. Wagner et al.

demonstrated that diethylpyrocarbonate (DEPC), which reacts almost selectively with histidine residues, inhibited Cu^{2+}-induced LDL oxidation (Wagner, P. *et al.*, 1997). The modification of histidine residues on LDL with DEPC also altered the LDL lipid peroxidation curve induced by Cu^{2+}. An increase in the lag phase was found. Moreover, the oxidation rate decreased in DEPC-LDL, and increasing the concentration of Cu^{2+} in the reaction mixture had little effect on the rate of lipid peroxidation, similar to the DPTVP results (Wagner, P. *et al.*, 1997). The lysine residues may also be involved in LDL oxidation and are also important for the receptor binding of oxLDL (Haberland, M.E. *et al.*, 1984; Parthasarathy, S. *et al.*, 1985). However, Gillotte et al. (Gillotte, K.L. *et al.*, 2000) showed that LDL with blockade of the lysine epsilon-amino groups, modified by reductive methylation, presented similar TBARS levels compared with native LDL when both were oxidized by copper. For this reason, DPTVP more likely interacts with histidine residues than with lysine residues. Ávila et al. (de Avila, D.S. *et al.*, 2006) showed that DPTVP inhibited ALA-D *in vitro*, an effect that involved the oxidation of its essential thiol groups. The tellurium compound increased the rate of DTT oxidation (de Avila, D.S. *et al.*, 2006). The modification of thiol groups from apolipoprotein B100 may alter the secondary and tertiary protein structures. Thus, changes in the conformation of apolipoprotein B100 might also play a role in altering Cu^{2+} binding by LDL. However, whether DPTVP interacts with lysine, histidine, or other amino acid residues remains to be examined. Likewise, whether such a selective interaction between DPTVP and LDL will be recognized by the apo B/E receptor and metabolized normally *in vivo* remains to be examined.

Ávila et al. recently reported that DPTVP had low toxicity in mice when administered intraperitoneally or subcutaneously for 12 days at doses as high as 500 μmol/kg (de Avila, D.S. *et al.*, 2006; Avila, D.S. *et al.*, 2007). We showed here that DPTVP (1-20 μM) was not able to damage aorta slices from rats exposed for 1 h (Fig. 7).

5. Conclusion

Altogether, our data showed that DPTVP has potential antioxidant effects against Cu^{2+}- and AAPH-induced LDL oxidation. This antioxidant activity may

contribute to the prevention of LDL oxidation *in vivo* and thus atherogenesis. DPTVP might present a dual mechanism that includes peroxyl scavenger activity and blockade of the copper-binding sites of LDL. DPTVP was nontoxic in rat aorta slices. These results are consistent with previous studies that demonstrated the antioxidant potential of DPTVP. However, the exact mechanism by which DPTVP blocks the copper-binding sites of LDL remains to be examined.

6. Acknowledgement

This work was supported by the FINEP research grant “Rede Instituto Brasileiro de Neurociência (IBN-Net)” # 01.06.0842-00 and INCT for Excitotoxicity and Neuroprotection- MCT/CNPq. J.B.T.R and F.A.A.S. receive a fellowship by CNPq. R.L.P. and R.P.B. receive a fellowship by CAPES. Additional support giving by FAPERGS and CAPES.

7. Declaration of interest:

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Table 1. Lag phase and maximum oxidation rate for conjugated diene formation in LDL and oxidation $t_{1/2}$ values for Trp fluorescence.

	5μM $CuSO_4$		1mM AAPH		Trp fluorescence
	lag phase	**oxidation rate**	**lag phase**	**oxidation rate**	**$t_{1/2}$**
Control	34,6 ± 4,96	0,227 ± 0,01	69,5 ± 1,8	0,195 ± 0,004	81,5 ± 2,66
0.5μM	49,7 ± 2,14**a	0,208 ± 0,007*a	75,8 ± 1,67	0,191 ± 0,012	106,9 ± 7,21 *
1μM	69,7 ± 1,7***b	0,205 ± 0,002*a	79 ± 1,13	0,200 ± 0,005	140,2 ± 8,2 *
2μM	116,2 ± 4,82***c	0,185 ± 0,005***b	94,3 ± 2,72**	0,194 ± 0,008	ND
3μM	172,5 ± 3,48***d	0,136 ± 0,002***c	103,4 ± 9,69***	0,182 ± 0,009	ND
5μM	ND	ND	131 ± 7,94***	0,179 ± 0,015	ND

LDL (50 μg/mL) was oxidized in the presence of 5 μM Cu^{2+} or 1 mM AAPH. Different concentrations of DPTVP were used. Different letters indicate statistically significant differences among different mean ($P < 0.05$). ND: Not determined.

* $p < 0.05$ compared to control

** $p < 0.001$ compared to control

*** $p < 0.0001$ compared to control

Table 2. Lag phase and maximum oxidation rate for conjugated diene formation in serum oxidation.

	lag phase	oxidation rate
Control	13,5 ± 1,41a	0,085 ± 0,0063a
0.5µM	28 ± 5,98a,b	0,078 ± 0,0048a
0.75µM	44,4 ± 13,95*b	0,075 ± 0,003a
1µM	68,4 ± 11,68**c	0,067 ± 0,0066#b
1.5µM	130,7 ± 18,13**d	0,059 ± 0,0103*b
2µM	ND	ND

Serum was oxidized in the presence of 30 µM Cu^{2+}. Different concentrations of DPTVP were used. Different letters indicate statistically significant differences among different mean ($p < 0.05$). ND: Not determined.

# $p < 0.05$ compared to control

* $p < 0.01$ compared to control

** $p < 0.001$ compared to control

Table 3. Effects of DPTVP concentrations on time-dependent changes in the thiobarbituric acid reactive substances (TBARS) production during LDL oxidation.

	TBARS (nmol MDA/20µg LDL)						
Time (min)	**LDL**	**$CuSO_4$**	**DPTVP 0.5µM**	**DPTVP 1µM**	**DPTVP 2µM**	**DPTVP 3µM**	**DPTVP 5µM**
0	0,22 ± 0,16	0,23 ± 0,14 **a**	0,23 ± 0,15 **a**	0,25 ± 0,15 **a**	0,32 ± 0,19 **a**	0,22 ± 0,16	0,21 ± 0,17
30	0,38 ± 0,3	0,41 ± 0,23 **a**	0,48 ± 0,37 **a**	0,38 ± 0,29 **a**	0,37 ± 0,32 **a**	0,44 ± 0,32	0,38 ± 0,34
60	0,35 ± 0,36 *	1,63 ± 0,44 **b**	0,72 ± 0,28 **a,***	0,47 ± 0,32 **a,***	0,47 ± 0,42 **a,***	0,43 ± 0,34 *	0,46 ± 0,32 *
90	0,45 ± 0,34 #	3,18 ± 0,72 **c**	2,18 ± 0,25 **b,#**	1,16 ± 0,03 **b,#**	0,57 ± 0,34 **a,#**	0,51 ± 0,35 #	0,54 ± 0,32 #
120	0,53 ± 0,35 #	3,14 ± 0,6 **c**	3,3 ± 0,45 **c**	2,28 ± 0,51 **c,***	0,62 ± 0,39 **a,#**	0,61 ± 0,42 #	0,53 ± 0,38 #
150	0,53 ± 0,31 #	3,32 ± 0,91 **c**	3,17 ± 0,48 **c**	2,91 ± 0,41 **d**	0,65 ± 0,26 **a,#**	0,52 ± 0,29 #	0,52 ± 0,23 #
180	0,55 ± 0,31 #	3,16 ± 0,63 **c**	3,16 ± 0,53 **c**	3,09 ± 0,56 **d**	1,15 ± 0,38 **a,#**	0,57 ± 0,32 #	0,53 ± 0,28 #
240	0,54 ± 0,3 #	3,15 ± 0,56 **c**	3,19 ± 0,5 **c**	3,18 ± 0,33 **d**	3,03 ± 0,32 **b**	0,74 ± 0,19 #	0,54 ± 0,26 #

LDL (50 µg protein/mL) was incubated at 37°C in the presence of 5 µM Cu^{2+}. Data are expressed as mean ± SD of three experiments. The values are expressed by nmol MDA/ 20µg protein LDL. Different letters indicate statistically significant differences among different mean ($p < 0.01$).

* $p < 0.01$ compared to $CuSO_4$ group

\# $p < 0.001$ compared to $CuSO_4$ group

Table 4. Effects of DPTVP concentrations on time-dependent changes in the thiobarbituric acid reactive substances (TBARS) production during serum oxidation.

	TBARS (nmol MDA/mL serum)					
Time (min)	**control**	**$CuSO_4$**	**DPTVP 1µM**	**DPTVP 2.5µM**	**DPTVP 5µM**	**DPTVP 10µM**
0	14,2 ± 14,1	40 ± 11,5 **a**	30,8 ± 17,3 **a**	38,1 ± 11,1 **a**	33,7 ± 16,8	30,2 ± 15,1
90	39,7 ± 23,1	86,6 ± 4,6 **a**	74,4 ± 5,4 **a**	60,7 ± 7,2 **a**	53,9 ± 13,3	51,8 ± 12,5
180	30,7 ± 4,3 #	190,2 ± 28,4 **b**	177,5 ± 16,9 **b**	107 ± 12,9 **a** *	65,7 ± 8,4 #	61,1 ± 6,3 #
360	32,3 ± 11,1 #	261,9 ± 95,2 **c**	255,2 ± 89,4 **c**	209,1 ± 89,4 **b**	68,5 ± 11 #	52,9 ± 6,7 #

Serum (diluted 4-fold) in PBS 10mM (pH7.4) was incubated at 37°C for 360min in the presence of 100 µM Cu^{2+}. Data are expressed as mean ± SD of three experiments. The values were expressed by nmol MDA/ mL serum. Different letters indicate statistically significant differences among different mean ($p < 0.01$).

* $p < 0.05$ compared to $CuSO_4$ group

# $p < 0.001$ compared to $CuSO_4$ group

8. Figures

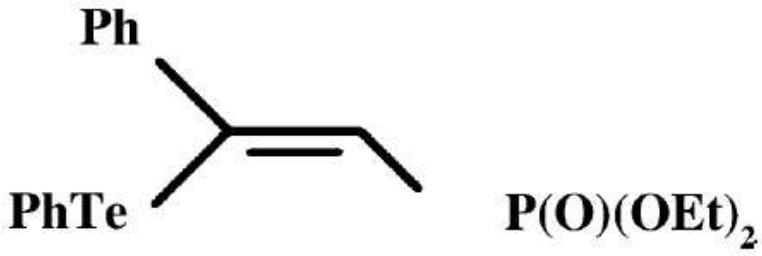
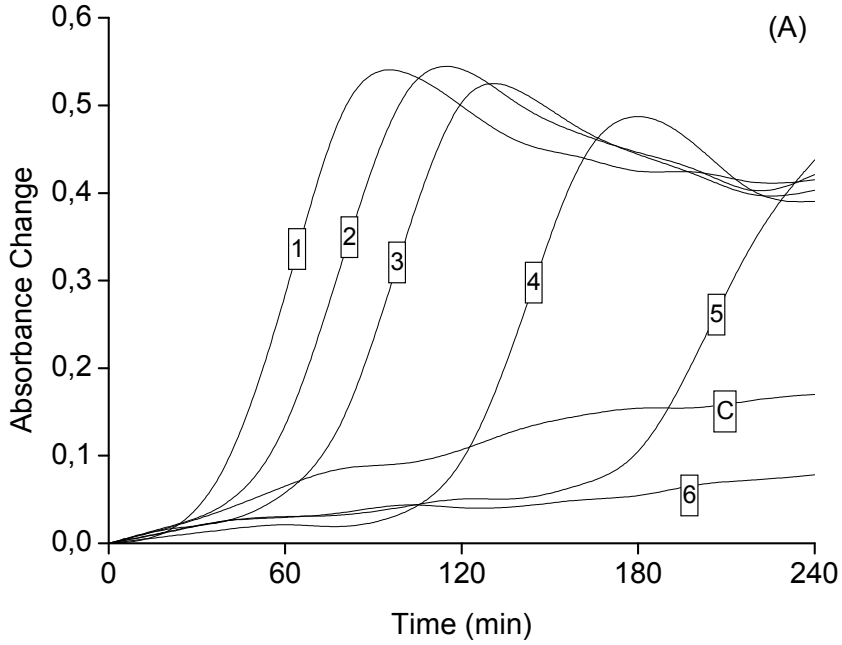
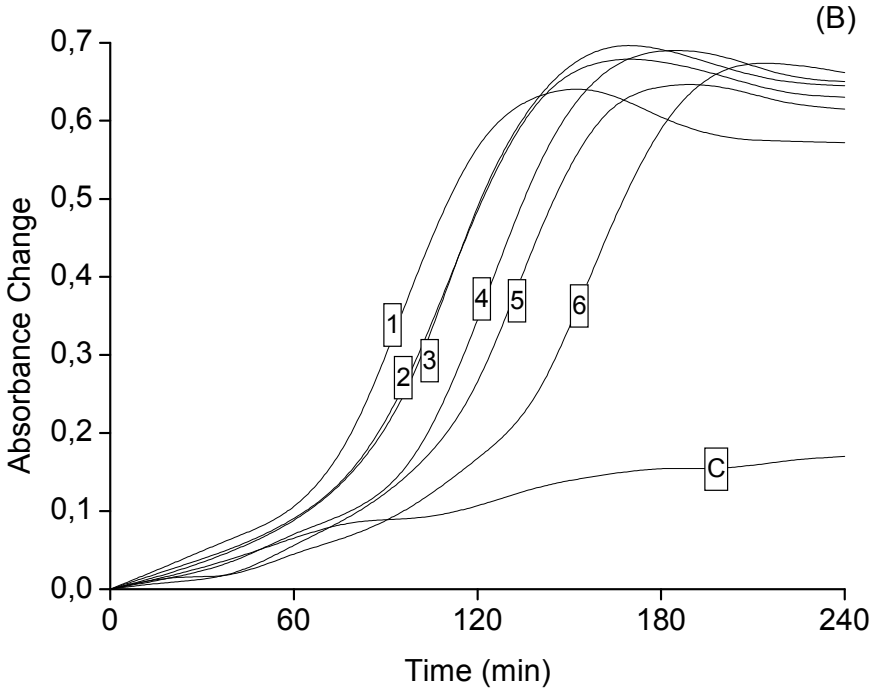


Figure 1. Structure of diethyl 2-phenyl-2 tellurophenyl vinylphosphonate.


(A)
Absorbance Change
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0
60
120
180
240
Time (min)
1
2
3
4
5
C
6


(B)
Absorbance Change
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0
60
120
180
240
Time (min)
1
2
3
4
5
6
C

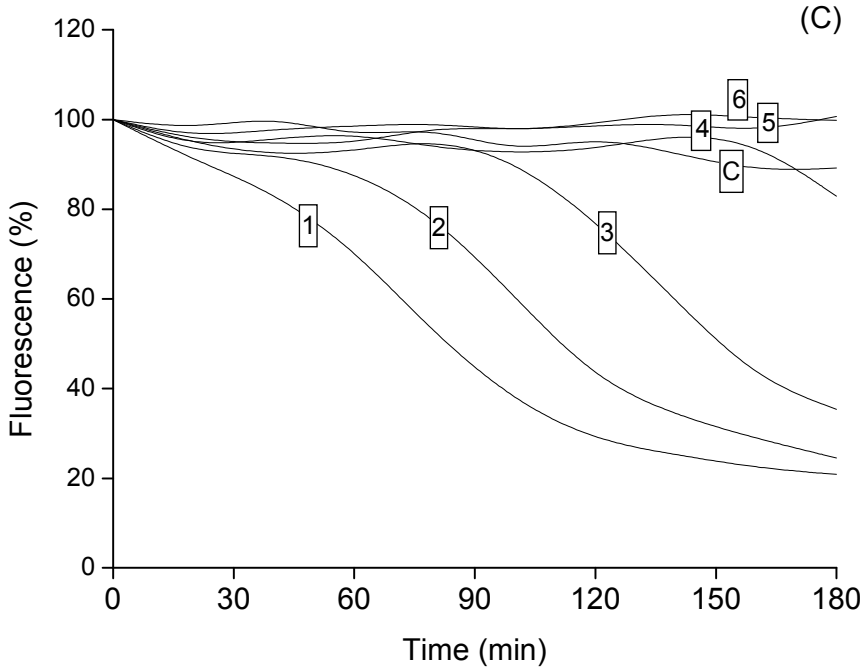


Figure 2. Effects of DPTVP on LDL oxidation. LDL (50 µg protein/mL) was incubated in PBS 10mM (pH 7.4) at 37°C in the presence of Cu^{2+} 5 µM (panel A) or AAPH 1mM (panel B). Conjugated diene formation was measured by determining the absorbance at 234 nm every 20 min. Decrease of Trp fluorescence during Cu^{2+}-mediated oxidation of LDL with different concentrations of DPTVP (panel C). LDL (50 µg protein/mL) was incubated in PBS with 5 µM Cu^{2+}. Fluorescence (Ex/Em = 282/331 nm) was measured at intervals of 20 min at 37°C. In all panels, incubation medium did not contain DPTVP (1) or contained 0.5 µM (2), 1 µM (3), 2 µM (4), 3 µM (5) or 5 µM (6) of DPTVP. (C) Control without oxidant agent and DPTVP. Experiments were repeated at least three times, showing similar results.

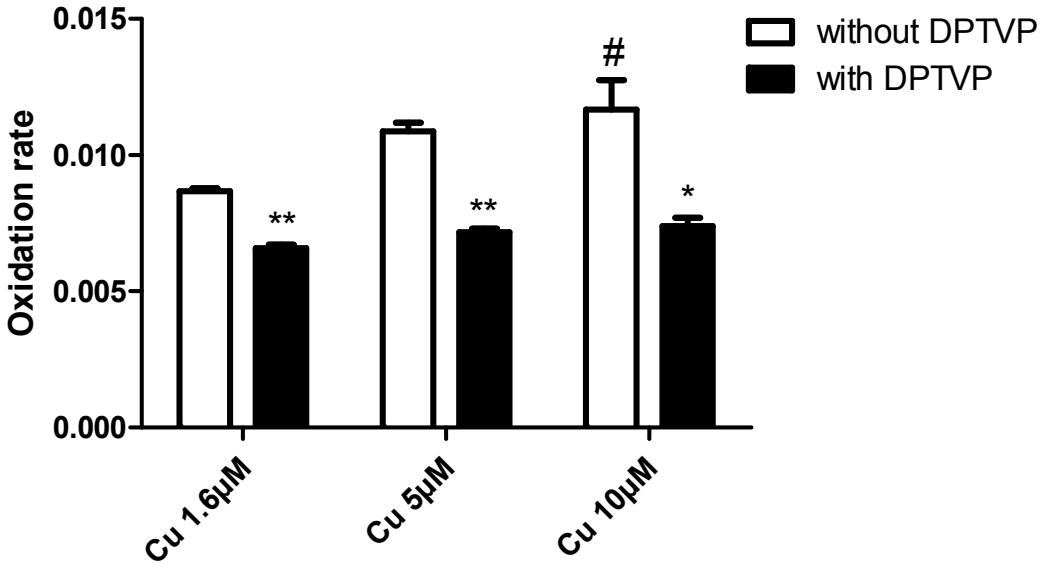


Figure 3. Effect of DPTVP on oxidation rate with different Cu^{2+} concentrations. LDL (50 µg protein/mL) was incubated in PBS 10mM (pH 7.4) at 37°C in the presence of Cu^{2+} at 1.6, 5 or 10 µM. DPTVP was used at 2 µM. * $p<0.05$ and ** $p<0.001$ compared to white bar in the same copper concentration. # $p<0.05$ compared to white bar at 1.6 µM copper concentration.

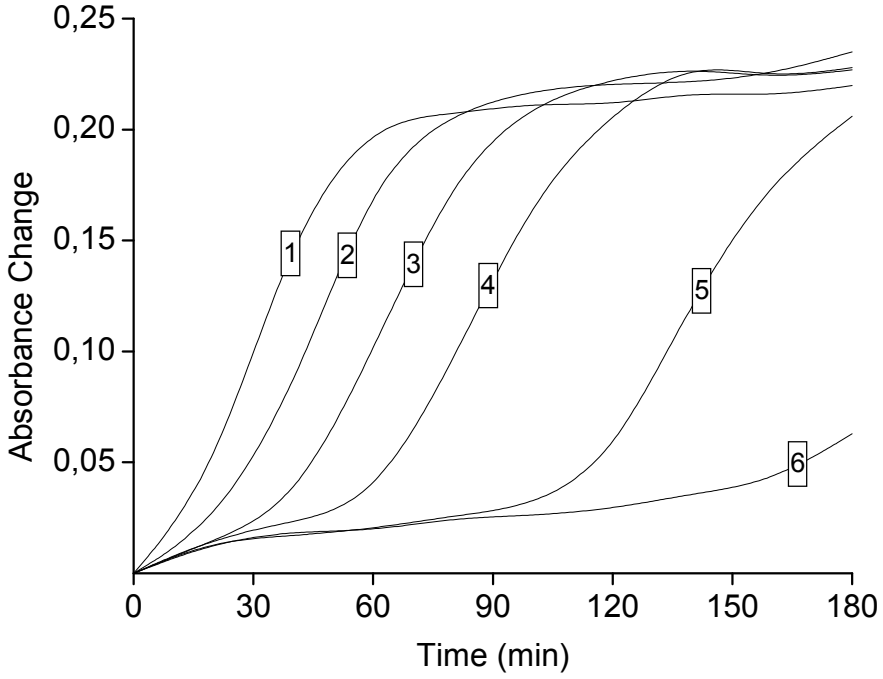


Figure 4. Effects of DPTVP concentrations on conjugated dienes formation during serum oxidation. Serum (diluted 100-fold) in PBS 10mM (pH7.4) was incubated at 37°C for 180min in the presence of 30 µM Cu^{2+}. Incubation medium did not contain DPTVP (1) or contained 0.5 µM (2), 0.75 µM (3), 1 µM (4), 1.5 µM (5) or 2 µM (6) of DPTVP. Conjugated diene formation was measured by determining the absorbance at 245 nm every 20 min. Experiments were repeated at least three times, showing similar results.

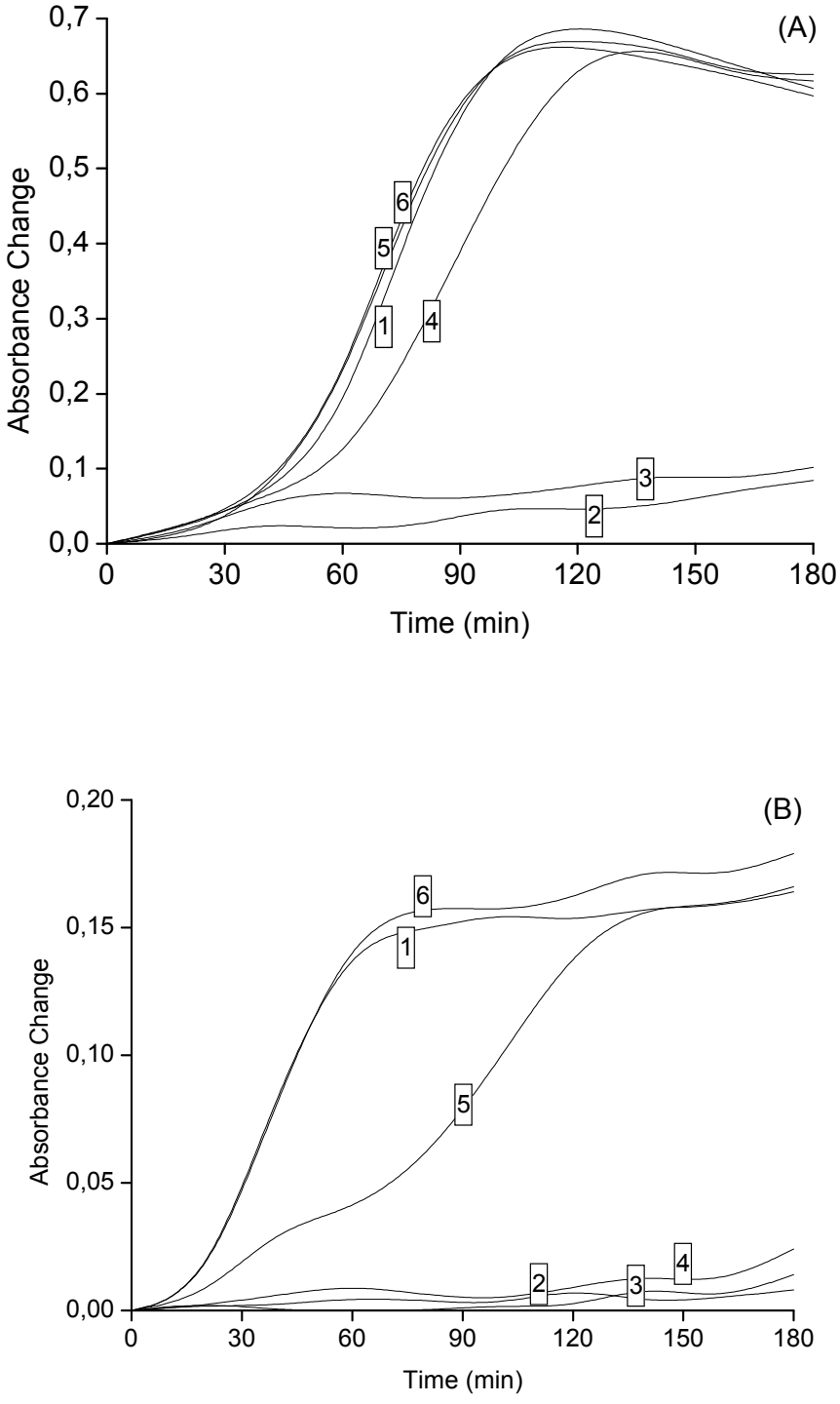


Figure 5. Effect of DPTVP when added at different times of LDL (panel A) or serum (panel B) oxidation. LDL (50 µg protein/mL) was incubated in PBS 10mM (pH 7.4) at 37°C in the presence of Cu^{2+} 5 µM and DPTVP 5 µM. Serum (diluted

100-fold) in PBS 10mM (pH 7.4) was incubated at 37°C for 180min in the presence of 30 μM Cu^{2+} and DPTVP 2μM. In panel A, incubation medium did not contain DPTVP (1) or DPTVP was added at 20 (2), 40 (3), 50 (4), 60 (5) or 80 (6) minutes. In panel B, incubation medium did not contain DPTVP (1) or DPTVP was added at zero (2), 10 (3), 20 (4), 30 (5) or 40 (6) minutes. Experiments were repeated at least three times, showing similar results.

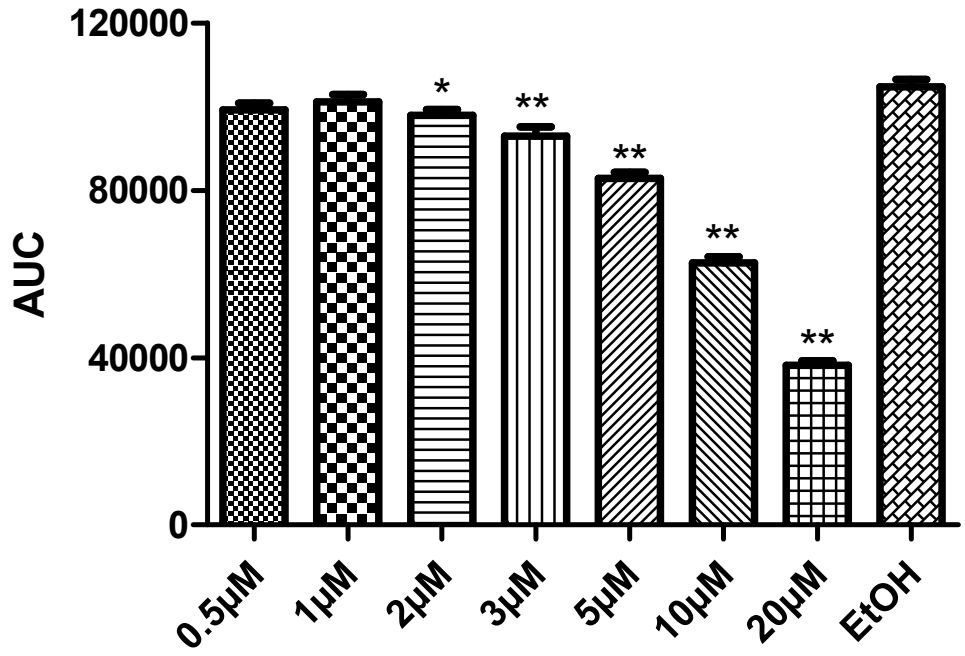


Figure 6. *In vitro* effect of DPTVP on total radical-trapping antioxidant potential (TRAP). The reaction mixture contained 10 mM AAPH, 1 mM luminol and DPTVP at different concentrations dissolved in 0.1 M glycine buffer (pH 8.6). The luminescence was measured every 5 minutes during 2 hours. The area under curve (AUC) was evaluated. The bars represent mean ± S.E.M. of five different experiments. * Indicates statistical difference with $P<0.05$ and ** refers to significance with $P<0.001$ by EtOH group.

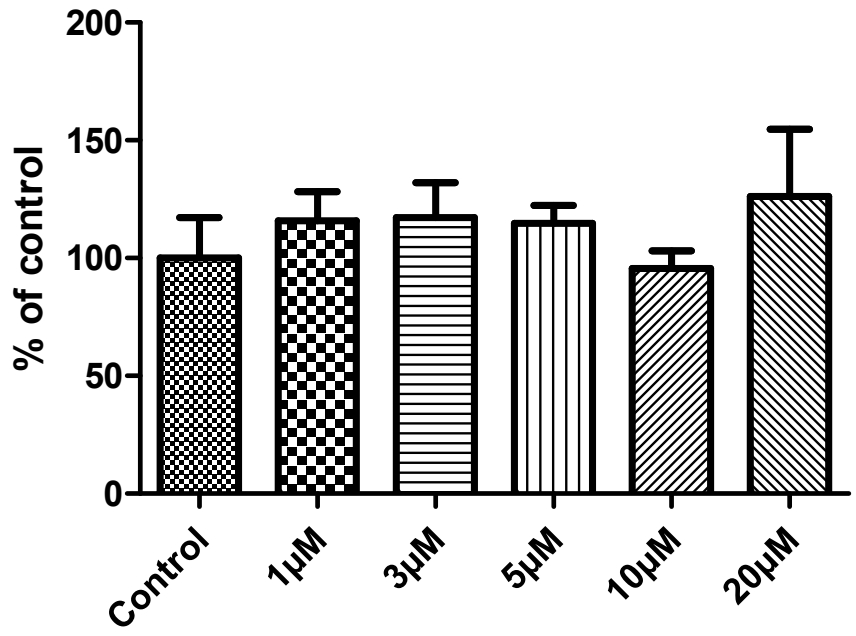


Figure 7. Aortic slices viability. Aortic slices were incubated in presence of DPTVP (1, 3, 5, 10 and 20 μM), and viability was measured by MTT assay. The values are mean±SEM of five independent experiments in duplicate ($P<0.05$).

9. References

Avila, D.S., et al. A biochemical and toxicological study with diethyl 2-phenyl-2-tellurophenyl vinylphosphonate in a sub-chronic intraperitoneal treatment in mice. **Life Sci,** V. 80, n. 20, p. 1865-1872, 2007.

Avila, D.S., et al. An organotellurium compound with antioxidant activity against excitotoxic agents without neurotoxic effects in brain of rats. **Brain Res Bull,** V. 76, n. 1-2, p. 114-123, 2008.

Avila, D.S., et al. Hepatoprotective activity of a vinylic telluride against acute exposure to acetaminophen. **Eur J Pharmacol,** V. 661, n. 1-3, p. 92-101, 2011.

Bem, A.F., et al. Diphenyl diselenide, a simple glutathione peroxidase mimetic, inhibits human LDL oxidation in vitro. **Atherosclerosis,** V. n. 2008.

Berliner, J.A., et al. Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. **Circulation,** V. 91, n. 9, p. 2488-2496, 1995.

Braga, A.L., et al. Stereoselective addition of sodium organyl chalcogenolates to alkynylphosphonates: synthesis of diethyl 2-(organyl)-2-(organochalcogenyl)vinylphosphonates. **Tetrahedron Lett.,** V. 41, n. p. 161-163, 2000.

Briviba, K., et al. Protection by organotellurium compounds against peroxynitrite-mediated oxidation and nitration reactions. **Biochem Pharmacol,** V. 55, n. 6, p. 817-823, 1998.

Cordova, F.M., et al. Lead stimulates ERK1/2 and p38MAPK phosphorylation in the hippocampus of immature rats. **Brain Res,** V. 998, n. 1, p. 65-72, 2004.

Cynshi, O., et al. Antiatherogenic effects of the antioxidant BO-653 in three different animal models. **Proc Natl Acad Sci U S A,** V. 95, n. 17, p. 10123-10128, 1998.

Daugherty, A., et al. Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. **J Clin Invest,** V. 94, n. 1, p. 437-444, 1994.

de Avila, D.S., et al. Diethyl 2-phenyl-2 tellurophenyl vinylphosphonate: an organotellurium compound with low toxicity. **Toxicology,** V. 224, n. 1-2, p. 100-107, 2006.

de Bem, A.F., et al. Diphenyl diselenide decreases serum levels of total cholesterol and tissue oxidative stress in cholesterol-fed rabbits. **Basic Clin Pharmacol Toxicol,** V. 105, n. 1, p. 17-23, 2009.

de Lima Portella, R., et al. Oximes as inhibitors of low density lipoprotein oxidation. **Life Sci,** V. 83, n. 25-26, p. 878-885, 2008.

Dresch, M.T., et al. Optimization and validation of an alternative method to evaluate total reactive antioxidant potential. **Anal Biochem,** V. 385, n. 1, p. 107-114, 2009.

Engman, L., et al. Organotellurium compounds as efficient retarders of lipid peroxidation in methanol. **Free Radic Biol Med,** V. 19, n. 4, p. 441-452, 1995.

Esterbauer, H., et al. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. **Free Radic Biol Med,** V. 13, n. 4, p. 341-390, 1992.

Esterbauer, H., et al. Continuous monitoring of in vitro oxidation of human low density lipoprotein. **Free Radic Res Commun,** V. 6, n. 1, p. 67-75, 1989.

Gieseg, S.P., Esterbauer, H. Low density lipoprotein is saturable by pro-oxidant copper. **FEBS Lett,** V. 343, n. 3, p. 188-194, 1994.

Giessauf, A., Steiner, E., Esterbauer, H. Early destruction of tryptophan residues of apolipoprotein B is a vitamin E-independent process during copper-mediated oxidation of LDL. **Biochim Biophys Acta,** V. 1256, n. 2, p. 221-232, 1995.

Gillotte, K.L., et al. Oxidized phospholipids, linked to apolipoprotein B of oxidized LDL, are ligands for macrophage scavenger receptors. **J Lipid Res,** V. 41, n. 5, p. 824-833, 2000.

Haberland, M.E., Olch, C.L., Folgelman, A.M. Role of lysines in mediating interaction of modified low density lipoproteins with the scavenger receptor of human monocyte macrophages. **J Biol Chem,** V. 259, n. 18, p. 11305-11311, 1984.

Halliwell, B. Oxidation of low-density lipoproteins: questions of initiation, propagation, and the effect of antioxidants. **Am J Clin Nutr,** V. 61, n. 3 Suppl, p. 670S-677S, 1995.

Jacob, C., et al. Water-soluble organotellurium compounds: catalytic protection against peroxynitrite and release of zinc from metallothionein. **Chem Res Toxicol,** V. 13, n. 1, p. 3-9, 2000.

Lapeyre, C., et al. Design, synthesis, and evaluation of pharmacological properties of cinnamic derivatives as antiatherogenic agents. **J Med Chem,** V. 48, n. 26, p. 8115-8124, 2005.

Lissi, E., Pascual, C., Del Castillo, M.D. Luminol luminescence induced by 2,2'-Azo-bis(2-amidinopropane) thermolysis. **Free Radic Res Commun,** V. 17, n. 5, p. 299-311, 1992.

Liu, Y., et al. Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. **J Neurochem,** V. 69, n. 2, p. 581-593, 1997.

Lopez-Alarcon, C., Speisky, H., Lissi, E. Antioxidant effect of 5-amino salicylic acid on copper-mediated LDL oxidation. **Biol Res,** V. 40, n. 2, p. 155-162, 2007.

Lowry, O.H., et al. Protein measurement with the Folin phenol reagent. **J Biol Chem,** V. 193, n. 1, p. 265-275, 1951.

Luiz da Silva, E., Tsushida, T., Terao, J. Inhibition of mammalian 15-lipoxygenase-dependent lipid peroxidation in low-density lipoprotein by quercetin and quercetin monoglucosides. **Arch Biochem Biophys,** V. 349, n. 2, p. 313-320, 1998.

Miranda, C.L., et al. Antioxidant and prooxidant actions of prenylated and nonprenylated chalcones and flavanones in vitro. **J Agric Food Chem,** V. 48, n. 9, p. 3876-3884, 2000.

Noguchi, N., et al. 2,3-Dihydro-5-hydroxy-2,2-dipentyl-4,6-di-tert-butylbenzofuran: design and evaluation as a novel radical-scavenging antioxidant against lipid peroxidation. **Arch Biochem Biophys,** V. 342, n. 2, p. 236-243, 1997.

Nogueira, C.W., Zeni, G., Rocha, J.B. Organoselenium and organotellurium compounds: toxicology and pharmacology. **Chem Rev,** V. 104, n. 12, p. 6255-6285, 2004.

Ohkawa, H., Ohishi, N., Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. **Anal Biochem,** V. 95, n. 2, p. 351-358, 1979.

Parthasarathy, S., Rankin, S.M. Role of oxidized low density lipoprotein in atherogenesis. **Prog Lipid Res,** V. 31, n. 2, p. 127-143, 1992.

Parthasarathy, S., et al. Essential role of phospholipase A2 activity in endothelial cell-induced modification of low density lipoprotein. **Proc Natl Acad Sci U S A,** V. 82, n. 9, p. 3000-3004, 1985.

Parthasarathy, S., Wieland, E., Steinberg, D. A role for endothelial cell lipoxygenase in the oxidative modification of low density lipoprotein. **Proc Natl Acad Sci U S A,** V. 86, n. 3, p. 1046-1050, 1989.

Pinchuk, I., Schnitzer, E., Lichtenberg, D. Kinetic analysis of copper-induced peroxidation of LDL. **Biochim Biophys Acta,** V. 1389, n. 2, p. 155-172, 1998.

Ramos, P., et al. Effect of temperature and phase transition on oxidation resistance of low density lipoprotein. **J Lipid Res,** V. 36, n. 10, p. 2113-2128, 1995.

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,** V. 1042, n. 2, p. 159-167, 1990.

Schnitzer, E., et al. Lipid oxidation in unfractionated serum and plasma. **Chem Phys Lipids,** V. 92, n. 2, p. 151-170, 1998.

Steinberg, D. Role of oxidized LDL and antioxidants in atherosclerosis. **Adv Exp Med Biol,** V. 369, n. p. 39-48, 1995.

Steinberg, D. Lewis A. Conner Memorial Lecture. Oxidative modification of LDL and atherogenesis. **Circulation,** V. 95, n. 4, p. 1062-1071, 1997.

Steinberg, D., et al. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. **N Engl J Med,** V. 320, n. 14, p. 915-924, 1989.

Steinberg, D., Witztum, J.L. Lipoproteins and atherogenesis. Current concepts. **JAMA,** V. 264, n. 23, p. 3047-3052, 1990.

Swain, J., Gutteridge, J.M. Prooxidant iron and copper, with ferroxidase and xanthine oxidase activities in human atherosclerotic material. **FEBS Lett,** V. 368, n. 3, p. 513-515, 1995.

Tardif, J.C. Clinical results with AGI-1067: a novel antioxidant vascular protectant. **Am J Cardiol,** V. 91, n. 3A, p. 41A-49A, 2003.

Tribble, D.L. AHA Science Advisory. Antioxidant consumption and risk of coronary heart disease: emphasison vitamin C, vitamin E, and beta-carotene: A statement for healthcare professionals from the American Heart Association. **Circulation,** V. 99, n. 4, p. 591-595, 1999.

Turchi, G., Alagona, G., Lubrano, V. Protective activity of plicatin B against human LDL oxidation induced in metal ion-dependent and -independent processes. Experimental and theoretical studies. **Phytomedicine,** V. 16, n. 11, p. 1014-1026, 2009a.

Turchi, G., Alagona, G., Lubrano, V. Protective activity of plicatin B against human LDL oxidation induced in metal ion-dependent and -independent processes. Experimental and theoretical studies. **Phytomedicine,** V. n. 2009b.

Verhoye, E., Langlois, M.R. Circulating oxidized low-density lipoprotein: a biomarker of atherosclerosis and cardiovascular risk? **Clin Chem Lab Med,** V. 47, n. 2, p. 128-137, 2009.

Wagner, P., Heinecke, J.W. Copper ions promote peroxidation of low density lipoprotein lipid by binding to histidine residues of apolipoprotein B100, but they are reduced at other sites on LDL. **Arterioscler Thromb Vasc Biol,** V. 17, n. 11, p. 3338-3346, 1997.

Witztum, J.L., Steinberg, D. The oxidative modification hypothesis of atherosclerosis: does it hold for humans? **Trends Cardiovasc Med,** V. 11, n. 3-4, p. 93-102, 2001.

Zeni, G., Braga, A.L., Stefani, H.A. Palladium-catalyzed coupling of sp(2)-hybridized tellurides. **Acc Chem Res,** V. 36, n. 10, p. 731-738, 2003.

Ziouzenkova, O., et al. Copper can promote oxidation of LDL by markedly different mechanisms. **Free Radic Biol Med,** V. 24, n. 4, p. 607-623, 1998.

5. DISCUSSÃO

Estudos genéticos, clínicos e farmacológicos envolvem os níveis elevados de LDL na patogênese das doenças ateroscleróticas vasculares, a principal causa de morte nos países industrializados (Brown, M.S. *et al.*, 1992). Paradoxalmente, a LDL nativa deixa de exercer os efeitos potencialmente aterogênicos *in vitro*, sugerindo que ela deve sofrer modificações para promover a doença vascular. De fato, muitas linhas de evidências sustentam a hipótese da oxidação da LDL, que sugerem que um dano oxidativo da LDL é o mecanismo principal para tornar as lipoproteínas aterogênicas (Witztum, J.L. *et al.*, 1991; Heinecke, J.W., 1998).

Experimentos *in vitro* mostraram que todos os principais tipos de células encontrados na lesão aterosclerótica (células endoteliais, células musculares lisas, monócitos e macrófagos) produzem intermediários reativos que oxidam a LDL (Heinecke, J.W. *et al.*, 1984; Morel, D.W. *et al.*, 1984; Steinbrecher, U.P. *et al.*, 1984). As modificações da LDL nestes sistemas necessitam de íons metálicos no meio, gerando produtos de oxidação lipídica, e são inibidas por antioxidantes lipossolúveis. Estes dados levam à hipótese que a oxidação lipídica é essencial para tornar a LDL aterogênica, uma proposta suportada pelos estudos bioquímicos e imunológicos mostrando que a LDL oxidada e os produtos da oxidação lipídica ligados às proteínas são encontradas nas lesões ateroscleróticas (Haberland, M.E. *et al.*, 1988; Rosenfeld, M.E. *et al.*, 1990).

Níveis elevados de íons metálicos podem estar presentes na lesão aterosclerótica, mas existe uma escassez de informações quantitativas e da natureza dos íons metálicos. Homogenatos preparados de tecidos da lesão aterosclerótica contêm íons metálicos cataliticamente ativos, indicando que estes metais podem estimular a oxidação da LDL *in vivo* na parede arterial, mas não pode ser completamente negado que os íons metálicos são gerados artificialmente durante o processo de homogeneização (Yoshida, H. *et al.*, 2010). No entanto, os resultados usando uma técnica minimamente invasiva, espectroscopia de ressonância paramagnética eletrônica (EPR) e espectrometria de massa acoplada ao plasma induzido (ICPMS) foram relatadas, mostrando uma correlação direta entre os níveis de ferro e o acúmulo de colesterol nas amostras de endarterectomia de

artérias carótidas humanas (Stadler, N. *et al.*, 2004). No entanto, a hemocromatose e a doença de Wilson aumentam os níveis de ferro e cobre na circulação sanguínea e nos tecidos, mas os pacientes com essas doenças não têm um risco aumentado para aterosclerose (Gaut, J.P. *et al.*, 2001). As doenças coronárias são menos extensas nos pacientes com hemocromatose, e estudos epidemiológicos têm mostrado que a relação entre os níveis de ferro no plasma e a aterosclerose é controversa, embora os estudos *in vitro* com culturas de células têm demonstrado que o tratamento com ferro pode aumentar o estresse oxidativo e a susceptibilidade da LDL à oxidação (Ascherio, A. *et al.*, 1994; Miller, M. *et al.*, 1994). A ceruloplasmina, uma proteína de 132 kDa que contém 7 átomos de cobre por molécula, foi considerada como um antioxidante, mas a sua potente atividade oxidante sobre a LDL tem sido verificada (Ehrenwald, E. *et al.*, 1994; Van Lenten, B.J. *et al.*, 1995; Chisolm, G.M., 3rd *et al.*, 1999).

Estudos com vários modelos de animais (coelhos, camundongos, hamsters, porcos-da-índia e macacos) e com diversos tipos de antioxidantes (vitamina E, probucol, análogos ao probucol e conenzima Q) têm demonstrado que todos antioxidantes apresentam efeitos protetores contra a aterosclerose (Witztum, J.L. *et al.*, 2001), embora os ensaios clínicos em humanos com vitamina E não sejam todos positivos. Os resultados negativos dos ensaios com antioxidantes em humanos podem ser devido ao uso do tipo errado de antioxidante e na dose errada e também, por um período muito curto (Witztum, J.L. *et al.*, 2001).

Os resultados com antioxidantes que foram positivos levantam diversas questões importantes sobre o desenho do estudo com antioxidantes. Primeiro, os estudos com resultados positivos selecionaram rigorosamente os candidatos para o tratamento com antioxidante, sendo uma população de pacientes específicos (Watanabe, K. *et al.*, 1996; Gokce, N. *et al.*, 1999). Isto é significante já que a maioria dos ensaios mal sucedidos com vitaminas antioxidantes usa populações de pacientes diversificadas e em diferentes estágios da doença, com carga de estresse oxidativo diferenciado, diluindo assim, a habilidade de verificar os benefícios do antioxidante estudado (Vivekananthan, D.P. *et al.*, 2003). É possível que nem todos os pacientes sejam amenizados com a mesma combinação de antioxidantes, tipo ou regime de tratamento, necessitando uma avaliação cuidadosa das mudanças específicas e temporais no estresse oxidativo (Thomson, M.J. *et al.*, 2007). Por isso

a necessidade de se encontrar novos agentes terapêuticos com atividade antioxidante para o tratamento das doenças cardiovasculares.

Durante o período colonial, o guaraná era vendido variadamente como fortificante, estimulante, tônico, antídoto para a febre, preventivo contra o endurecimento das artérias e para tratar enxaquecas. O guaraná foi considerado especialmente eficaz no tratamento de diarréia e disenteria (Smith, N. *et al.*, 2010). O guaraná é rico em metilxantinas como a cafeína, teobromina e teofilina e contém taninos, saponinas, catequinas, epicatequinas, proantocianidinas, bem como traços de muitos outros compostos (Belliardo, F. *et al.*, 1985).

Nossos resultados com o guaraná mostraram que as pessoas que consumiam habitualmente guaraná tiveram os níveis de dienos conjugados reduzidos comparado às pessoas que não consumiam guaraná. Esses resultados são consistentes com os resultados achados nos estudos *in vitro*, que demonstraram que o extrato de guaraná tem capacidade de reduzir a oxidação da porção lipídica e protéica da LDL. Além disso, o guaraná apresenta uma capacidade *scavenger* de radical hidroxil como determinado no ensaio de TRAP. Esses efeitos antioxidantes estão relacionados com a grande quantidade de compostos fenólicos presentes no extrato de guaraná. Outros efeitos do guaraná que podem ajudar na prevenção do processo aterogênico, incluem os efeitos no metabolismo lipídico (Lima, W.P. *et al.*, 2005), na perda de peso (Boozer, C.N. *et al.*, 2001), no aumento do gasto basal de energia (Berube-Parent, S. *et al.*, 2005), além da baixa prevalência de hipertensão, síndrome metabólica e redução nos níveis de colesterol e de AOPP nos indivíduos que consomem guaraná (Costa Krewer, C. *et al.*, 2011).

As tiosemicarbazonas formam uma importante classe de compostos por causa de suas importantes propriedades farmacológicas, tais como atividades tripanocida (Papanastasiou, I. *et al.*, 2008), antitubercular (Sriram, D. *et al.*, 2006) e antitumoral (Scovill, J.P. *et al.*, 1982; Huang, H. *et al.*, 2010). Derivados de tiosemicarbazonas têm mostrado atividades *scavenger* de radicais superóxido e hidroxil, inibição de peroxidação lipídica, proteção da oxidação de LDL, além da atividade quelante (Prathima, B. *et al.*, 2010; Zhong, Z. *et al.*, 2010; Barcelos, R.P. *et al.*, 2011).

Os resultados com a tiosemicarbazona SPTS, apesar de serem bastante preliminares, mostraram que ela tem um efeito antioxidante para vários agentes oxidantes, como íons Cu^{2+}, AAPH e SNP. A SPTS foi capaz de prevenir a oxidação

da porção lipídica e protéica da LDL, bem como preveniu danos oxidativos em tecidos de ratos. As tiosemicarbazonas possuem uma propriedade quelante bem caracterizada na literatura, devido ao seu sistema de coordenação O, N, S (Yu, Y. *et al.*, 2009). Além disso, sua substituição fenila na porção N terminal torna a molécula um bom *scavenger* de radicais (Raja, D.S. *et al.*, 2011). Com isso, esses estudos preliminares tornam a SPTS um composto promissor para ser usado no tratamento de doenças relacionadas com o estresse oxidativo, assim como a aterosclerose.

O composto orgânico de telúrio DPTVP já foi demonstrado possuir atividade *scavenger* de H_2O_2, DPPH• e NO• (Avila, D.S. *et al.*, 2011). Neste estudo, mostramos que o DPTVP tem a habilidade de inibir a oxidação das porções lipídica e proteica da LDL, induzidas por íons Cu^{2+} e AAPH. Essa atividade *scavenger* de radicais está ligada à propriedade do telúrio passar de um estado divalente para um estado tetravalente (Nogueira, C.W. *et al.*, 2004). Ávila et al. propuseram um mecanismo pelo qual o DPTVP pode agir com *scavenger* de ROS e RNS (Figura 5). O DPTVP tem um grupo fosfato que e um grupo retirador de elétrons. Este grupo causa um decréscimo da densidade eletrônica no átomo de telúrio (Te), o qual se torna Te^{+}, uma espécie eletrofílica. Considerando que as espécies reativas têm elétrons desemparelhados, eles propuseram que o Te^{+} pode aceitar este elétron, tornando-se teluróxido (Te IV), similarmente ao que é descrito para diversos compostos orgânicos de telúrio com estrutura simétrica ou não (Andersson, C.M. *et al.*, 1994; Briviba, K. *et al.*, 1998; Braga, A.L. *et al.*, 2009). No entanto, o DPTVP parece interagir com a porção protéica da LDL, tendo um mecanismo adicional contra a oxidação induzida por íons Cu^{2+}. Além disso, o DPTVP não causou danos em células de artérias de ratos, o que vai ao encontro com os dados achados previamente por Ávila et al., mostrando que o DPTVP não apresenta toxicidade para camundongos quando administrados intraperitonealmente e subcutaneamente por 12 dias em altas doses (500 µmol/kg) (Avila, D.S. *et al.*, 2007; Avila, D.S. *et al.*, 2008).

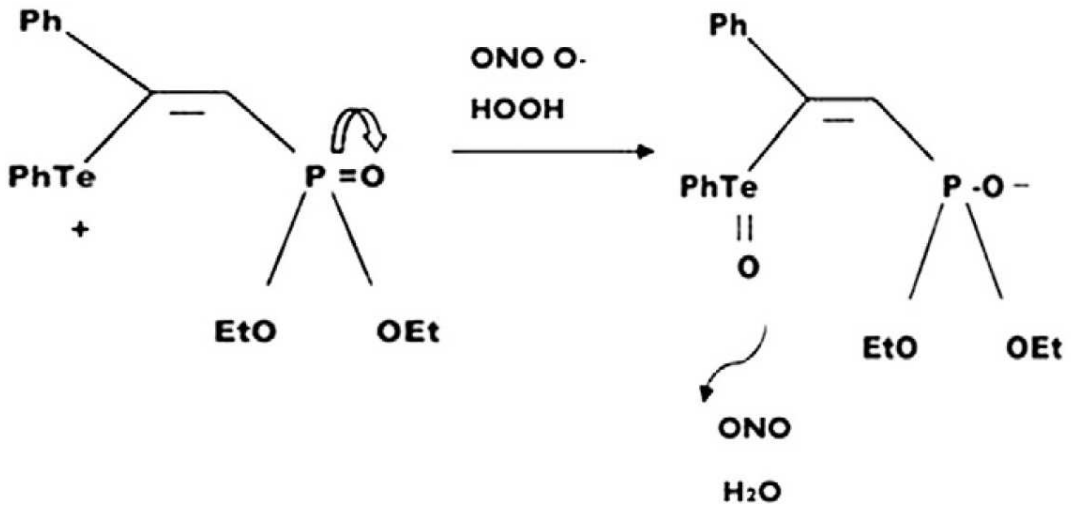


Figura 5 – Hipótese do mecanismo de ação da atividade *scavenger* do DPTPV contra $ONOO^-$ e H_2O_2. (Figura extraída de Ávila et al. 2011)

6. CONCLUSÕES

De acordo com os resultados apresentados nesta tese, podemos inferir que:

- O guaraná:
  - Diminui a quantidade de dienos conjugados de indivíduos idosos que consomem guaraná comparado aos indivíduos que não o consomem;
  - Em baixas concentrações (1-5 µg/mL) ele pode suprimir a produção de dienos conjugados e TBARS, previne a destruição do triptofano e tem alta atividade *scavenger* de radicais *in vitro*;
  - Esses efeitos foram relacionados principalmente com as altas quantidades de compostos fenólicos;
- O salicilaldeído-4-feniltiosemicarbazona:
  - Protege a LDL contra oxidação induzida por Cu^{2+} e AAPH *in vitro* em concentrações tão baixas quanto 1 µM;
  - Previne a oxidação da porção lipídica e protéica da LDL;
  - Possui alta atividade *scavenger* de radicais e pode prevenir a formação de TBARS induzida por nitroprussiato de sódio em diferentes tecidos de ratos e por Cu^{2+} em LDL e soro humano;
  - Esses efeitos foram atribuídos em parte à atividade quelante do composto e a capacidade *scavenger* de radicais;
- O 2-fenil-2-telurofenil vinilfosfonato:
  - Previne a oxidação de LDL e soro humano contra a oxidação induzida por Cu^{2+} e AAPH;
  - Previne a oxidação da porção lipídica e protéica da LDL;
  - Apresenta uma boa atividade *scavenger* de radicais e previne a produção de TBARS em LDL e soro humano;
  - Não apresenta toxicidade em células de artérias de ratos *in vitro*;
  - Esses efeitos foram atribuídos à combinação da alta capacidade *scavenger* de radicais e ao fato do composto possivelmente interferir com a ligação dos íons Cu^{2+} com a porção protéica (apoB-100) da LDL;

Considerando todos os resultados apresentados nesta tese, podemos concluir que, apesar dos resultados serem bastante preliminares, o guaraná, o SPTS e o

DPTVP têm grande potencial antioxidante, principalmente na prevenção de modificações oxidativas em lipoproteínas de baixa densidade. Dentre os três compostos, o guaraná é o que apresenta estudos mais avançados, sendo que já foi demonstrado não apresentar toxicidade alguma para humanos. Sendo assim, o guaraná é o mais promissor destes compostos, podendo ser usado para estudos *in vivo* com humanos. Considerando os três compostos, precisa-se de estudos adicionais para verificar os efeitos destes na prevenção da aterogênese em modelos de animais, bem como outros possíveis efeitos com potencial antiaterogênico.

7. PERSPECTIVAS

De acordo com os resultados apresentados nesta tese, poderíamos avançar mais os estudos com relação aos mecanismos antioxidantes, efeitos toxicológicos e antiaterogênicos em animais. Para isso, poderíamos realizar estudos com os seguintes objetivos:

- Avaliar os efeitos toxicológicos e a farmacodinâmica do SPTS e DPTVP em animais, administrados em diferentes vias;
- Investigar os efeitos dos compostos na oxidação de LDL frente a outros agentes oxidantes importantes para o processo aterogênico, como NADPH oxidase, mieloperoxidase, lipoxigenase e peroxinitrito;
- Verificar a propriedade dos compostos de inibirem a formação de células espumosas em culturas de macrófagos, bem como os mecanismos que isso pode ser afetado;
- Avaliar o efeito dos compostos sobre as células endoteliais quanto à influência na expressão de moléculas de adesão, na liberação de fatores inflamatórios e na alteração de enzimas, como a óxido nítrico sintase e as metaloproteinases;
- Verificar a capacidade dos compostos na alteração dos níveis de colesterol e triglicerídeos, assim como o processo aterogênico em animais;
- Verificar a atividade dos compostos isolados do extrato de guaraná em parâmetros que podem influenciar no processo aterogênico.

8. REFERÊNCIAS BIBLIOGRÁFICAS

Andersson, C.M., et al. Diaryl tellurides as inhibitors of lipid peroxidation in biological and chemical systems. **Free Radic Res**, V. 20, n. 6, p. 401-410, 1994.

Angelo, P.C., et al. Guarana (Paullinia cupana var. sorbilis), an anciently consumed stimulant from the Amazon rain forest: the seeded-fruit transcriptome. **Plant Cell Rep,** V. 27, n. 1, p. 117-124, 2008.

Ascherio, A., Willett, W.C. Are body iron stores related to the risk of coronary heart disease? **N Engl J Med**, V. 330, n. 16, p. 1152-1154, 1994.

Avila, D.S., et al. A biochemical and toxicological study with diethyl 2-phenyl-2-tellurophenyl vinylphosphonate in a sub-chronic intraperitoneal treatment in mice. **Life Sci**, V. 80, n. 20, p. 1865-1872, 2007.

Avila, D.S., et al. An organotellurium compound with antioxidant activity against excitotoxic agents without neurotoxic effects in brain of rats. **Brain Res Bull**, V. 76, n. 1-2, p. 114-123, 2008.

Avila, D.S., et al. Hepatoprotective activity of a vinylic telluride against acute exposure to acetaminophen. **Eur J Pharmacol**, V. 661, n. 1-3, p. 92-101, 2011.

Barcelos, R.P., et al. Thiosemicarbazone derivate protects from AAPH and Cu2+ - induced LDL oxidation. **Life Sci**, V. 89, n. 1-2, p. 20-28, 2011.

Basile, A., et al. Antibacterial and antioxidant activities of ethanol extract from Paullinia cupana Mart. **J Ethnopharmacol,** V. 102, n. 1, p. 32-36, 2005.

Belliardo, F., Martelli, A., Valle, M.G. HPLC determination of caffeine and theophylline in Paullinia cupana Kunth (guarana) and Cola spp. samples. **Z Lebensm Unters Forsch**, V. 180, n. 5, p. 398-401, 1985.

Beraldo, H., Gambino, D. The wide pharmacological versatility of semicarbazones, thiosemicarba-zones and their metal complexes. **Mini Rev Med Chem,** V. 4, n. 1, p. 31-39, 2004.

Berube-Parent, S., et al. Effects of encapsulated green tea and Guarana extracts containing a mixture of epigallocatechin-3-gallate and caffeine on 24 h energy expenditure and fat oxidation in men. **Br J Nutr**, V. 94, n. 3, p. 432-436, 2005.

Boozer, C.N., et al. An herbal supplement containing Ma Huang-Guarana for weight loss: a randomized, double-blind trial. **Int J Obes Relat Metab Disord**, V. 25, n. 3, p. 316-324, 2001.

Braga, A.L., et al. Synthesis of telluroamino acid derivatives with remarkable GPx like activity. **Org Biomol Chem**, V. 7, n. 1, p. 43-45, 2009.

Briviba, K., et al. Protection by organotellurium compounds against peroxynitrite-mediated oxidation and nitration reactions. **Biochem Pharmacol**, V. 55, n. 6, p. 817-823, 1998.

Brown, M.S., Goldstein, J.L. Koch's postulates for cholesterol. **Cell**, V. 71, n. 2, p. 187-188, 1992.

Brown, M.S., Goldstein, J.L. Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. **Annu Rev Biochem,** V. 52, n. p. 223-261, 1983.

Bydlowski, S.P., D'Amico, E.A., Chamone, D.A. An aqueous extract of guarana (Paullinia cupana) decreases platelet thromboxane synthesis. **Braz J Med Biol Res,** V. 24, n. 4, p. 421-424, 1991.

Bydlowski, S.P., Yunker, R.L., Subbiah, M.T. A novel property of an aqueous guarana extract (Paullinia cupana): inhibition of platelet aggregation in vitro and in vivo. **Braz J Med Biol Res,** V. 21, n. 3, p. 535-538, 1988.

Campos, A.R., et al. Acute effects of guarana (Paullinia cupana Mart.) on mouse behaviour in forced swimming and open field tests. **Phytother Res,** V. 19, n. 5, p. 441-443, 2005.

Cherubini, A., et al. Role of antioxidants in atherosclerosis: epidemiological and clinical update. **Curr Pharm Des,** V. 11, n. 16, p. 2017-2032, 2005.

Chisolm, G.M., 3rd, et al. The oxidation of lipoproteins by monocytes-macrophages. Biochemical and biological mechanisms. **J Biol Chem**, V. 274, n. 37, p. 25959-25962, 1999.

Costa Krewer, C., et al. Habitual Intake of Guarana and Metabolic Morbidities: An Epidemiological Study of an Elderly Amazonian Population. **Phytother Res**, V. n. 2011.

Cushing, S.D., et al. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. **Proc Natl Acad Sci U S A,** V. 87, n. 13, p. 5134-5138, 1990.

da Fonseca, C.A., et al. Genotoxic and mutagenic effects of guarana (Paullinia cupana) in prokaryotic organisms. **Mutat Res,** V. 321, n. 3, p. 165-173, 1994.

DeConti, R.C., et al. Clinical and pharmacological studies with 5-hydroxy-2-formylpyridine thiosemicarbazone. **Cancer Res,** V. 32, n. 7, p. 1455-1462, 1972.

Ehara, S., et al. Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes. **Circulation,** V. 103, n. 15, p. 1955-1960, 2001.

Ehrenwald, E., Chisolm, G.M., Fox, P.L. Intact human ceruloplasmin oxidatively modifies low density lipoprotein. **J Clin Invest**, V. 93, n. 4, p. 1493-1501, 1994.

Espinola, E.B., et al. Pharmacological activity of Guarana (Paullinia cupana Mart.) in laboratory animals. **J Ethnopharmacol,** V. 55, n. 3, p. 223-229, 1997.

Fox, P.L., et al. Ceruloplasmin and cardiovascular disease. **Free Radic Biol Med,** V. 28, n. 12, p. 1735-1744, 2000.

French, F.A., Blanz, E.J., Jr. The carcinostatic activity of alpha-(N)-heterocyclic carboxaldehyde thiosemicarbazones. II. 3-Hydroxypyridine-2-carboxaldehyde thiosemicarbazone. **Cancer Res,** V. 26, n. 8, p. 1638-1640, 1966.

Fukumasu, H., et al. Chemopreventive effects of Paullinia cupana Mart var. sorbilis, the guarana, on mouse hepatocarcinogenesis. **Cancer Lett,** V. 233, n. 1, p. 158-164, 2006.

Gaut, J.P., Heinecke, J.W. Mechanisms for oxidizing low-density lipoprotein. Insights from patterns of oxidation products in the artery wall and from mouse models of atherosclerosis. **Trends Cardiovasc Med**, V. 11, n. 3-4, p. 103-112, 2001.

Gey, K.F., Brubacher, G.B., Stahelin, H.B. Plasma levels of antioxidant vitamins in relation to ischemic heart disease and cancer. **Am J Clin Nutr,** V. 45, n. 5 Suppl, p. 1368-1377, 1987a.

Gey, K.F., et al. Inverse correlation between plasma vitamin E and mortality from ischemic heart disease in cross-cultural epidemiology. **Am J Clin Nutr,** V. 53, n. 1 Suppl, p. 326S-334S, 1991.

Gey, K.F., et al. Relationship of plasma level of vitamin C to mortality from ischemic heart disease. **Ann N Y Acad Sci,** V. 498, n. p. 110-123, 1987b.

Gey, K.F., Puska, P. Plasma vitamins E and A inversely correlated to mortality from ischemic heart disease in cross-cultural epidemiology. **Ann N Y Acad Sci,** V. 570, n. p. 268-282, 1989.

Gokce, N., et al. Long-term ascorbic acid administration reverses endothelial vasomotor dysfunction in patients with coronary artery disease. **Circulation**, V. 99, n. 25, p. 3234-3240, 1999.

Haberland, M.E., Fong, D., Cheng, L. Malondialdehyde-altered protein occurs in atheroma of Watanabe heritable hyperlipidemic rabbits. **Science**, V. 241, n. 4862, p. 215-218, 1988.

Heinecke, J.W. Oxidants and antioxidants in the pathogenesis of atherosclerosis: implications for the oxidized low density lipoprotein hypothesis. **Atherosclerosis**, V. 141, n. 1, p. 1-15, 1998.

Heinecke, J.W., Rosen, H., Chait, A. Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. **J Clin Invest**, V. 74, n. 5, p. 1890-1894, 1984.

Hessler, J.R., et al. Lipoprotein oxidation and lipoprotein-induced cytotoxicity. **Arteriosclerosis,** V. 3, n. 3, p. 215-222, 1983.

Horkko, S., et al. Immunological responses to oxidized LDL. **Free Radic Biol Med,** V. 28, n. 12, p. 1771-1779, 2000.

Huang, H., et al. A series of alpha-heterocyclic carboxaldehyde thiosemicarbazones inhibit topoisomerase IIalpha catalytic activity. **J Med Chem**, V. 53, n. 8, p. 3048-3064, 2010.

Hulthe, J., Fagerberg, B. Circulating oxidized LDL is associated with subclinical atherosclerosis development and inflammatory cytokines (AIR Study). **Arterioscler Thromb Vasc Biol,** V. 22, n. 7, p. 1162-1167, 2002.

Ishitani, L.H., et al. Desigualdade social e mortalidade precoce por doenças cardiovasculares no Brasil. **Rev Saúde Pública,** V. 40, n. 4, p. 684-691, 2006.

Itabe, H. Oxidized low-density lipoproteins: what is understood and what remains to be clarified. **Biol Pharm Bull,** V. 26, n. 1, p. 1-9, 2003.

Jimoh, F.O., Sofidiya, M.O., Afolayan, A.J. Antioxidant properties of the methanol extracts from the leaves of Paullinia pinnata. **J Med Food,** V. 10, n. 4, p. 707-711, 2007.

Kalinowski, D.S., Richardson, D.R. The evolution of iron chelators for the treatment of iron overload disease and cancer. **Pharmacol Rev,** V. 57, n. 4, p. 547-583, 2005.

Kardinaal, A.F., et al. Antioxidants in adipose tissue and risk of myocardial infarction: the EURAMIC Study. **Lancet,** V. 342, n. 8884, p. 1379-1384, 1993.

Keaney, J.F., Jr. Atherosclerosis: from lesion formation to plaque activation and endothelial dysfunction. **Mol Aspects Med,** V. 21, n. 4-5, p. 99-166, 2000.

Kennedy, D.O., et al. Improved cognitive performance in human volunteers following administration of guarana (Paullinia cupana) extract: comparison and interaction with Panax ginseng. **Pharmacol Biochem Behav,** V. 79, n. 3, p. 401-411, 2004.

Khan, B.V., et al. Modified low density lipoprotein and its constituents augment cytokine-activated vascular cell adhesion molecule-1 gene expression in human vascular endothelial cells. **J Clin Invest,** V. 95, n. 3, p. 1262-1270, 1995.

Kohlmeier, L., et al. Lycopene and myocardial infarction risk in the EURAMIC Study. **Am J Epidemiol,** V. 146, n. 8, p. 618-626, 1997.

Krakoff, I.H., et al. Clinical trial of 5-hydroxypicolinaldehyde thiosemicarbazone (5-HP; NSC-107392), with special reference to its iron-chelating properties. **Cancer Chemother Rep,** V. 58, n. 2, p. 207-212, 1974.

Leite, R.P., et al. Protective effect of Guarana (Paullinia cupana var. sorbilis) pre-treatment on cadmium-induced damages in adult wistar testis. **Biol Trace Elem Res,** V. 141, n. 1-3, p. 262-274, 2011.

Levitan, I., Volkov, S., Subbaiah, P.V. Oxidized LDL: diversity, patterns of recognition, and pathophysiology. **Antioxid Redox Signal,** V. 13, n. 1, p. 39-75, 2010.

Libby, P. Inflammation in atherosclerosis. **Nature,** V. 420, n. 6917, p. 868-874, 2002.

Lima, W.P., et al. Lipid metabolism in trained rats: effect of guarana (Paullinia cupana Mart.) supplementation. **Clin Nutr**, V. 24, n. 6, p. 1019-1028, 2005.

Mashima, R., Witting, P.K., Stocker, R. Oxidants and antioxidants in atherosclerosis. **Curr Opin Lipidol,** V. 12, n. 4, p. 411-418, 2001.

Matsuura, E., et al. Oxidative modification of low-density lipoprotein and immune regulation of atherosclerosis. **Prog Lipid Res,** V. 45, n. 6, p. 466-486, 2006.

Mattei, R., et al. Guarana (Paullinia cupana): toxic behavioral effects in laboratory animals and antioxidants activity in vitro. **J Ethnopharmacol,** V. 60, n. 2, p. 111-116, 1998.

Metso, S., et al. Circulating oxidized low-density lipoprotein and common carotid artery intima-media thickness in a random sample of middle-aged men. **J Biomed Sci,** V. 11, n. 3, p. 356-361, 2004.

Meyers, D.G. The iron hypothesis--does iron cause atherosclerosis? **Clin Cardiol,** V. 19, n. 12, p. 925-929, 1996.

Miller, M., Hutchins, G.M. Hemochromatosis, multiorgan hemosiderosis, and coronary artery disease. **JAMA**, V. 272, n. 3, p. 231-233, 1994.

Morel, D.W., DiCorleto, P.E., Chisolm, G.M. Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation. **Arteriosclerosis**, V. 4, n. 4, p. 357-364, 1984.

Navab, M., et al. The Yin and Yang of oxidation in the development of the fatty streak. A review based on the 1994 George Lyman Duff Memorial Lecture. **Arterioscler Thromb Vasc Biol,** V. 16, n. 7, p. 831-842, 1996.

Nishi, K., et al. Oxidized LDL in carotid plaques and plasma associates with plaque instability. **Arterioscler Thromb Vasc Biol,** V. 22, n. 10, p. 1649-1654, 2002.

Nogueira, C.W., Zeni, G., Rocha, J.B. Organoselenium and organotellurium compounds: toxicology and pharmacology. **Chem Rev**, V. 104, n. 12, p. 6255-6285, 2004.

Oliveira, G.M.M., Klein, C.H., Souza e Silva, N.A. Mortalidade por doenças cardiovasculares em três estados do Brasil de 1980 a 2002. **Rev Panam Salud Publica,** V. 19, n. 2, p. 85–93, 2006.

Opala, T., et al. Efficacy of 12 weeks supplementation of a botanical extract-based weight loss formula on body weight, body composition and blood chemistry in healthy, overweight subjects--a randomised double-blind placebo-controlled clinical trial. **Eur J Med Res,** V. 11, n. 8, p. 343-350, 2006.

Osterud, B., Bjorklid, E. Role of monocytes in atherogenesis. **Physiol Rev,** V. 83, n. 4, p. 1069-1112, 2003.

Otobone, F.J., et al. Effect of lyophilized extracts from guarana seeds [Paullinia cupana var. sorbilis (Mart.) Ducke] on behavioral profiles in rats. **Phytother Res,** V. 21, n. 6, p. 531-535, 2007.

Palinski, W., et al. Low density lipoprotein undergoes oxidative modification in vivo. **Proc Natl Acad Sci U S A,** V. 86, n. 4, p. 1372-1376, 1989.

Pandeya, S.N., et al. Synthesis, antibacterial, antifungal and anti-HIV activities of Schiff and Mannich bases derived from isatin derivatives and N-[4-(4'-chlorophenyl)thiazol-2-yl] thiosemicarbazide. **Eur J Pharm Sci,** V. 9, n. 1, p. 25-31, 1999.

Papanastasiou, I., et al. Design, synthesis, and trypanocidal activity of new aminoadamantane derivatives. **J Med Chem**, V. 51, n. 5, p. 1496-1500, 2008.

Parthasarathy, S., et al. Oxidants and antioxidants in atherogenesis. An appraisal. **J Lipid Res,** V. 40, n. 12, p. 2143-2157, 1999.

Patel, R.P., et al. Mechanisms of the pro- and anti-oxidant actions of nitric oxide in atherosclerosis. **Cardiovasc Res,** V. 47, n. 3, p. 465-474, 2000.

Pedrido, R., et al. Pentadentate thiosemicarbazones as versatile chelating systems. A comparative structural study of their metallic complexes. **Dalton Trans,** V. n. 47, p. 6776-6787, 2008.

Pinheiro, C.E., et al. [Effect of guarana and Stevia Rebaudiana Bertoni (leaves) extracts, and stevioside, on the fermentation and synthesis of extracellular insoluble polysaccharides of dental plaque]. **Rev Odontol Univ Sao Paulo,** V. 1, n. 4, p. 9-13, 1987.

Prathima, B., et al. Copper(II) and nickel(II) complexes of benzyloxybenzaldehyde-4-phenyl-3-thiosemicarbazone: Synthesis, characterization and biological activity. **Spectrochim Acta A Mol Biomol Spectrosc**, V. 77, n. 1, p. 248-252, 2010.

Raja, D.S., et al. Effect of terminal N-substitution in 2-oxo-1,2-dihydroquinoline-3-carbaldehyde thiosemicarbazones on the mode of coordination, structure, interaction with protein, radical scavenging and cytotoxic activity of copper(II) complexes. **Dalton Trans**, V. 40, n. 17, p. 4548-4559, 2011.

Rimm, E.B., et al. Vitamin E consumption and the risk of coronary heart disease in men. **N Engl J Med,** V. 328, n. 20, p. 1450-1456, 1993.

Rosenfeld, M.E., et al. Distribution of oxidation specific lipid-protein adducts and apolipoprotein B in atherosclerotic lesions of varying severity from WHHL rabbits. **Arteriosclerosis**, V. 10, n. 3, p. 336-349, 1990.

Sartorelli, A.C., Booth, B.A. Inhibition of the growth of sarcoma 180 ascites cells by combinations of inhibitors of nucleic acid biosynthesis and the cupric chelate

of kethoxal bis-(thiosemicarbazone). **Cancer Res,** V. 27, n. 9, p. 1614-1619, 1967.

Scovill, J.P., Klayman, D.L., Franchino, C.F. 2-Acetylpyridine thiosemicarbazones. 4. Complexes with transition metals as antimalarial and antileukemic agents. **J Med Chem**, V. 25, n. 10, p. 1261-1264, 1982.

Smith, N., Atroch, A.L. Guarana's Journey from Regional Tonic to Aphrodisiac and Global Energy Drink. **Evid Based Complement Alternat Med**, V. 7, n. 3, p. 279-282, 2010.

Smith, S.C., Jr., et al. Atherosclerotic Vascular Disease Conference: Writing Group II: risk factors. **Circulation,** V. 109, n. 21, p. 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,** V. 34, n. 6, p. 550-555, 1989.

Sriram, D., et al. Discovery of new antitubercular oxazolyl thiosemicarbazones. **J Med Chem**, V. 49, n. 12, p. 3448-3450, 2006.

Stadler, N., Lindner, R.A., Davies, M.J. Direct detection and quantification of transition metal ions in human atherosclerotic plaques: evidence for the presence of elevated levels of iron and copper. **Arterioscler Thromb Vasc Biol**, V. 24, n. 5, p. 949-954, 2004.

Stampfer, M.J., et al. Vitamin E consumption and the risk of coronary disease in women. **N Engl J Med,** V. 328, n. 20, p. 1444-1449, 1993.

Steinberg, D. Low density lipoprotein oxidation and its pathobiological significance. **J Biol Chem,** V. 272, n. 34, p. 20963-20966, 1997.

Steinberg, D., Witztum, J.L. Is the oxidative modification hypothesis relevant to human atherosclerosis? Do the antioxidant trials conducted to date refute the hypothesis? **Circulation,** V. 105, n. 17, p. 2107-2111, 2002.

Steinberg, D., Witztum, J.L., Lipoproteins, lipoprotein oxidation, and atherogenesis., in: Chien, K.R., (Ed.), Molecular Basis of Cardiovascular Disease., W.B. Saunders Co., **Philadelphia**, 1999, pp. 458–475.

Steinbrecher, U.P., et al. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. **Proc Natl Acad Sci U S A**, V. 81, n. 12, p. 3883-3887, 1984.

Stocker, R., Keaney, J.F. New insights on oxidative stress in the artery wall. **Journal of Thrombosis and Haemostasis,** V. 3, n. 8, p. 1825-1834, 2005.

Stocker, R., Keaney, J.F., Jr. Role of oxidative modifications in atherosclerosis. **Physiol Rev,** V. 84, n. 4, p. 1381-1478, 2004.

Street, D.A., et al. Serum antioxidants and myocardial infarction. Are low levels of carotenoids and alpha-tocopherol risk factors for myocardial infarction? **Circulation,** V. 90, n. 3, p. 1154-1161, 1994.

Takei, A., Huang, Y., Lopes-Virella, M.F. Expression of adhesion molecules by human endothelial cells exposed to oxidized low density lipoprotein. Influences of degree of oxidation and location of oxidized LDL. **Atherosclerosis,** V. 154, n. 1, p. 79-86, 2001.

Thomson, M.J., Puntmann, V., Kaski, J.C. Atherosclerosis and oxidant stress: the end of the road for antioxidant vitamin treatment? **Cardiovasc Drugs Ther**, V. 21, n. 3, p. 195-210, 2007.

Van Lenten, B.J., et al. Lipid-induced changes in intracellular iron homeostasis in vitro and in vivo. **J Clin Invest**, V. 95, n. 5, p. 2104-2110, 1995.

Verlangieri, A.J., et al. Fruit and vegetable consumption and cardiovascular mortality. **Med Hypotheses,** V. 16, n. 1, p. 7-15, 1985.

Villa-Colinayo, V., et al. Genetics of atherosclerosis: the search for genes acting at the level of the vessel wall. **Curr Atheroscler Rep,** V. 2, n. 5, p. 380-389, 2000.

Vivekananthan, D.P., et al. Use of antioxidant vitamins for the prevention of cardiovascular disease: meta-analysis of randomised trials. **Lancet**, V. 361, n. 9374, p. 2017-2023, 2003.

Wallenfeldt, K., et al. Oxidized low-density lipoprotein in plasma is a prognostic marker of subclinical atherosclerosis development in clinically healthy men. **J Intern Med,** V. 256, n. 5, p. 413-420, 2004.

Watanabe, K., et al. Preventive effects of probucol on restenosis after percutaneous transluminal coronary angioplasty. **Am Heart J**, V. 132, n. 1 Pt 1, p. 23-29, 1996.

Wen, Y., Leake, D.S. Low density lipoprotein undergoes oxidation within lysosomes in cells. **Circ Res,** V. 100, n. 9, p. 1337-1343, 2007.

Wiseman, S.A., Balentine, D.A., Frei, B. Antioxidants in tea. **Crit Rev Food Sci Nutr,** V. 37, n. 8, p. 705-718, 1997.

Witztum, J.L., Steinberg, D. Role of oxidized low density lipoprotein in atherogenesis. **J Clin Invest**, V. 88, n. 6, p. 1785-1792, 1991.

Witztum, J.L., Steinberg, D. The oxidative modification hypothesis of atherosclerosis: does it hold for humans? **Trends Cardiovasc Med**, V. 11, n. 3-4, p. 93-102, 2001.

Yamaguti-Sasaki, E., et al. Antioxidant capacity and in vitro prevention of dental plaque formation by extracts and condensed tannins of Paullinia cupana. **Molecules,** V. 12, n. 8, p. 1950-1963, 2007.

Yla-Herttuala, S., et al. Lipoproteins in normal and atherosclerotic aorta. **Eur Heart J,** V. 11 Suppl E, n. p. 88-99, 1990.

Yoshida, H., Kisugi, R. Mechanisms of LDL oxidation. **Clin Chim Acta**, V. 411, n. 23-24, p. 1875-1882, 2010.

Yu, Y., et al. Thiosemicarbazones from the old to new: iron chelators that are more than just ribonucleotide reductase inhibitors. **J Med Chem**, V. 52, n. 17, p. 5271-5294, 2009.

Zhong, Z., et al. The preparation and antioxidant activity of 2-[phenylhydrazine (or hydrazine)-thiosemicarbazone]-chitosan. **Int J Biol Macromol**, V. 47, n. 2, p. 93-97, 2010.