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AÇÃO PROTETORA DA *Carya illinoensis* SOBRE A TOXICIDADE INDUZIDA POR CICLOFOSFAMIDA EM RATOS

DISSERTAÇÃO DE MESTRADO

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Santa Maria, RS, Brasil
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AÇÃO PROTETORA DA *Carya illinoensis* SOBRE A TOXICIDADE INDUZIDA POR CICLOFOSFAMIDA EM RATOS

por

Dalila Moter Benvegnú

Dissertação apresentada ao Programa de Pós-Graduação em Farmacologia, Área de Concentração em Neuropsicofarmacologia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Farmacologia.**

Orientador: Profª. Drª. Marilise Escobar Bürger

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elaborada por
Dalila Moter Benvegnú

como requisito parcial para obtenção do grau de
Mestre em Farmacologia

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"Pode-se vencer pela inteligência,

pela habilidade ou pela sorte,

mas nunca sem trabalho."

(A. Destoef)

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Farmacologia
Universidade Federal de Santa Maria, RS, Brasil

AÇÃO PROTETORA DA *Carya illinoensis* SOBRE A TOXICIDADE INDUZIDA POR CICLOFOSFAMIDA EM RATOS

Autora: Dalila Moter Benvegnú
Orientadora: Marilise Escobar Bürger
Data e Local da Defesa: Santa Maria, 29 de janeiro de 2010

A ciclofosfamida (CP) é um agente alquilante muito usado na terapia anticâncer e imunossupressora. Assim como outros quimioterápicos, este fármaco pode causar danos aos tecidos saudáveis, gerando toxicidade em múltiplos órgãos. Desta forma, o estudo de substâncias naturais capazes de prevenir ou minimizar a toxicidade decorrente do tratamento quimioterápico vem ganhando sua importância. A casca da noz pecã (*Carya illinoensis*), é um sub-produto de origem vegetal de baixo custo e com elevado potencial antioxidante, constituindo uma substância promissora. O objetivo deste estudo foi verificar se o extrato aquoso bruto do vegetal citado apresenta efeito protetor contra os danos teciduais múltiplos induzidos pela CP. Ratos Wistar receberam água potável ou extrato aquoso bruto (EAB – 5%) da casca da noz pecã, *ad libitum,* no lugar da água de beber até o final do experimento. No trigésimo dia, a metade de cada grupo recebeu uma administração única de veículo ou CP 200 mg/kg-ip. Após 7 dias, os animais foram sacrificados por exsanguinação, para obtenção de plasma e eritrócitos, enquanto alguns tecidos como coração, fígado, rim, testículos e bexiga foram removidos. Parâmetros de estresse oxidativo (EO) foram avaliados através da peroxidação lipídica (LPO), medida por meio da formação de espécies reativas ao ácido tiobarbitúrico (TBARS) e glutationa reduzida (GSH) em todos os tecidos avaliados, bem como a enzima catalase (CAT) no coração, fígado, rim e testículos. A enzima superóxido dismutase (SOD) testicular e os níveis plasmáticos de vitamina C (VIT C) também foram determinados. A função testicular foi determinada através dos níveis da enzima lactato desidrogenase (LDH). A bexiga foi submetida à avaliação macroscópica e histopatológica, já que é um tecido intensamente danificado pela CP. Os resultados obtidos demonstraram que o tratamento com CP aumentou os níveis de TBARS e diminuiu os níveis de GSH em todos os tecidos avaliados. A atividade da SOD testicular apresentou-se aumentada, bem como a atividade da CAT no coração. Nos demais órgãos, a atividade desta última enzima encontrou-se diminuída. Os níveis da enzima LDH testicular e os níveis plasmáticos de VIT C também se apresentaram reduzidos. Por último, as bexigas dos ratos tratados com CP mostraram coloração escura e diversas alterações histológicas, tais como: espessamento das camadas, hemorragia, edema, infiltração leucocitária e proliferação vascular. O co-tratamento com EAB foi capaz de prevenir o aumento do TBARS e diminuição do GSH em todos os tecidos, exceto coração e plasma, respectivamente. O extrato também preveniu modificações na atividade da SOD testicular e da CAT em todos os órgãos, exceto no rim. Além disso, o extrato também preveniu as alterações nos níveis de

VIT C plasmática e LDH testicular, bem como as modificações macroscópicas e histopatológicas na bexiga dos ratos tratados com CP. Os resultados apresentados aqui evidenciam os efeitos protetores do EAB das casca da noz pecã sobre a toxicidade generalizada da CP. Deste modo, este sub-produto da indústria da noz pode ser considerado para minimizar os efeitos indesejáveis relacionados ao tratamento com CP, melhorando a qualidade de vida dos pacientes que necessitam deste quimioterápico.

Palavras-chave: ciclofosfamida, *Carya illinoensis,* noz pecã, estresse oxidativo, antioxidante.

ABSTRACT

Master Dissertation
Graduate Program in Pharmacology
Federal University of Santa Maria, RS, Brasil

ACTION OF *Carya illinoensis* ON THE CYCLOPHOSPHAMIDE INDUCED TOXICITY IN RATS

Author: Dalila Moter Benvegnú
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Date and place of defense: January 29th, 2010, Santa Maria

Cyclophosphamide (CP) is an alkylating agent widely used in anticancer and immunosuppression therapy. Like other chemotherapies, this drug can induce damage in healthy tissues, generating multiple organ toxicity. The study of natural substances able to prevent or reduce the toxicity induced by the chemotherapy is very important. Pecan nut shell (*Carya illinoensis)* is a vegetal by-product with low cost and elevated antioxidant potential and therefore can be considered a promissory substance. The aim of this study was to verify whether the cited antioxidant protects against multiple tissue damage induced by CP. Wistar rats received water or pecan shell aqueous extract (AE - 5%) *ad libitum* in place of drinking water up to the end of the experiment. On day 30, half of each group received a single administration of vehicle or CP 200 mg/kg-ip. After 7 days, the animals were sacrificed by exsanguination. The blood was collected to obtain plasma and erythrocytes. Vital tissues as heart, liver, kidney, testis and bladder were removed. The occurrence of oxidative stress (OS) was observed through the determination of lipid peroxidation (LP), measured by the thiobarbituric acid reactive substances (TBARS) formation and reduced glutathione (GSH) in all removed tissues, the enzyme catalase (CAT) in the heart, liver, kidney and testis, the enzyme superoxide dismutase (SOD) in the testis and the plasmatic vitamin C (VIT C) levels. Furthermore, the testicular function was evaluated by the enzyme lactate deydrogenase (LDH) and the macroscopic and histopathological analysis of the bladder was carried out. Results demonstrated that the CP treatment increased TBARS and decreased GSH levels in all studied tissues. Moreover, the activities of SOD in the testis and CAT in the heart were increased. However, in the other organs, the activity of the latter was diminished. The testicular LDH and plasmatic VIT C levels were also decreased. Finally, the bladder of the CP treated rats showed dark color and various histological alterations, such as thickening of the lines, hemorrhage, edema, leukocyte infiltration and vascular proliferation. The co-treatment with AE was able to prevent the TBARS increase and GSH depletion in all tissues, except heart and plasma, respectively. The extract also prevented changes in the testicular SOD and CAT activity in all organs, except the kidney. Besides, the extract prevented alteration in plasmatic VIT C and testicular LDH as well as various visual and histological changes in the rat bladder. These findings provided important evidences of the protective effect of pecan shell AE against the general organic toxicity induced by CP. Therefore, this natural substance may be

considered to minimize adverse effects related with this chemotherapy, improving the life quality of patients who need to use this type of drug.

Key-words: cyclophosphamide, *Carya illinoensis,* pecan nut, oxidative stress, antioxidant.

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LISTA DE ABREVIATURAS

CAT – catalase
CP – ciclofosfamida
EAB – extrato aquoso bruto
EO – estresse oxidativo
EROs – espécies reativas de oxigênio
GPx – glutationa peroxidase
GSH – glutationa reduzida
GSSG – glutationa oxidada
LPO – peroxidação lipídica ou lipoperoxidação
MDA – malondialdeído
RL – radicais livres
SOD – superóxido dismutase
TBA – ácido tiobarbitúrico
TBARS – substâncias reativas ao ácido tiobarbitúrico
VIT C – vitamina C

SUMÁRIO

APRESENTAÇÃO

Esta dissertação apresenta os resultados na forma de manuscritos, os quais se encontram no ítem **MANUSCRITOS CIENTÍFICOS**. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas encontram-se nos próprios manuscritos e representam a íntegra deste estudo.

Ao fim desta dissertação encontram-se os ítens **DISCUSSÃO** e **CONCLUSÕES**, nos quais há interpretações e comentários gerais sobre os manuscritos científicos contidos neste estudo.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO, REVISÃO BIBLIOGRÁFICA, DISCUSSÃO** e **CONCLUSÕES** desta dissertação.

1 INTRODUÇÃO

A ciclofosfamida (CP) é um agente alquilante amplamente utilizado para o tratamento de vários tipos de câncer, atuando ainda como fármaco imunossupressor para transplante de órgãos, artrite reumatóide, lúpus eritematoso sistêmico, esclerose múltipla, entre outros (PERINI et al., 2007). A CP é considerada um pró-fármaco, visto que necessita sofrer ativação hepática para gerar os metabólitos ativos: mostarda fosforamida e acroleína (SLADEK, 1971; LUDEMAN, 1999). Os efeitos antineoplásicos são associados à mostarda fosforamida, enquanto que a acroleína é a responsável pelos efeitos tóxicos (KUMAR et al., 2006).

A maior limitação da quimioterapia do câncer é a injúria que ocorre no tecido normal, conduzindo a uma toxicidade orgânica múltipla (FRAISER et al., 1991; BUKOWSKI, 1999). Já está bem documentado que o tratamento com altas doses terapêuticas de CP gera toxicidade em vários órgãos, tais como, coração, fígado, rins e testículos (MYTHILI et al., 2004; 2006; 2007; SELVAKUMAR et al. 2004; 2005a; 2005b; 2006; SENTHILKUMAR et al., 2006a; 2006b; SUGUMAR & ABRAHAM, 2007). Entretanto, a toxicidade urológica, evidenciada através do quadro da cistite hemorrágica é o maior fator limitante para o seu uso (LEVINE & RICHIE, 1989).

O mecanismo de toxicidade da CP ainda é pouco compreendido. No entanto, alguns estudos relacionam esta toxicidade ao desenvolvimento do estresse oxidativo (EO), principalmente devido à geração de espécies reativas de oxigênio (EROs) (HAQUE et al., 2001, DAS et al., 2002 GHOSH et al., 2002). Na presença de dano induzido pela CP é observado um aumento da LPO e uma depleção de moléculas antioxidantes, incluindo GSH, CAT e SOD (MYTHILI et al., 2004; SELVAKUMAR et al., 2005a; 2005b; 2006)**.**

Como a faixa de seletividade dos agentes quimioterápicos é bem estreita, durante o tratamento oncológico é essencial a citoproteção de tecidos não injuriados (KLEIN & MUGGIA, 1999). Neste sentido, estudos clínicos mostraram que o co-tratamento de pacientes com quimioterápicos padrões e antioxidantes tornou esses mais resistentes ao tratamento, tendo um tempo de sobrevivência prolongado em

relação aos pacientes tratados somente com quimioterápicos (JAAKKOLA et al., 1992; SINGH & LIPPMAN, 1998).

Atualmente existe um grande interesse na pesquisa de compostos naturais que possam minimizar a toxicidade de antineoplásicos em células saudáveis, sem comprometer sua atividade antitumoral. Desta forma, o estudo etnofarmacológico auxilia na busca de novos produtos, com custos reduzidos e menor incidência de efeitos colaterais, contribuindo para amenizar várias doenças relacionadas à geração de EROs e ao desenvolvimento do EO.

E dentro da etnofarmacologia está a *Carya illinoensis.* Essa planta pertencente à família Junglandaceae é originária do sul dos Estados Unidos e norte do México, posteriormente alcançando a América do Sul (HANCOCK, 1997). Essa árvore é conhecida popularmente como nogueira e seu fruto como noz pecã. As nozes são fontes de ácidos graxos monoinsaturados e apresentam uma elevada capacidade antioxidante, pelo fato de possuirem alto teor de compostos fenólicos e taninos condensados (VILLARREAL-LOZOYA et al., 2007).

O processamento da noz pecã resulta em 40-50% de cascas, subproduto de cor avermelhada intensa e de difícil degradação. Recentemente foi demonstrado por Villarreal-Lozoya et al. (2007) que a casca apresenta níveis maiores de compostos antioxidantes e taninos condensados que a própria noz, apresentando dessa forma, um potencial antioxidante promissor. Popularmente, o uso do chá da casca da noz pecã é muito difundido para o tratamento de diferentes problemas de saúde, incluindo patologias relacionadas ao EO e como depurativo do sangue. Entretanto, ainda não existem relatos científicos que comprovam os efeitos descritos popularmente.

Devido ao amplo uso da CP na quimioterapia do câncer e da imunossupressão, (DOLLERY, 1999) e pela sua elevada toxicidade capaz de desencadear efeitos deletérios em diferentes tecidos e órgãos (BUKOWSKI, 1999), existe a necessidade da busca de terapias adicionais que possam ser associadas a este fármaco, reduzindo sua toxicidade. Além disso, como a casca da noz pecã é um produto natural acessível, de baixo custo e com elevado potencial antioxidante, estudos de seu papel protetor e antioxidante se fazem necessários.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar os possíveis benefícios do extrato aquoso bruto (EAB) da casca da noz pecã sobre os efeitos tóxicos induzidos pelo quimioterápico ciclofosfamida (CP) em ratos.

2.2 Objetivos específicos

- Avaliar os diferentes tecidos de ratos tratados com CP e/ou EAB em relação a indicadores de estresse oxidativo, tais como:

- determinação dos níveis de peroxidação lipídica no fígado, rim, coração, testículos, plasma e eritrócitos;

- verificação dos níveis de glutationa reduzida no fígado, rim, coração, testículos, plasma e eritrócitos;

- avaliação da atividade da enzima antioxidante catalase no fígado, rim, coração e testículos;

- avaliação da atividade da enzima antioxidante superóxido dismutase nos testículos;

- determinação dos níveis plasmáticos de vitamina C.

- Monitorar a função testicular dos mesmos ratos através da verificação dos níveis da enzima lactato desidrogenase testicular.

- Avaliar a morfologia da bexiga dos referidos animais por meio de:

- análise macroscópica com auxílio de uma escala de dano;

- análise histopatológica.

3 REVISÃO BIBLIOGRÁFICA

3.1 Ciclofosfamida

3.1.1 Aspectos Gerais

A mostarda alquilante ciclofosfamida (CP), ou 2-[bis(2-cloroetil)amino]-2-oxo-1,3,2-oxazafosforinana, é um composto organofosforado amplamente usado como agente antineoplásico. Desde sua descoberta, em 1958, este quimioterápico tem se tornado o mais utilizado na classe dos agentes alquilantes (DOS SANTOS et al., 2007). O mecanismo de ação proposto para este agente citotóxico é a sua interferência na replicação do DNA e na transcrição do RNA, resultando em interrupção da função do ácido nucléico (KALLAMA & HEMMINKI, 1986).

Devido as suas propriedades anticâncer e imunossupressora este fármaco passou a ter um amplo espectro de usos clínicos. Desta forma o seu uso vai desde doenças neoplásicas até transplantes de órgãos e diversas desordens não-malignas (DOLLERY, 1999). No que diz respeito a tumores malignos o quimioterápico tem sua utilização em leucemias agudas e crônicas, mieloma múltiplo, linfomas, tumores de células germinativas, meduloblastomas, carcinomas de mama, pulmão e cérvice (WANG et al., 2007). Além disso, atua também em outras doenças não malignas como a artrite reumatóide, a vasculite e ainda, na preparação para o transplante de medula óssea (GOLDBERG et al., 1986; DOLLERY, 1999) .

A CP é um pró-fármaco, não apresentando desta forma atividade contra células cancerosas in vitro (WANG et al., 2007). Ela necessita chegar ao sistema microssomal hepático citocromo P450 oxidase de função mista para ativar seus metabólitos que passam a entrar na circulação. Os metabólitos gerados são a 4-hidroxiciclofosfamida e a aldofosfamida (Figura 1). Nas células tumorais a aldofosfamida sofre clivagem espontânea, levando a formação da mostarda fosforamida e da acroleína. (HARDMAN & LIMBIRD, 2003). Os efeitos

antineoplásicos são associados à mostarda fosforamida, enquanto que a acroleína é a responsável pelos efeitos tóxicos (KUMAR et al., 2006). A acroleína é um aldeído α-β insaturado, altamente eletrofílico, capaz de inibir a proliferação celular e induzir apoptose devido à geração de espécies reativas de oxigênio (EROs) (ALLISON, 2000). Além disso, também é relatada a sua atividade mutagênica em células de mamíferos, bem como a interferência no sistema de defesa antioxidante (ARUMUGAM et al., 1997).

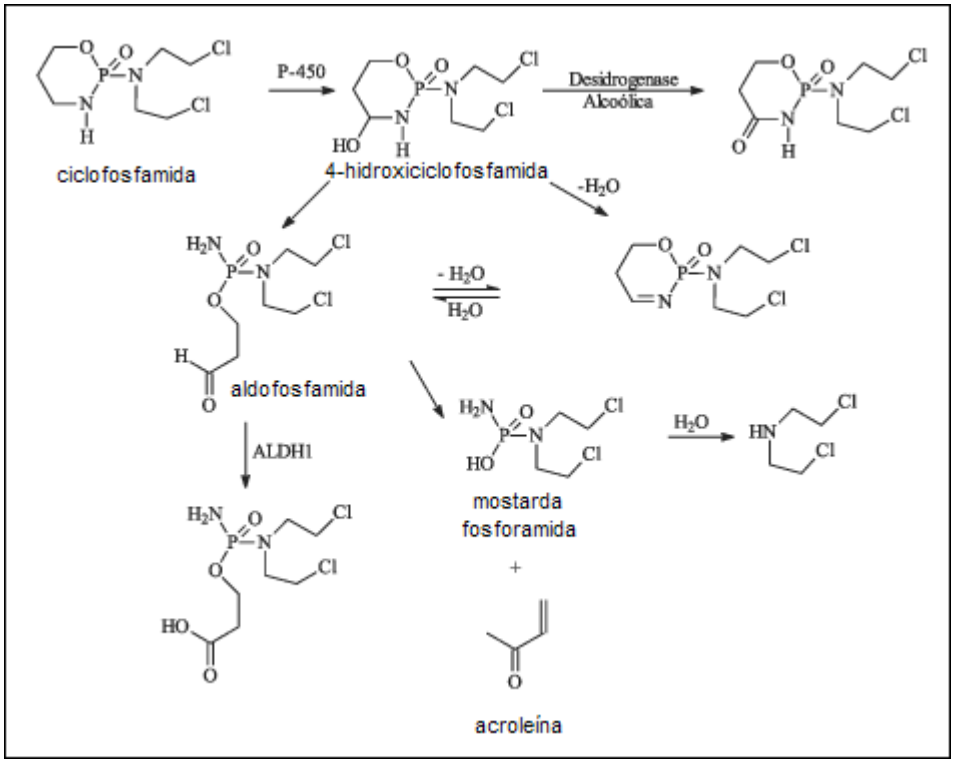

Figura 1. Mecanismo de degradação oxidativa da ciclofosfamida no DNA para formação de metabólitos reativos (adaptado de dos Santos et al., 2007).

3.1.2 Efeitos Colaterais

A maior limitação da quimioterapia do câncer é a injúria que ocorre no tecido normal, conduzindo a uma toxicidade orgânica múltipla (FRAISER et al., 1991; BUKOWSKI, 1999). Com o uso da CP foi documentada uma variedade de efeitos colaterais, os quais incluem náusea, vômito, ulceração da mucosa gástrica, fibrose

pulmonar, toxicidade hepática e renal, linfocitopenia, depressão hematopoiética, alopecia, dano cardíaco, cistite hemorrágica, antidiurese, toxicidade reprodutiva e teratogenicidade (FLEMING, 1997).

Doses terapêuticas altas de CP poderiam causar uma cardiotoxicidade letal que é uma combinação de sintomas e sinais de miopericardite, o que leva a complicações fatais, tais como, insuficiência cardíaca congestiva, arritmias, e depressão miocárdica. Os efeitos cardiotóxicos agudos são caracterizados morfologicamente por necrose e hemorragia, ocorrendo tardiamente o desenvolvimento de fibrose (MYTHILI et al., 2004). Alterações primárias e/ou secundárias no metabolismo lipídico em diferentes rotas e sob várias condições conduzem ao acúmulo de lipídios no miocárdio e à cardiomiopatia lipotóxica (MYTHILI et al., 2006).

Os efeitos colaterais no aparelho urinário são o maior fator de limitação para o uso deste agente citotóxico. A cistite hemorrágica é um efeito colateral frequentemente observado, estando presente em 75% dos pacientes tratados com o fármaco. Os sintomas relacionados aos danos urinários são relatados como edema da mucosa, hemorragia, ulceração, fibrose, necrose e refluxo urinário (LEVINE & RITCHIE, 1989).

Além disso, a ativação hepática da CP ao produzir os metabólitos tóxicos pode gerar danos nos tecidos renais e hepáticos (ABRAHAM & SUGUMAR, 2008). A nefrotoxicidade pode ser evidenciada por danos nos túbulos proximais e uma significante diminuição da condutância renal (KLETA, 1996; ROSSI, 1997). Já as desordens hepáticas são observadas quando há um maior aumento na dose terapêutica da CP (SRIOVER, 1989).

Em relação à área reprodutiva, sabe-se que o fármaco descrito induz alterações na função testicular, promovendo infertilidade através de oligospermia, azoospermia e atrofia tubular seminífera (CAMARGO, et al., 2006). Na mulher pode causar defeitos congênitos, fibrose ovariana e supressão da menstruação (DOS SANTOS et al., 2007). Além disso, já está bem documentado a teratogenicidade da CP, através de estudos que demonstram má formação fetal em camundongos, ratos, hamsters, coelhos e humanos (UJHAZY et al., 1993; SHAH et al., 1996; WEXLER, 1998; KANG et al., 2003; KIM et al., 2003). Alguns estudos em roedores mostraram retardo de crescimento, anomalias múltiplas, incluindo anencefalia e defeitos nos membros e esqueleto (CHAHOUD et al., 2002; KANG et al., 2003; KIM et al., 2003).

O mecanismo de patogênese da CP ainda não está totalmente elucidado. Entretanto, a causa pode ser devido ao seu metabólito acroleína, o qual interfere com os antioxidantes dos tecidos, além de produzir um aumento das EROs, culminando assim com o desenvolvimento do EO (KUMAR et al., 2006). Desta forma, o EO desempenha um papel fundamental no dano tecidual induzido pela CP (HAQUE et al., 2003; MANDA & BHATIA, 2003).

3.2 Espécies Reativas de Oxigênio

Radicais livres (RL) são espécies químicas altamente reativas que contêm um ou mais elétrons não-pareados em sua órbita de valência (HALLIWELL & GUTTERIDGE, 1999). São produzidos naturalmente em nosso organismo através de processos metabólicos oxidativos e, muitas vezes, de extrema utilidade em situações em que há necessidade de ativação do sistema imune; na desintoxicação de drogas; e na produção de óxido nítrico (SCHNEIDER & OLIVEIRA, 2004). As reações de oxidação são essenciais no metabolismo normal dos organismos aeróbicos, principalmente porque o oxigênio atua como aceptor do último elétron no sistema de fluxo de elétrons, sendo responsável pela geração de energia via fosforilação oxidativa (STOREY, 1996). O oxigênio é utilizado pela enzima mitocondrial citocromo oxidase em um processo de redução tetravalente que resulta na formação de água (FRIDOVICH, 1986). Estima-se que em torno de 98% do oxigênio consumido em organismos aeróbios seja reduzido desta forma, sem a geração paralela de RL (CHANCE et al., 1979). Entretanto, sob condições normais, uma pequena quantidade de oxigênio pode ser reduzida por um, dois ou três elétrons somente, gerando o radical superóxido ($O_2^{-\bullet}$), peróxido de hidrogênio (H_2O_2) e o radical hidroxil ($OH^{\bullet}$), respectivamente (CADET & KAHLER, 1994). Estas substâncias, junto ao oxigênio singlete são denominadas EROs, diferindo do oxigênio no seu estado fundamental e caracterizando-se pela acentuada reatividade e habilidade em promover mudanças oxidativas no interior celular (PRYOR, 1986).

As EROs são bem conhecidas por desempenharem um papel duplo como espécies deletérias e benéficas. Os efeitos benéficos das EROS ocorrem em concentrações baixas a moderadas e envolvem papéis fisiológicos nas respostas

celulares, como por exemplo, na defesa contra agentes infecciosos, no funcionamento de diversas vias de sinalização celular, e na indução de resposta mitogênica (VALKO et al., 2007). Em contraste, em altas concentrações as EROs podem danificar membranas ou outras estruturas lipídicas celulares, ocasionar modificações nas proteínas, alterando a estrutura terciária e provocando a perda de função, fragmentação e ligações cruzadas; e no DNA induzir alterações, que podem ser reparadas pelos mecanismos de reparo ou induzir mutações (HALLIWELL & GUTTERIDGE, 1999). A extensão e os tipos de danos causados pelas EROs depende tanto da sua quantidade como da qualidade ou natureza, bem como das defesas antioxidantes celulares (DAVIES, 1991).

O ânion superóxido é uma espécie menos reativa, pois ele perde a habilidade de penetrar as membranas lipídicas e permanece no compartimento onde foi produzido. Diferentemente, a molécula de peróxido de hidrogênio é altamente capaz de penetrar as membranas biológicas assim como o radical hidroxil que possui forte reatividade com biomoléculas (NORDBERG & ARNÉR, 2001). O radical hidroxil é o mais reativo pelo fato da busca imediata de sua estabilidade. Desta forma, este radical transforma as moléculas circundantes em radicais, que, por sua vez, também precisam estabilizar-se. E é esta sequência de eventos que origina reações em cadeia com os constituintes celulares (HALLIWELL & GUTTERIDGE, 1999).

3.3 Defesas Antioxidantes

Existem distintas estratégias celulares de defesa contra as substâncias pró-oxidantes e essas podem ser representadas por três categorias: defesas preventivas, interceptativas e de reparo. As defesas preventivas impedem a formação das EROs por meios físicos ou bioquímicos. Já as interceptativas atuam impedindo que as EROs danifiquem as diversas estruturas celulares. E por último, estão as defesas de reparo que são capazes de restaurar danos que já tenham ocorrido (reparo do DNA, modificações no “turnover” de lipídios, proteólise) (SIES, 1993).

Os mecanismos de defesa antioxidante são encarregados de manter baixas as concentrações das EROs e para isso, atuam prevenindo a formação dessas

espécies ou capturando-as, uma vez que se formaram. O sistema de defesa antioxidante enzimático inclui as enzimas superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx) (Figura 2).

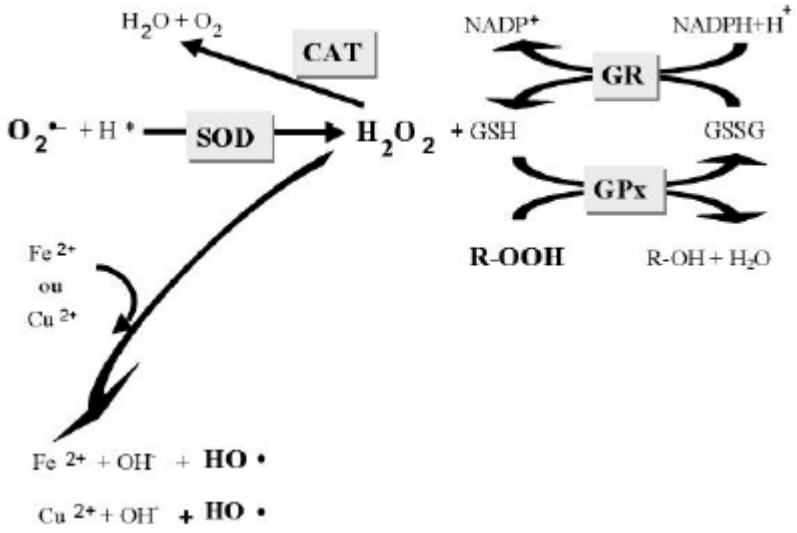

Figura 2. Via de formação de espécies pró-oxidantes e vias de detoxificação pelo sistema de defesa antioxidante (adaptado de Cravo, 2006).

A SOD corresponde a uma família de enzimas com diferentes grupos prostéticos em sua composição e atua sobre o radical superóxido convertendo-o em peróxido de hidrogênio. Nos sistemas eucariontes existem duas formas de SOD: uma forma citosólica, SOD-Cu/Zn, que contém o íon cobre no seu sítio ativo, o qual participa da reação, e o íon zinco, que tem função de estabilização molecular; e outra localizada primariamente na mitocôndria, a SOD-Mn, que possui o íon manganês em seu sítio ativo, o qual participa na reação de dismutação (FERREIRA & MATSUBARA, 1997). Além dessas, existe a SOD extracelular que também contém cobre e zinco e está ligada à superfície das células (HALLIWELL & GUTTERIDGE, 1999).

A GPx juntamente com a CAT catalisam a transformação de peróxido de hidrogênio em água e oxigênio (LOHR et al., 2003). A CAT facilita a remoção do peróxido de hidrogênio, metabolizando-o em oxigênio molecular e água; já a GPx catalisa o metabolismo de um grande número de hidroperóxidos orgânicos, além de converter o peróxido de hidrogênio a água, envolvendo a oxidação concomitante da

glutationa reduzida (GSH) para sua forma oxidada (GSSG). A GPx pode estar localizada no citosol, bem como na matriz mitocondrial, ao passo que a CAT está geralmente restrita aos peroxissomos (HALLIWELL & GUTTERIDGE, 1999). Muitos estudos sugerem que a CAT é mais efetiva quando as concentrações intracelulares de peróxido de hidrogênio são muito elevadas. De maneira oposta, pequenos aumentos no peróxido de hidrogênio parecem ser mais bem controlados pela GPx (HERMES-LIMA, 2004).

O sistema não enzimático por sua vez, inclui compostos sintetizados no organismo como bilirrubina, ceruloplasmina, hormônios sexuais, melatonina, coenzima Q, ácido úrico ou ainda substâncias que podem ser provenientes da dieta (SCHNEIDER & OLIVEIRA, 2004). Esse sistema ainda pode ser dividido em componentes lipossolúveis e hidrossolúveis.

No grupo dos componentes lipossolúveis merece destaque o α-tocoferol (vitamina E), cuja atividade antioxidante deve-se principalmente a sua capacidade de capturar radicais peroxil, alquil e alcoxil, e de modo importante o oxigênio singlete, protegendo as membranas celulares (BURTON et al., 1983). Dentro desse grupo também estão os carotenóides, substâncias que atuam como capturadores principalmente de lipoperóxidos, tendo importância principal o β-caroteno, que é precursor da vitamina A (KRINSKY, 1989).

Em relação aos antioxidantes hidrossolúveis são citados os compostos fenólicos, o ácido ascórbico e a glutationa. Os flavonóides são derivados de plantas e constituem os principais compostos fenólicos com ação antioxidante (HANASAKI, 1994). O ácido ascórbico (vitamina C) é um capturador de radicais de amplo espectro, desempenhando uma função essencial no compartimento aquoso (PACKER et al., 1979). Ainda, é considerado um dos antioxidantes mais importantes em tecidos de mamíferos (BANHEGYI et al., 1997), protegendo os lipídios plasmáticos contra a peroxidação e regenerando o α-tocoferol (PADH, 2005). Outra defesa fundamental é a glutationa reduzida (GSH), complexo que apresenta um potencial regulador do estado redox celular (YUAN et al., 1991). Além disso, desempenha um importante papel na homeostase celular (VALKO et al., 2007), estando envolvida em determinados processos de sinalização celular, como a apoptose (SIES, 1999) e também ajudando na regeneração de outros antioxidantes como as vitaminas E e C (MEISTER, 1994). O complexo GSH possui grupos

sulfidrílicos funcionais que atuam na manutenção desses mesmos grupos presentes em outras moléculas, detoxificando desta forma os xenobióticos (GUL et al., 2000).

3.4 Estresse Oxidativo

Halliwell e Gutteridge (1999) definiram o EO como o desequilíbrio no balanço entre agentes pró-oxidantes e agentes antioxidantes com a potencialidade de exercer efeitos deletérios. Na condição do EO as EROs podem estar aumentadas sem o concomitante aumento das defesas; as proteções podem estar reduzidas sem o aumento das EROs; ou a situação mais crítica, onde o aumento da concentração das EROs vem acompanhado de uma redução paralela das defesas correspondentes (SIES, 1986; AMSTAD & CERUTTI, 1990).

Dependendo da extensão, o EO pode levar à morte celular por apoptose, pois as EROs como o ânion superóxido e o radical hidroxil exercem seus efeitos tóxicos por gerarem a lipoperoxidação, a oxidação de carboidratos e proteínas (HALLIWELL, 1994) e os defeitos no DNA (SIMONIAN & COYLE, 1996).

O interesse relativo nos danos ocasionados pelo EO cresceu enormemente durante as últimas décadas, principalmente pelas evidências ou suspeitas vinculando as EROs a diversas patologias humanas (JENKINS & GOLDFARB, 1993). Destacam-se especialmente aquelas relacionadas com o sistema imunológico, aparelho respiratório, cardiovascular, neurológico e da visão, ademais de processos ligados à carcinogênese e ao processo de envelhecimento (JI, 1993; HALLIWELL & GUTERIDGE, 1999).

3.4.1 Peroxidação Lipídica

O ataque dos lipídios das membranas celulares pelas EROs recebe o nome de peroxidação lipídica ou lipoperoxidação (LPO). Este processo pode levar à modificação nos lipídios, que consequentemente levará à perda das características das membranas biológicas, tornando-as menos firmes e flexíveis e criando fendas

iônicas que alteram sua permeabilidade, favorecendo o trânsito indiscriminado de metabólitos e detritos celulares, provocando sua ruptura e lise (JOSEPHY, 1997; TIMBRELL, 2000).

A LPO pode ser iniciada por qualquer radical livre primário que tenha reatividade suficiente para extrair um átomo de hidrogênio de um grupo metileno reativo de um ácido graxo insaturado. Na primeira fase do processo de LPO, as cadeias altamente vulneráveis dos ácidos graxos poliinsaturados são atacadas por RL formando hidroperóxidos lipídicos. Estes hidroperóxidos lipídicos são moléculas razoavelmente estáveis sob condições fisiológicas, mas sua decomposição é catalisada por metais de transição formando radicais alcoxil e peroxil. O radical alcoxil é análogo altamente reativo do radical hidroxil e pode propagar as reações de LPO (JANERO, 1990).

A intensidade da peroxidação lipídica pode ser avaliada de acordo com os níveis dos produtos primários ou ainda com os produtos finais da peroxidação, como por exemplo, o malondialdeído (MDA) que é ensaiado com o ácido tiobarbitúrico (TBA) e expresso em substâncias reativas ao ácido tiobarbitúrico (TBARS) (RICE-EVANS et al., 1991; HALLIWELL & GUTTERIDGE, 1999). Assim, a reação do MDA com o TBA é um dos marcadores de EO mais utilizados (LIU et al., 1997).

3.5 *Carya illinoensis*

Carya illinoensis (Wangenh) K. Koch é uma árvore pertencente à família Juglandaceae, nativa do Sul dos Estados Unidos e norte do México, que teve a sua distribuição expandida até a América do Sul (HANCOCK, 1997). Essa planta é popularmente conhecida como nogueira e o seu produto como noz pecã. Nas regiões sul e sudeste do Brasil, encontram-se extensas plantações de nogueiras introduzidas no país pelos imigrantes norte-americanos (JOLY, 1993). Essa árvore apresenta folhas caducas e pode atingir grande porte, superando 40 m de altura (HANCOCK, 1997).

A própria composição da noz pecã foi um estímulo para o aumento do seu consumo, visto que apresenta proteínas, ácidos graxos monoinsaturados, fibras, esteróis e micronutrientes como tocoferol (RAJARAM et al., 2001). Em um estudo

realizado por Wu et al. (2004) onde foram analisados diversos alimentos e vegetais comuns nos Estados Unidos, a noz pecã mostrou a maior capacidade antioxidante e conteúdo fenólico dentro do grupo das nozes, ingressando entre alimentos com maior conteúdo fenólico. Os polifenóis são bem reconhecidos pelas suas propriedades antioxidantes decorrentes do mecanismo de redução da geração de ânion superóxido (ROBAK & GRYGLEWSKI, 1988), radicais hidroxil (HUSAIN et al., 1987) e peroxil (TOREL et al., 1986). Aqui, merece destaque o ácido gálico, polifenol presente em abundância na noz. Estudos recentes mostram que essa substância apresenta efeitos terapêuticos promissores (LU et al., 2006), principalmente por sua atividade como sequestrador de RL (SAWA et al., 1999) preservando desta forma a viabilidade celular (LI et al., 2000). Proantocianidinas e taninos condensados também estão relatados na noz pecã (POLLES et al., 1981). Esses compostos apresentam atividades biológicas devido as suas propriedades antioxidantes e antimutagênicas (GRIMMER et al., 1992). De acordo com Polles et al. (1981), a concentração de taninos em pecãs está na faixa de 0.699 a 1.71%.

Desta forma, as nozes fornecem um método de dieta potencial no combate aos danos oxidativos, reduzindo o risco de doenças crônicas, por sua capacidade antioxidante (KRIS-ETHERTON et al., 2001). Além disso, estudos epidemiológicos e clínicos demostraram que as nozes, incluindo a pecã, foram capazes de reduzir os níveis de colesterol sanguíneo, diminuindo assim a incidência de doenças cardiovasculares. Ainda, que esses efeitos se devem ao menos em parte pela proteção antioxidante fornecida (TOROBIAN et al., 2009).

A casca da noz pecã, que corresponde a 40%-50% de toda a castanha (WORLEY, 1994), é um subproduto de cor avermelhada intensa, de difícil degradação e que pode constituir uma fonte alternativa de compostos com alta capacidade antioxidante (VILLARREAL-LOYOZA et al, 2007). Estudos recentes da casca demonstraram que o seu conteúdo de compostos fenólicos totais e taninos condensados foi superior em relação ao conteúdo encontrado na própria noz (VILLARREAL-LOZOYA et al, 2007), o que a torna uma nova fonte antioxidante. Desse modo, esse subproduto passou a ser utilizado popularmente sob a forma de infusão (chá) para o tratamento de vários distúrbios. O uso do mesmo tem demonstrado utilidade no tratamento de diversos estados patológicos, geralmente relacionados à produção de EROs e EO, tal como alguns tipos de câncer e de doenças crônicas como Alzheimer, Parkinson e outras desordens

neurodegenerativas. Ainda, como depurativo sanguíneo, utilizado para reduzir até mesmo a toxicidade decorrente da nicotina. No entanto, esses dados são baseados no conhecimento empírico, não havendo ainda estudos documentados que comprovam esses usos populares.

Tendo em vista que o EO está relacionado a diversas patologias e à ação de vários xenobióticos, dentre eles a ciclofosfamida, a busca por novas fontes antioxidantes capazes de prevenir ou minimizar esse quadro se faz necessária. Assim sendo, a casca da noz pecã surge como uma alternativa promissora, já que possui elevada atividade antioxidante e já é utilizada popularmente, sob a forma de chá, como detoxificante. Ademais, é uma substância natural, de fácil acesso e baixo custo.

4 MANUSCRITOS CIENTÍFICOS

Os resultados inseridos nesta dissertação apresentam-se sob a forma de manuscritos científicos, os quais se encontram aqui estruturados. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios manuscritos. Os manuscritos estão dispostos da mesma maneira que foram submetidos.

Manuscrito 1

4.1 Extrato aquoso da casca da noz pecã [(*Carya illinoensis*) Wangenh.) K. Koch] exerce proteção contra o dano oxidativo induzido pela ciclofosfamida em testículos de ratos.

Aqueous extract of pecan nut shell [(*Carya illinoensis*) Wangenh.) K. Koch] exerts protection against the oxidative damage induced by cyclophosphamide in rat testis

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Aqueous extract of pecan nut shell [(*Carya illinoensis*) (Wangenh.) K. Koch] exerts protection against the oxidative damage induced by cyclophosphamide in rat testis

Short Title: Benefits of pecan shells on cyclophosphamide-induced testis damage

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ABSTRACT

Cyclophosphamide (CP) is a chemotherapy that exerts toxic effects on the reproductive system. The present study investigated the protective effect of pecan nut (*Carya illinoensis)* shell aqueous extract (AE) on lipid peroxidation (LP) and antioxidant defenses in testis of rats CP-treated. Wistar rats received water or AE (5%) *ad libitum* in the place of drinking water up to the end of the experiment. On day 30, half of each group received a single administration of vehicle or CP 200 mg/kg-ip. After 7 days, the animals were sacrificed and the testis removed. Rats treated with CP had loss of body and testis weight, which was not prevented by AE. The CP reduced the catalase (CAT) activity and lactate dehydrogenase (LDH) and gluthatione (GSH) levels, while LP levels and superoxide dismutase (SOD) activity were increased. In contrast, co-treatment with pecan shell AE totally prevented the decrease of LDH levels and CAT activity and partially prevented the depletion of GSH levels. Besides, it totally prevented the increase in SOD activity and LP levels. These findings showed the protective role of pecan shell AE in the testicular toxicity CP-induced and the use of this phytotherapy may be considered to minimize deleterious effects related to this chemotherapy.

1. Introduction

Cyclophosphamide (CP) is an alkylating agent extensively used for the treatment of cancers as well as an immunosuppressive drug for organ transplantation, rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis, among others[1]. Both therapeutic and toxic effects of CP require metabolic activation by P450 hepatic microsomal cytochrome, generating phosphoramide mustard and acrolein. Has been described that acrolein is mutagenic to mammalian cells, interferes in the antioxidant defense system, and produces cellular toxicity by the generation of reactive oxygen species (ROS) [2]. In this sense, a wide range of side effects has been related to CP treatment, including reproductive toxicity in humans and experimental animals. Of particular importance, CP treatment in patients has been associated with oligozoospermia/azoospermia, disturbance in gonadotropin secretion, testicular damage as well as the low blood level of testosterone and antioxidant defenses [3].

Experimentally, the use of this chemotherapy has showed biochemical and histological alterations in testis and epididymis of rats, decrease of reproductive organ weight, and impaired fertility [4]. Furthermore, Velez de la Calle et al. observed anatomical and functional abnormalities in testis of rats treated with CP, including disruption of the seminiferous tubular structure, tubular atrophy, vacuolization of Sertoli cells, reduction in the population of primary spermatocytes, and immature spermatids [5].

The cellular mechanisms by which CP causes testicular injury are poorly understood; however, numerous studies have shown that CP is associated with the induction of oxidative stress (OS) by the generation of free radicals and ROS [6].

Under normal physiological conditions, ROS are generated in testicular sub cellular compartments, particularly in the mitochondria, being subsequently scavenged by the antioxidant defense system of the corresponding cellular compartments [7]. Furthermore, the mitochondrial membrane is rich in polyunsaturated fatty acids, contains low amounts of antioxidants, and therefore is more susceptible to lipid peroxidation (LP). Currently, there is an increasing interest in searching natural products capable of minimizing the toxicity of the chemotherapy to healthy cells without compromising its antineoplasic activity. In this sense, foods or plants, traditionally used, are an important source of new products, with lower costs and low incidence of undesirable side effects contributing in alleviating various diseases related to ROS generation.

Carya illinonensis (Wangenh) K. Koch (Juglandaceae) is a tree native to the southern United States and northern Mexico, but its distribution reaches South America as well. This specie is popularly known as pecan, whose nuts are rich sources of monounsaturated fatty acids stimulating their consumption [8]. Pecan has the highest antioxidant capacity and total extractable phenolic content within the nut group [9]. With this feature, it has ranked pecans among the foods with highest phenolic content [10]. It was recently demonstrated that pecan shells have higher levels of total phenolics and condensed tannins than kernels [9] and these phytochemical compounds are related to antioxidant properties. Pecan processing results in a great amount (40-50%) of shells (the hard cover that surrounds the kernel) that represent an alternative source of antioxidant compounds [9]. Popularly, the pecan shells are used in tea form as depurative of blood, useful to reduce the toxicity consequent of tobacco nicotine and of pharmacotherapy. In fact, this popular use of pecan shells is an empirical knowledge, and therefore is not documented. Further researches are

needed to confirm and validate its medicinal use. Experimentally, several laboratories have studied the effect of some natural compounds on the OS caused by anticancer drugs [11], particularly the CP [2,6]. Considering the close relation between the CP-induced toxicity and the development of OS, this study was performed to evaluate the effects of pecan shell aqueous extract on the CP-induced oxidative damage and the antioxidant status in testis of rats.

2. Materials and methods

2.1. Drugs and chemicals

Cyclophosphamide (Genuxal®, Asta Médica, Brazil) was donated by 'Hospital Universitário' from the 'Universidade Federal de Santa Maria' (HUSM), RS, Brazil and was dissolved in distilled water just before use.

The raw material was kindly donated by Pecantea, a pecan nut company. Voucher specimens of *C. illinoensis* were deposit at the Departamento de Ciências Biológicas Herbarium (SMDB-11.528), UFSM, Santa Maria, RS, Brazil. This vegetal specie came from commercial crops and not from natural sources, thus ensuring the respect for vegetal biodiversity.

2.2. Preparation of pecan shell aqueous extract

Shells of *C. illinoensis* were let overnight at 40°C in a hot air oven and fine powdered. The aqueous extract (AE) of the shells was freshly prepared by infusion (5%, 90°C), filtered using filter paper and cooled to room temperature. During this procedure, the extract was protected from light. The fresh extract was daily offered to the animals (*ad libitum*).

The determination of the dry weight was also carried out by gravimetry and the yield obtained was 5.4%.

2.3. Phytochemical characterization

The extract of pecan shells was standardized through of characterization of total phenolic compounds (TP) content by colorimetric method according to Budini et al. [12], and condensed tannin (CT) content in accordance with Villarreal-Lozoya et al. [9], which showed high levels of total phenolic compound (192.36 mg of gallic acid equivalent/g) and condensed tannins (58.43±2.25 mg catechin equivalent/ g). Determination of antioxidant capacity (AC) *in vitro* with pecan shell AE were conducted by ABTS assay [13], whose results showed 2218.8±2.5 µmol of TEAC (Trolox Equivalent Antioxidant Capacity)/g and by DPPH assay [14], whose results showed 529.4±7.6mg and 794.1±11.4mg of Trolox equivalent/g, through of reaction for 30min and 24h, respectively. These analyses were preliminarily assayed in our laboratory, and provided support for the continuation of *in vivo* studies performed here.

Further qualitative analysis of pecan shell AE were performed by high performance liquid chromatography (HPLC). HPLC system (Shimadzu Corporation, Kyoto, Japan) was equipped with two Shimadzu LC-10 ATVP reciprocating pumps, a variable UV-VIS detector (Shimadzu SPD-10 AVP) and a Winchrom integrator (Winchrom). Reverse phase chromatographic analysis was carried out in isocratic conditions using RP C-18 HPLC column (250 x 4.6 mm id, particle size 5 µm, Luna 5µ C-18 (2), Phenomenex, USA) at 25°C. Running conditions included injection volume: 20 µl, mobile phase: methanol:water (80:20, v/v), 0.4 % acetic acid, flow rate: 1 ml/min and detection at 290 nm. Samples were filtered through organic solvent compatible membrane filters (Pore size 0.20 µm, Millipore) prior to injection in

sample loop. Gallic acid was used as standard. The content of gallic acid was calculated by comparing peak area of reference compound with those in the samples run under similar elution conditions.

The major constituent of the pecan shell AE, represented here, was gallic acid (GA), corresponding to 1.8g/100g of AE (Figure 1 and 2).

2.4. Animals

Wistar adult male rats (330±40g) were kept in Plexiglas cages with free access to food and water in a room with controlled temperature (22–23°C) and on a 12 h-light/dark cycle with lights on at 7:00 a.m. The animals were maintained and used in accordance to the rules of the Committee on Care and Use of Experimental Animal of the Federal University of Santa Maria, RS, Brazil, as well in accordance to the guide for the care and use of laboratory animals (NIH Publication No. 80-23; revised 1978).

2.5. Experimental protocol

CP-induced testis toxicity: After 2 weeks of acclimatization, 28 rats were randomly allocated into four experimental groups (n=7), designated as control (C), cyclophosphamide-treated (CP), pecan nut shell aqueous extract (AE) and extract plus CP-treated (AE+CP). The fresh aqueous extract of pecan shells (AE and AE+CP groups) or tap water (C and CP groups) was daily offered to animals. The rats received the pecan shell AE *ad libitum*, similarly to tea, which is popularly used to treat different diseases. Since no data have been found in the literature, the choice of the extract concentration was based on previous studies performed in our laboratory, which showed that acute administration of the extract presents antioxidant properties. After 30 days of treatment with pecan shell AE or vehicle, the groups designated as CP and AE+CP received a single dose of CP (200 mg/kg body weight-i.p.), while C

and AE groups were injected with vehicle (distilled water) and maintained with the oral treatment (pecan shell AE or vehicle) *ad libitum* for another 7 days. After the sacrifice, the testis were removed and homogenized in 10 volumes (10mL/g tissue) of 10mM Tris-HCl buffer (pH−7.4). The homogenates were centrifuged at 5000 rpm for 20 min and the supernatants were used for the assays.

2.6. Lipid peroxidation (LP)

LP in the testis tissue was measured by estimating the formation of thiobarbituric acid reactive substances (TBARS), in accordance with Ohkawa et al.[15]. Tissue supernatant (10% w/v) was boiled (100ºC) with TBA (0.67%) in acid medium for 60 min, cooled and centrifuged. The absorbance of the supernatant was read at 535nm. Results were expressed as nmol MDA (malondialdehyde)/g tissue.

2.7. Estimation of antioxidants

Reduced gluthatione (GSH) levels

Testicular GSH content was determined after the reaction with 5,5'-dithio-bis-(2-nitrobenzoic acid) and the yellow color developed was read at 412 nm according to Boyne and Ellman [16], with modifications described by Jacques-Silva et al. [17]. A standard curve using cysteine was used to calculate the content of GSH in tissue samples, expressed as μmol GSH/g tissue.

Superoxide dismutase (SOD) enzyme activity

SOD was assayed spectrophotometrically as described by Misra and Fridovich [18]. Briefly, epinephrine rapidly auto oxidizes at pH 10.2 producing adrenochrome, a pink colored product that can be detected at 480 nm. The addition of samples

containing SOD inhibits the auto-oxidation of epinephrine. The rate of inhibition was monitored during 120 s at intervals of 15 s. The amount of enzyme required to produce 50% inhibition at 40°C was defined as one unit of enzyme activity. The SOD activity was expressed as U/mg of protein. Protein content was determined by the Lowry et al. method [19].

Catalase (CAT) enzyme activity

CAT activity was spectrophotometrically quantified in testis tissue by the method of Aebi [20], which involves monitoring the disappearance of H_2O_2 in the presence of cell homogenate (pH 7 at 25°C) at 240 nm. The enzymatic activity was expressed in µmol H_2O_2/min/g tissue.

2.8. Lactate dehydrogenase (LDH) enzyme levels

LDH catalyzes the reversible reaction of lactate-pyruvate in the presence of NAD, which is reduced to NADH. Next, NADH is measured by enzymatic action, which is directly proportional to the testis tissue LDH activity. The LDH levels were estimated by a kit using a spectrophotometer (500 nm). LDH levels were expressed in µM of NADH/min/g tissue.

2.9. Statistical analysis

Results were expressed as mean ± S.E.M. Data were analyzed by Two-way ANOVA followed by Duncan's Post Hoc tests when appropriated. (*Statistica* software package for Windows version 8.0 was used). Values of $P<0.05$ were considered statistically significant for all comparisons made.

3. Results

Effects of pecan shell AE and CP treatment on the variation (gain/ loss) of body weight (%) and the testis weight are shown in Table 1.

Two way ANOVA of body weight revealed a significant main effect of CP treatment [$F(1,24)=59.8$, $P<0.000$] and a significant interaction between CP x pecan shell AE [$F(1,24)=5.79$, $P<0.05$]. Univariate ANOVA followed by Duncan's multiple range tests showed that CP decreased the body weight (CP and AE+CP groups), when compared to control group and AE did not prevent this effect. The AE and C groups showed similar gain of body weight. Two way ANOVA revealed a significant main effect of CP [$F(1,24)=30.6$, $P<0.001$] for testis weight and a significant interaction CP x pecan shell AE [$F(1,24)=4.6$, $P<0.05$]. Univariate ANOVA followed by Duncan's multiple range tests revealed that CP reduced the testis weight and that the AE did not prevent the effect of CP. In fact, the testis weight of the AE plus CP treated groups was significantly minor than the control and similar to CP group, while the AE group showed testis weight higher than AE+CP and similar to control groups.

Effects of pecan shell AE and cyclophosphamide treatment on LDH and TBARS levels in testis are shown in Figure 3.

Two way ANOVA of LDH levels revealed a significant main effect of pecan shell AE [F (1,24)=9.4; $P<0.05$] and a significant interaction between AE and CP [F (1,24)=4.9; $P<0.05$]. Univariate analysis showed that the cyclophosphamide treatment (CP group) reduced the LDH levels in relation to control group (C), being that this effect was prevented by co-treatment with pecan shell AE (AE+CP group). In fact, the groups treated with the extract (AE and AE+CP) presented LDH levels similar to control group (C) (Fig. 3A).

Two way ANOVA of TBARS levels revealed a significant main effect of cyclophosphamide [F (1,24)=11.1; P<0.05] and pecan shell AE [F (1,24)=22.9; P<0.001]. Post hoc analysis indicated a significant increase on TBARS levels in CP group when compared to the other groups. The pecan shell AE (AE group) decreased TBARS levels in relation to control (C group), and also prevented (AE+CP group) the increase of TBARS CP-induced (CP group). In fact, the co-treatment (AE+CP) showed TBARS levels similar to control group (C) (Fig. 3B).

Effects of pecan shell AE and cyclophosphamide treatment on the GSH levels and SOD and CAT activity in testis are shown in Figure 4.

Two way ANOVA of GSH levels revealed a significant effect of cyclophosphamide [F (1,24)=106.9; P<0.001] and a significant interaction between CP and AE [F (1,24)=8.9; P<0.05]. One-way ANOVA followed by Duncan's test showed that the CP decreased the GSH levels in relation to C group, being that this effect was partially prevented by co-treatment with pecan shell AE (AE+CP group). The extract (AE group) did not change the basal GSH level and was similar to control group (Fig. 4A)

Two way ANOVA of SOD activity revealed a significant main effect of pecan shell AE [F (1,24)=5.8; P<0.05] and a significant interaction between AE and CP [F (1,24)=9.6; P<0.05]. One-way ANOVA revealed that cyclophosphamide increased the SOD activity when compared to control (C group). The pecan shell AE (AE group) did not change the basal levels of SOD, but prevented the effect of CP (AE+CP group), indicating that SOD levels were similar to control group (Fig. 4B)

Two way ANOVA of CAT activity revealed a significant interaction between AE and CP [F (1,24)=13.5; P<0.05]. Duncan's multiple range tests showed that cyclophosphamide reduced the activity of CAT when compared to control (C group),

being that this effect was prevented by co-treatment with pecan shell AE (AE+CP group). In fact, the catalase activity was similar in the C, AE and AE plus CP groups (Fig. 4C).

4. Discussion

Alkylating agents are widely used drugs in chemotherapy and strongly related to healthy tissue toxicity. The loss of body weight caused by CP shows some measurable signs of general toxicity [21] and this effect was also observed here. Besides the loss of 32% of body weight, CP exerts impairment in reproductive organs, whose toxic effects were confirmed in our findings through the lower weight of testis (6.5%). The effects of CP on the loss of body weight and testis weight were not changed by the co-treatment with pecan shell AE.

LDH is an essential enzyme for the testicular membrane and has been used as a chemical marker for the germ cell status in seminiferous epithelium [22]. Testicular toxicity can be diagnosed by the levels of this enzyme [23], whose metabolism involves processes of energy generation needed for the survival, differentiation and motility of cells. In Sertoli cells, this enzyme catalyzes the reduction of pyruvate to lactate and vice versa. This process is needed for the metabolic activities that occur in spermatocytes and spermatids [24]. Immature sperms and particularly spermatids have very low glycolytic potential and thus they are highly dependent on the lactate utilization catalyzed by LDH, whose amount increases with the testis maturity. When the levels of this enzyme are reduced, an alteration in spermatogenesis, depletion of germ cells, and cell impaired function occur [25]. In this study, we observed a reduction of 6.7% of LDH levels in testicular tissue of rats treated with CP. This testicular toxicity was prevented by the co-treatment with pecan shell AE possibly due to its

antioxidant activity. Oxidative mechanisms have been related with reduced levels of testicular LDH [26], and the use of the antioxidants can minimize this toxicity.

The toxicity of CP occurs after the biotransformation process by liver cytochrome P450, whose cytotoxic metabolites are distributed by systemic circulation and are related to the development of OS and tissue damage in different organs. Of particular importance to the reproductive health, different experimental studies involving oxidative mechanisms have discussed the clinic limitation of CP [6]. In this sense, CP and its metabolite acrolein cause inactivation of microsomal enzymes resulting in increased ROS generation and LP development; particularly important in the testis, whose tissue is very vulnerable to oxidative injury [27]. Here, we observed an increase of 66% of rat testis LP. This result may be related to an increase of ROS and/or a decrease in antioxidant defenses. Similarly, other laboratories have reported the CP-induced testicular toxicity to OS development [26]. In fact, in our findings, the co-treatment with pecan shell AE decreased 51.4% of LP CP-induced, showing the antioxidant therapeutic potential of this vegetal.

Considering the OS, the major and first line of defense against ROS induced organ injury is the GSH, whose depletion may trigger tissue injury processes [28]. GSH is one of the most important molecules in the cellular defense against chemically reactive toxic compounds or OS, constituting a major redox system of the cell. The action of GSH occurs by direct interaction of –SH group with ROS or it can be involved in the enzymatic detoxification reaction for ROS, as a cofactor or coenzyme [29]. CP is an alkylant agent that produces ROS that may interact with thiol groups of proteins, oxidizing them to disulfides. GSH may ultimately result in covalent binding of CP reactive metabolites to critical cellular macromolecules, with consequent cytotoxicity [30]. The depletion of testis GSH content observed in this experiment may

be attributed to the direct conjugation of CP and/or its metabolites with free or protein bound –SH groups, thus interfering with the antioxidant functions. Recently, Tsai-Turton et al. observed tissue damages CP-induced, OS, and GSH depletion [31]. In the present study, the CP-induced OS occurred concomitantly with a depletion of 49% of testicular GSH levels, but the co-treatment with pecan shell AE, showing a depletion of just 31%. Therefore, a current interest has been focused on compounds that act as antioxidants and are capable of stimulating GSH synthesis, like pecan shell AE, which in the present study partially prevented the decrease in the GSH levels.

Besides GSH, SOD constitutes an important biological defense that acts through the dismutation of superoxide radicals to H_2O_2 and molecular oxygen. Spermatozoa are particularly susceptible to peroxide damage because they contain high concentration of polyunsaturated fatty acids, exhibit no capacity for membrane repair, and possess a significant ability to generate ROS, predominantly superoxide anion and hydrogen peroxide [32]. Interestingly, in our findings, SOD activity was increased in the testis of the animals treated with CP, possibly to compensate the ROS generation and the consequent OS induced by this drug. Selvakumar et al. showed a decrease of testicular SOD activity after 10 weeks of CP treatment [26]. These results are not contrary to ours, which were performed after 7 days of treatment. In fact, it is not clear why the CP increased the testicular SOD activity. Since this effect may be temporary, more studies are necessary.

CAT is another important antioxidant enzyme that prevents the generation of ROS by converting hydrogen peroxide into water and non-reactive oxygen species, protecting cells from oxidative damage [33]. CAT is likely to be the major antioxidant enzyme responsible for H_2O_2 degradation [34], mainly in the membrane testis, where CAT confers complete protection against H_2O_2 [35]. Here, CP causes a reduction in the

testis CAT activity, and this effect may reflect in the incapacity of eliminating the H_2O_2 produced by SOD. When the CAT activity is insufficient to degrade H_2O_2, more H_2O_2 could be converted into hydroxyl radicals, contributing to the development of OS caused by CP. The CP treatment caused a decrease of 28% of CAT activity and the co-treatment with pecan shell AE prevented this effect.

Natural products are a source of innovative substances that can be fractionated and isolated, leading to a new drug and a new industrial therapeutic arsenal. When a new chemical substance is isolated from the crude extract of medicinal plants, it becomes a new drug with side effects. On the other hand, the popular knowledge may be extremely useful to minimize side effects related to conventional pharmacotherapy as well as contribute with the therapeutic effects. In this sense, the use of the natural compounds, as tea or beverage in their integral form, can be especially beneficial when associated to conventional medicines, mainly by their high antioxidant potential. In fact, plants are an important source of phenolic compounds, which are synthesized as secondary metabolites during normal development in response to stress, among other conditions. Of particular importance to our findings, GA (3,4,5-trihydroxybenzoic acid) was the more abundant polyphenol observed in pecan shells. Recently, this compound showed healthy potentially effect, mainly by its scavenger activity on superoxide anion, hydroxyl radicals, singlet oxygen or peroxyl radical, preserving the cellular viability[36]. In this sense, we hypothesized that the foremost responsible by the beneficial effects observed here is the GA, mainly by its substantial presence in pecan shells. In fact, the presence of hydroxyl groups, mainly at the *para* position is especially efficient for antioxidant activity of GA [36]. In accordance with our results, the pecan shells are popularly used

a detoxifying agent, which may be associated to chemotherapy, contributing to prevent or reduce the side effects arising of this pharmacological treatment.

In conclusion, our findings confirm the involvement of the LP and/or the decrease of antioxidant defenses on the CP-induced testicular damage. Pecan shell AE is a natural product with a potent protective effect against the testicular toxicity of this alkylant agent, possibly due to its high content of polyphenols, mainly the gallic acid. This innovative biotherapy is being demonstrated for the first time, and may contribute for decreasing the toxicity related to CP treatment.

Acknowledgments

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Legends of Figures and Tables

Figure 1. Chemical structure of gallic acid (3,4,5-trihydroxybenzoic acid).

Figure 2. High performance liquid chromatogram of gallic acid (GA) authentic standard (A) and pecan shell AE (B). *t*R = 3.68 min and 1.8g GA/100g pecan shell AE (monitored at 290 nm).

Table 1. Effects of pecan shell aqueous extract (AE) on the % of gain/loss of body weight and testis weight of rats treated with cyclophosphamide (CP) (values are expressed as mean ± S.E.M., n=7).

* Indicates a significant difference from C group for $P<0.05$; $^{+}$ Indicates a significant difference from AE plus CP group for $P<0.05$.

Figure 3. Effects of pecan shell aqueous extract (AE) on lactate dehydrogenase (LDH) (A) and lipid peroxidation (LP) (B) levels in testis of rats treated with cyclophosphamide (CP) (values are expressed as mean ± S.E.M., n=7).

* Indicates a significant difference from C group for $P<0.05$; $^{+}$ Indicates a significant difference from AE plus CP group for $P<0.05$.

Figure 4. Effects of pecan shell aqueous extract (AE) on the levels of reduced glutathione (GSH) (A), superoxide dismutase (SOD) (B) and catalase (CAT) activities in testis of rats treated with cyclophosphamide (CP) (values are expressed as mean ± S.E.M.; n=7).

* Indicates a significant difference from C group for $P<0.05$; $^{+}$ Indicates a significant difference from AE plus CP group for $P<0.05$.

Figure 1:

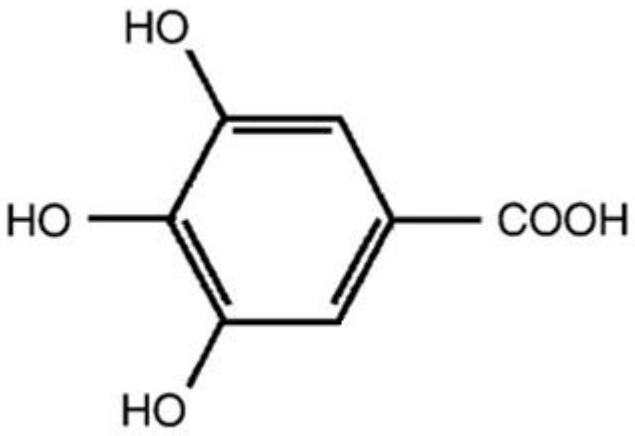


Figure 2:

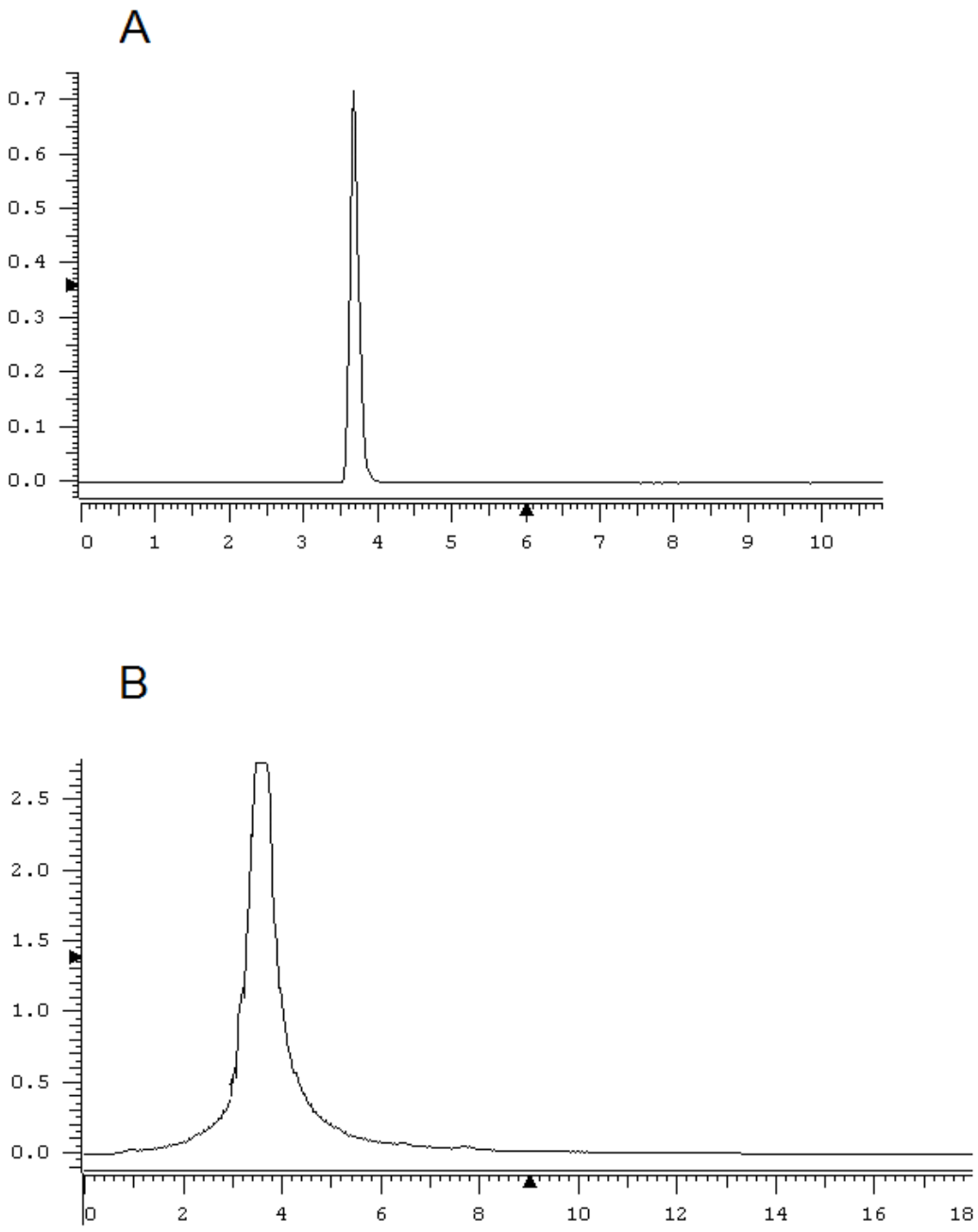

A
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 1 2 3 4 5 6 7 8 9 10
B
2.5
2.0
1.5
1.0
0.5
0.0
0 2 4 6 8 10 12 14 16 18

Table 1:

Groups	% variation body weight	Testicular weight (g)
C	7.56 ± 0.63	3.38 ± 0.08
AE	5.47 ± 1.54 $^{+}$	3.52 ± 0.06 $^{+}$
CP	-31.90 ± 2.29 *	3.16 ± 0.03 *
AE+CP	-26.93 ± 1.08 *	3.04 ± 0.05 *

Figure 3:

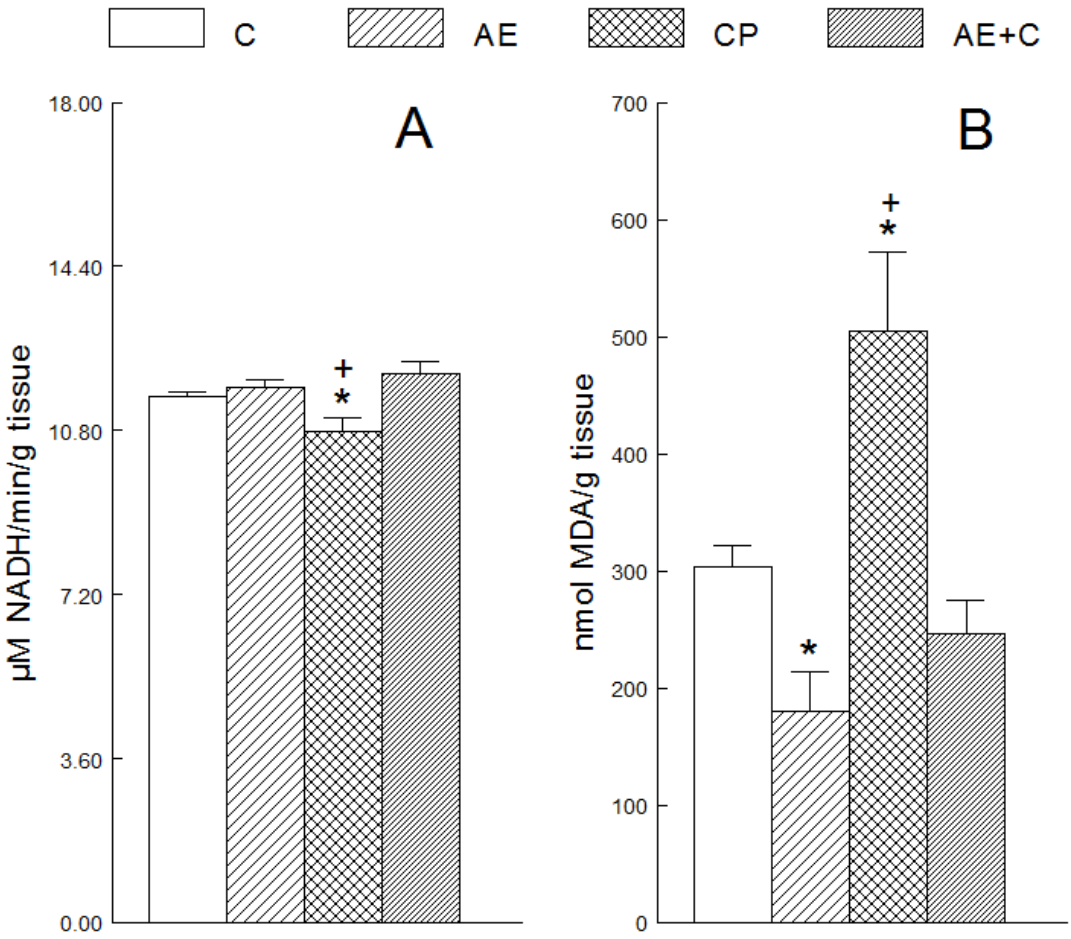
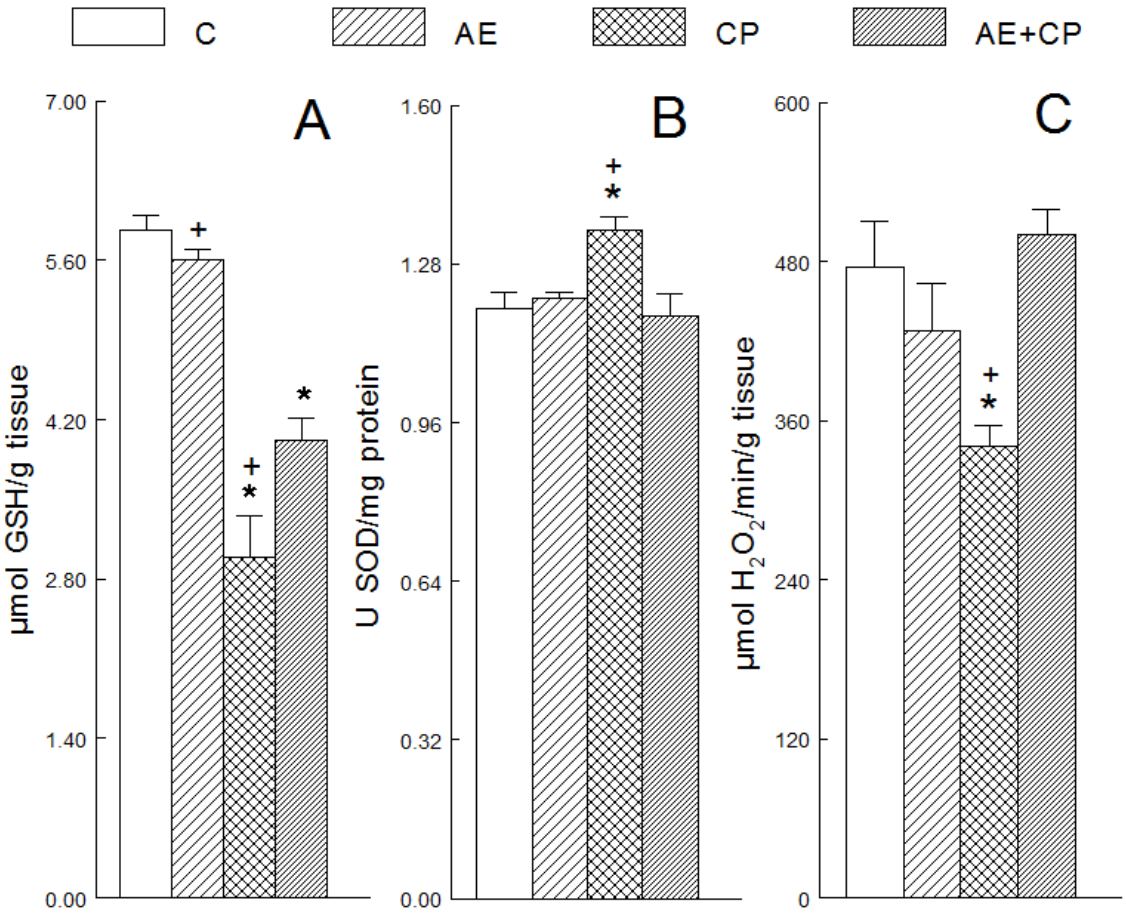

C
AE
CP
AE+C
A
B
μM NADH/min/g tissue
nmol MDA/g tissue
18.00
14.40
10.80
7.20
3.60
0.00
700
600
500
400
300
200
100
0
+
*

Figure 4:


C
AE
CP
AE+CP
A
B
C
µmol GSH/g tissue
U SOD/mg protein
µmol H_2O_2/min/g tissue
7.00
5.60
4.20
2.80
1.40
0.00
1.60
1.28
0.96
0.64
0.32
0.00
600
480
360
240
120
0
+
*

Manuscrito 2

4.2 Efeito protetor de um sub-produto da indústria de noz pecã (*Carya illinoensis*) sobre a toxicidade induzida pela ciclofosfamida em ratos

Protective effects of a by-product of the pecan nut industry (*Carya illinoensis*) on the toxicity induced by cyclophosphamide in rats

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Protective effects of a by-product of the pecan nut industry (*Carya illinoensis*) on the toxicity induced by cyclophosphamide in rats

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Abstract

The present study was carried out to investigate the antioxidant effects of pecan nut (*Carya illinoensis)* shell aqueous extract (AE) on toxic effects induced by cyclophosphamide (CP) in heart, kidney, liver, bladder, plasma and erythrocytes of rats. Rats were treated with water or pecan shell AE (5%) *ad libitum* in the place of drinking water for 37 days up to the end of the experiment. On day 30, half of each group received a single administration of vehicle or 200 mg/kg-ip of CP. After 7 days, the rats were sacrificed and the organs removed. Rats treated with CP had increase of lipid peroxidation (LP) and decrease in reduced glutathione (GSH) levels in all structures analyzed. Catalase (CAT) activity was elevated in heart and diminished in liver and kidney. Besides, CP treatment decreased plasmatic vitamin C (VIT C) levels and induced severe bladder macroscopical and microscopical damages. In contrast, co-treatment with pecan shell AE prevented the LP development and the GSH depletion in all structures, except in the heart and plasma, respectively. CAT activity in the heart and liver remained unchanged as well the plasmatic VIT C levels. Finally, AE was able to prevent the injury CP induced in the bladder. These findings revealed the antioxidant and protective role of pecan shell AE in the multiple organ toxicity CP-induced and may be considered a supplementation in this pharmacotherapy.

Key-words: cyclophosphamide, *Carya illinoensis,* pecan nut shells, oxidative stress, antioxidant.

1. Introduction

Carya illinoensis (Wangenh.) K. Koch (Juglandaceae), popularly known as pecan, is a tree native to the southern United States and northern Mexico, which reaches South America (Hancock, 1997). Pecan nuts are a source of monounsaturated fatty acids, what stimulate their cultivation and consumption (Rajaram et al., 2001). Pecan processing results in a great amount (40-50%) of shells, and this by-product of pecan industry represents a promising source of antioxidant compounds (Worley, 1994). In this sense, recently Villarreal-Lozoya et al., (2007) showed that pecan shells have higher levels of phenolic compounds and condensed tannin, which showed a greater antioxidant capacity than the kernels. In fact, pecan shells are popularly used in tea form to prevent different pathologies, mainly those related to xenobiotic toxicity and to treat diseases related to tobacco nicotine (www.pecantea.com.br). Food and its derivatives have been widely used to maintain the functional integrity of the body and are an important source of new products with lower costs and low incidence of undesirable side effects, often associated with drug treatments (Velioglu et al., 1998).

Cyclophosphamide (CP) is an antineoplastic drug widely used in the treatment of cancer and immunosuppression induction before organ transplantation (Zincke and Woods, 1977). The therapeutic effects of CP are associated with the phosphoramide mustard, while the acrolein is linked with its toxic side effects (Kern and Kehrer, 2002). These metabolites are generated by the hepatic microsomal cytochrome P450 mixed functional oxidase system (Sladek, 1971). The major limitation of CP treatment is the damage to normal tissues, leading to multiple organ toxicity (Bukowski, 1999; Fraiser et al., 1991). Besides lethal cardiotoxicity (Shanholtz, 2001), the CP treatment is related to hepatotoxicity (Snover et al., 1989; Shulman et al., 1980), nephrotoxicity (Rossi, 1997), and urotoxicity (Abraham and Sugumar, 2008, Appelbaum et al., 1976). The bladder is especially affected by CP treatment, whose deleterious effects include mucosal edema, hemorrhage ulceration, fibrosis, necrosis, contracture, and vesico-ureteral reflux (Levine and Richie, 1989). In fact, most of the side-effects of CP, including the urotoxicity, are related to reactive oxygen species (ROS) generation (Korkmaz et al., 2007), which have been implicated in the action of many cytostatic drugs (Kalyanaraman et a., 1989) as CP. CP is closely related to oxidative stress (OS) and tissue damage by the increase of

lipid peroxidation (LP) and depletion of the antioxidant agents (Haque et al., 2003), such as glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) (Dorr and Lagel, 1994; Patel 1987; Patel and Block, 1985).

In this sense, biological compounds with antioxidant properties may contribute to the protection of tissues against deleterious effects of ROS. Multiple clinical studies have suggested that the use of antioxidant supplements in combination with chemotherapy can prolong the survival time of patients compared with expected outcome without supplementation (Manda and Bhatia, 2003; Singh and Lippman, 1998; Sudharsan et al. 2006). Thus, natural antioxidants, especially from plants, foods or nutritional supplements have become an important research issue at a worldwide level.

Considering that the pecan nut shell is a natural product widely used in folkloric medicine to treat xenobiotic toxic effects and that its chemical composition is rich in polyphenols as flavonoids and condensed tannins, the present study was performed to evaluate the protective effects of pecan shell extract on the toxicity CP-induced in vital tissues of rats.

2. Materials and methods

2.1. Drugs and chemicals

Cyclophosphamide (Genuxal®, Asta Médica, Brazil) was donated by 'Hospital Universitário' from the 'Universidade Federal de Santa Maria' (HUSM), RS, Brazil and was dissolved in distilled water just before use.

2.2. Vegetal material

The raw material was kindly donated by a pecan processing company, which also makes the processing of the nut shells for the preparation of tea. This product is sold in supermarkets, with permission of the Ministry of Agriculture, Brazil.

2.3. Aqueous extract preparation

Shells of *C. illinoensis* were let overnight at 40°C in a hot air oven and fine powdered. The aqueous extract (AE) of the shells was freshly prepared by infusion (5%, 90°C), filtered using filter paper and cooled to room temperature. During this procedure, the extract was protected from light. Since experimental data were not

found in the literature, the choice of the extract concentration was based on preliminary studies performed in our laboratory, which showed the high potential antioxidant of the pecan shell extract.

2.4. Characterization of Aqueous Extract

Further qualitative and quantitative analyses of *C. illinoensis* shell aqueous extract were performed using HPLC (condition: column XTerra RP-18 (4.6 x 250 mm, 5 µM); eluent: methanol:water (80:20), 0.4% acetic acid; flow rate: 1ml/min; detection: UV at 290 nm). The major constituent of the AE detected was gallic acid (GA), corresponding to 1.8g/100g of extract (Figure 1).

2.5. Animals

Wistar adult male rats (330±40g) were used. Groups of seven animals were kept in Plexiglas cages with free access to food and water in a room with controlled temperature (22–23 °C) and on a 12 h-light/dark cycle with lights on at 7:00 a.m. Animals were maintained and used in accordance to the rules of the Committee on Care and Use of Experimental Animal of the Federal University of Santa Maria, RS, Brazil.

2.6. Experimental protocol

After 2 weeks of acclimatization, 28 rats were randomly allocated into four experimental groups (n=7), designated as control (C), cyclophosphamide-treated (CP), pecan nut shell aqueous extract (AE) and extract plus CP-treated (AE+CP). The fresh aqueous extract of pecan shells (AE and AE+CP groups) or tap water (C and CP groups) was daily offered to animals *ad libitum*, similarly to tea, which is popularly used. After 30 days of treatment with pecan shell AE or vehicle, the groups designated as CP and AE+CP received a single dose of CP (200 mg/kg body weight-i.p.), while C and AE groups were injected with vehicle (distilled water) and maintained with the oral treatment (pecan shell AE or vehicle) for another 7 days. On day 8, the animals were anesthetized with thiopental (50 mg/kg/mL, i.p.) and sacrificed by exsanguination (the blood was collected by cardiac puncture). The blood was centrifuged for separation in plasma and erythrocytes used in the evaluations described below. The heart, liver and kidneys were removed, homogenized in 10 volumes (10mL/g tissue) of 10mM Tris-HCl buffer (pH−7.4), and

centrifuged (5000 rpm/20min) for biochemical evaluations. After removal, the bladder was observed macroscopically and fixed in 10% buffered formaldehyde for subsequent histological analysis. This procedure was especially done with the bladder because the urotoxicity is the most serious side effect related to CP treatment.

2.7. Thiobarbituric acid reactive species (TBARS) levels

TBARS assay measures the lipid peroxidation (LP) which occurs by excessive ROS generation. LP was estimated through the pink chromogen produced by the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA) at 100ºC, measured spectrophotometrically at 535 nm. In plasma and erythrocytes, TBARS was estimated by the method described by Lapenna et al. (2001), after modifications (Jacques-Silva et al., 2001). In the tissues, TBARS levels were determined in accordance with Ohkawa et al. (1979). Results were expressed as nmol MDA/mL plasma, nmol MDA/mL erythrocytes and nmol MDA/g tissue, respectively.

2.8. Estimation of antioxidants

2.8.1. Reduced gluthatione (GSH) levels

GSH tissue content was determined after reaction with 5,5'-dithiobis-(2-nitrobenzoic acid); the yellow color developed was read at 412 nm, in accordance with Boyne and Ellman (1972), after modifications (Jacques-Silva et al., 2001). A standard curve using cysteine was used to calculate the content of GSH in tissue samples, expressed as μmol GSH/g tissue.

Red blood cell pellets (RBC) obtained after centrifugation of whole blood were hemolyzed with 10% triton solution and the protein fraction was precipitated with 20% trichloroacetic acid followed by centrifugation. The colorimetric assay was carried out as described. A standard curve using glutathione was constructed in order to calculate the content of GSH, expressed as nmol GSH/mL erythrocytes.

GSH was assayed in plasma using the same method of Boyne and Ellman (1972). A standard curve using glutathione was also utilized. Results were expressed as nmol GSH/mL plasma.

2.8.2. Vitamin C

Plasma vitamin C (VIT C) was estimated as described by Galley et al. (1996) with some modifications (Jacques-Silva et al., 2001). This method produces an orange chromogen by the reaction with dinitrophenylhydrazine at 37ºC, measured spectrophotometrically at 520 nm. A standard curve using ascorbic acid was used to calculate the content of VIT C and expressed as μg VIT C/mL plasma.

2.8.3. Catalase (CAT) enzyme activity

CAT activity was spectrophotometrically quantified in tissues by the method of Aebi (1984) which involves monitoring the disappearance of H_2O_2 in the presence of cell homogenate (pH 7 at 25°C) at 240 nm. The enzymatic activity was expressed in μmol H_2O_2/min/g tissue.

2.9. Macroscopically analysis

Bladder tissues were analyzed by three observers and scored according to a damage scale of 0 (normal) to 4 (severe changes). The damage was scored according to the tissue color intensity.

2.10. Histological evaluation

After fixation (minimum time 18-24 h), the bladder tissues were trimmed into 2-4 mm thick sections for processing and sectioning. The tissues were embedded in paraffin and at least four cross-sections 4-5 μm thick were taken from each bladder and stained with hematoxylin-eosin (H & E). Histopatological examination was performed by a pathologist and microphotographs were taken using the software Win TV2000.

2.11. Statistical analysis

Levene's test was applied to verify the variances homogeneity. Parametric data were analyzed by two-way ANOVA followed by Duncan's multiple range test when appropriate. Values were expressed as mean± S.E.M. Nonparametric data were analyzed by Kruskal-Wallis analysis of variance followed by two-tailed Mann-Whitney U test when appropriate, and expressed as median±quartiles. Statistica software package for Windows version 8.0 was used and values of $P<0.05$ were considered statistically significant for all comparisons made. Values of TBARS of all

tissues were expressed as median±quartile. Values of GSH, VIT C levels and CAT activity showed normal distribution and therefore were expressed as mean±S.E.M.

3. Results.

Treatments with cyclophosphamide (CP), pecan shell AE (AE) or the combination of cyclophosphamide plus pecan shell AE (CP+AE) on the oxidative stress parameters of heart, liver, kidney, plasma and erythrocytes are shown in Figures 2-6, respectively.

Heart: The CP treatment increased the TBARS levels in heart in relation to C group, and this effect was not prevented by the extract. In fact, the co-treated group showed similar TBARS levels to CP group, while the AE treated group showed similar values to C group (Fig. 2A). CP decreased the GSH levels in heart when compared to control, and this effect was prevented by the extract of pecan shell. The extract alone increased the GSH levels when compared to C group (Fig. 2B). Rats treated with CP presented a significant increase in CAT activity in relation to control. Alone, pecan shell extract did not change CAT activity, but prevented the effect of CP in heart tissue when administered concomitantly (Fig. 2C).

Liver: The CP treatment increased the hepatic TBARS levels when compared to control, and this effect was partially prevented by the co-treatment with the extract. The extract treated group (AE) showed similar values to control (Fig. 3A). CP significantly decreased the hepatic GSH levels in relation to C group, and this effect was partially prevented by the co-treatment with pecan shell extract. The extract alone did not change the liver GSH levels, which were similar to C group (Fig. 3B). Rats treated with CP presented a significant decrease in liver CAT activity in relation to C group, and this effect was partially prevented by the extract. The AE group showed higher CAT activity than control (Fig. 3C)

Kidney: The CP treatment increased the renal TBARS levels in relation to C group, and this effect was partially prevented by the extract. In fact, the co-treated group showed higher renal TBARS levels than the C group, while the AE group showed similar values to control (Fig. 4A). CP decreased the GSH levels in kidney when compared to control, and this effect was partially prevented by the co-treatment with pecan shell extract. The extract alone did not change the GSH levels when compared to control (Fig. 4B). The CP treatment significantly decreased the renal

CAT activity when compared to C group. Alone, the extract increased the CAT activity in relation to control, but did not prevent the CP effect when administered together (Fig. 4C).

Plasma: The CP treatment increased the TBARS levels in plasma, and the co-treatment prevented this effect. The extract alone did not change the TBARS levels (Fig. 5A). CP reduced the GSH levels in plasma when compared to control, and the pecan shell extract did not prevent this effect. GSH levels were not changed by the extract treatment (Fig. 5B). The CP treatment reduced the plasmatic VIT C levels when compared to control, and the extract prevented this effect. Pecan shell extract showed VIT C levels similar to control (Fig. 5C).

Erythrocytes: The CP treatment increased the TBARS levels in relation to control and this effect was partially prevented by the co-treatment with pecan shell extract. In fact, the co-treated group showed higher TBARS levels in erythrocytes than the C group. The extract alone decreased the TBARS levels in erythrocytes, when compared to control (Fig. 6A). The treatment with CP decreased the GSH levels in erythrocytes in relation to control. The co-treatment with pecan shell extract partially prevented this effect of CP and the extract alone did not alter the erythrocytes GSH levels (Fig. 6B).

Treatments with cyclophosphamide (CP), pecan shell AE (AE) or the combination of cyclophosphamide plus pecan shell AE (CP+AE) on the macroscopical evaluation of the bladder are shown in Table 1 and the histopathological evaluations are shown in Table 2 and Figure 7.

CP treatment induced damages in bladder, which were prevented by pecan shell extract. The co-treated group showed damage scores similar to control. The extract alone did not modify the scores of bladder (Table 1).

Urinary bladders from control group showed normal cytology, showing transitional epithelial lining; narrow lamina propria with epithelial lining in folds and normal muscle layer (Table 2; Figures 7A and 7B). Rats treated with CP showed widened lamina propria with severe degree of hemorrhage and edema as well as moderate leukocyte infiltration and vascular proliferation. We can also observe a thickening of muscle layer in these tissues (Figures 7E and 7F). The co-treatment with pecan shell extract showed an improvement in bladder tissues because they

presented mild thickening of lamina propria without edema and vascular proliferation as well as low degree of hemorrhage and leukocyte infiltration. Only the muscle layer did not show any changes in this group (Figures 7G and 7H). Pecan shell extract did not alter the histology of bladder tissue in relation to control (Figures 7C and 7D).

4. Discussion

The most common effect of the chemotherapeutic agents is the cytotoxicity in different tissues, which is related to an increase in the free radicals (FR) (Lee et al., 1996). In our study, due to the LP induced by CP, the TBARS levels were increased in heart, liver, kidney, plasma, and blood red cells. These effects might be due to the increased production of FR and/or decreased antioxidant defense system. Animals co-treated with pecan shells AE plus CP showed lower TBARS values in liver, kidney, plasma, and blood red cells, indicating reduced levels of LP in these tissues. In this sense, the OS CP induced was prevented or attenuated by pecan shell extract. This hypothesis is in accordance with previous experiments performed in our laboratory, when the pecan shells showed high *in vitro* and *ex vivo* antioxidant potential (submitted results).

The effects of pecan shell AE on the oxidative damages induced by CP may be explained by the presence of phenolic compounds and condensed tannins in pecan shells (Prado et al., 2009; Villarreal-Lozoya et al., 2007). Of particular importance to our findings, GA (3,4,5-trihydroxybenzoic acid) was the most abundant polyphenol observed in pecan shells . Recently, Lu et al. (2006) attributed scavenger activity to this compound on superoxide anion, hydroxyl radicals, singlet oxygen or peroxyl radical, showing healthy potential. In fact, the presence of hydroxyl groups, mainly at the *para* position is especially efficient for the antioxidant activity of GA (Lu et al., 2006).

LP mediated by excessive ROS production not neutralized is showed here through an increase of 60.2% of heart TBARS levels. Other authors also observed LP development in heart tissue of rats treated with CP (Mythili et al., 2004, Senthilkumar et al., 2006). In this sense, cardiac muscle is particularly susceptible to FR injury, mainly because it contains low levels of enzymatic antioxidants and GSH (Olson et al., 1981; Takacs et al., 1992). In our findings, the CP treatment reduced around 37.6% of GSH levels and increased 73% of CAT activity in heart tissue. Recently it was reported that the alkylant agents are potent inactivators of glutathione

reductase (GR) (Ren and Slattery, 1999). In fact, the alkylant metabolites of CP can react with sulphydryl groups of GR, and in turn reduce the regeneration of GSH from GSSG (Senthilkumar et al., 2006). The same authors reported that the CP decreases the heart CAT activity, while we observed an increase of its activity. In fact, these experimental paradigms were performed through different doses of CP and therefore cannot be considered contradictories. Thus, the heart enzymatic changes demonstrated here were prevented by pecan shell extract, showing its antioxidant potential. The cardiotoxicity of CP treatment is a serious side effect of CP treatment, mainly by lethal cardiotoxicity described after high therapeutic doses of this chemotherapy (Shanholtz, 2001), emphasizing the need for novel compounds, as plants or foods, which would protect the normal tissue from chemotherapy-induced toxicity.

Liver disorders were observed when the therapeutic dose of CP needs to be increased (Atkinson et al., 1987; Shulman et al., 1980; Snover et al., 1989). Here we could show the liver toxicity by CP treatment, evidenced by an increase of 116.7% in LP and a decrease of antioxidant defenses of 84.9% for GSH levels and 58.12% for CAT activity. Other authors also found similar findings (Bhattacharya et al., 2003; Premkumara et al., 2001), confirming the toxicity of CP in the liver. Pecan shell extract showed beneficial effects through the prevention of these outcomes in the liver.

CP treatment is also related to nephrotoxicity (Kopecna, 2001), which has been attributed to acrolein metabolite (Horvath et al., 1992). Recently, Sugumar and Abraham (2007) observed an increase of 47% of renal LP and a decrease of 77% of GSH levels. The mechanism of CP induced renal damage is limited, and OS is thought to play a central role in these events (Haque et al., 2003). In this sense, we found similar results observed by an increase of TBARS (266.8%) in renal tissue, besides the lowest levels of GSH (74.4%) and CAT activity (32.9%), demonstrating the wide renal damage mediated by CP. An important finding in the present study is that the high antioxidant property of pecan shell extract (Prado et al., 2009) was able to prevent the LP and restore the GSH levels CP induced in this vital tissue.

The blood can also be affected by CP treatment, which might be observed in plasma and erythrocytes serving as markers of body damage (Ascensao et al., 2007). Here, CP treatment increased 42.4% of plasma TBARS levels and the levels of GSH and VIT C were reduced in 56.9% and 26.3%, respectively. Low levels of

plasmatic VIT C during CP treatment may increase the susceptibility of tissues to ROS damage (Lee, 1999). Here, pecan shell extract preserved the plasmatic VIT C levels and prevented the LP, strengthening its antioxidant potential.

Blood red cell membrane is susceptible to OS due to its high content of polyunsaturated fatty acids, which are vulnerable to oxidative damage (Manoharan et al., 2004). Recently, a study showed an increase of TBARS and a decrease of GSH in erythrocytes of rats treated with CP, which were reversed by the antioxidant properties of the squalene (Senthilkumar et al. 2006). These results are according to our findings which showed that the pecan shell extract preserved the LP and GSH levels modified by CP in erythrocytes.

One of the major side effects of CP administration is urotoxicity (Bischel, 1979). Since the bladder is the primary storage organ for urine, the content of CP metabolites is higher than the other areas of urinary tract, increasing the sensitivity of the bladder to oxidative damage (Datta et al. 1998). These deleterious effects of CP include urothelial damage, edema, necrosis, ulceration, hemorrhage, neovascularization, and leukocyte infiltration (Bischel, 1979). In our findings, the CP induced accentuated damage to the endothelial layer, edema and hemorrhage, as well as moderate vascular proliferation and leukocyte infiltration in the bladder. The co-treatment with pecan shell extract showed once again its high antioxidant potential observed through the absence of these deleterious effects of CP on the bladder. The use of antioxidant compounds sounds promising alternatives to prevent CP induced urotoxicity. In fact, Mesna (2-mercaptoethanesulfonic acid) is a thiol clinically used as uroprotective compound (Cavalletti et al., 1986), but this medicine decreases the incidence of cystitis in only 5% (Korkmaz et al., 2007). This modest benefit of Mesna on the urotoxicity CP induced leads to the search of more potent compounds that may prevent these damages.

In this sense, clinical studies have shown that patients who receive antioxidants with the standard chemotherapy tolerate the treatment better and have prolonged survival time compared with expected outcome without the antioxidant supplements (Jaakkola et al., 1992; Singh et al. 1998). Moreover, chemotherapy and antioxidants may enhance the effectiveness of the treatment (Conklin, 2004; Klimberg, 1996). Then, natural antioxidants may offer comparatively safer alternatives to synthetic antioxidants, which may cause serious or unacceptable adverse side effects (Ali, 2003). This way, the natural antioxidant studied here

revealed a potent effect to minimize the chemotherapy damage caused in healthy tissues.

We know that innovative substances can be fractionated and isolated leading to a new drug and a new industrial therapeutic arsenal. However, when a new chemical substance is isolated from the crude extract of plants it becomes a new drug with side effects. On the other hand, the popular knowledge may be extremely useful to minimize side effects related to conventional pharmacotherapy as well as to contribute with the therapeutic effects. In this sense, the use of natural compounds such as foods or beverages in their integral form, as demonstrated here, can be especially beneficial when associated to medicines.

Pecan shell is a by-product of the pecan nut industry with high antioxidant potential which was effective to reduce the CP damage on vital tissues. This extract may be useful to prevent deleterious effects related to this chemotherapy. From these observations, it is possible to conclude that CP treatment results in pronounced damage in heart, liver, kidney, blood and bladder mediated by OS due to its toxic metabolites. Pecan shell extract showed protective effect against the general toxicity of CP, and its chemical constituents deserve further studies.

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Legends for Figures

Figure 1- High performance liquid chromatogram of gallic acid (GA) authentic standard (A) and pecan shell AE (B). *t*R = 3.68 min and 1.8g GA/100g pecan shell AE (monitored at 290 nm).

Figure 2 - Effects of pecan shell AE on TBARS (A) and GSH (B) levels and CAT activity (C) in heart of rats treated with CP.
*Indicates a significant difference from C group ($P<0.05$); $^{+}$Indicates a significant difference from AE plus CP group ($P<0.05$).

Figure 3 - Effects of pecan shell AE on TBARS (A) and GSH (B) levels and CAT activity (C) in liver of rats treated with CP.
*Indicates a significant difference from C group ($P<0.05$); $^{+}$Indicates a significant difference from AE plus CP group ($P<0.05$).

Figure 4 - Effects of pecan shell AE on TBARS (A) and GSH (B) levels and CAT activity (C) in kidney of rats treated with CP.
*Indicates a significant difference from C group ($P<0.05$); $^{+}$Indicates a significant difference from AE plus CP group ($P<0.05$).

Figure 5 - Effects of pecan shell AE on TBARS (A), GSH (B) and VIT C (C) levels in plasma of rats treated with CP.
*Indicates a significant difference from C group ($P<0.05$); $^{+}$Indicates a significant difference from AE plus CP group ($P<0.05$).

Figure 6 - Effects of pecan shell AE on TBARS (A) and GSH (B) levels in erythrocytes of rats treated with CP.
*Indicates a significant difference from C group ($P<0.05$); $^{+}$Indicates a significant difference from AE plus CP group ($P<0.05$).

Figure 7 - Effects of pecan shell AE on histological changes in the urinary bladder of rats treated with CP (values are expressed as mean±S.E.M., n=7). The left column

represents low magnification (40X) and the right column represents high magnification (100X). A and B–control group; C and D–pecan shell AE group; E and F–cyclophosphamide group; G and H–pecan shell AE plus cyclophosphamide group.

Legends for Tables

Table 1. Effects of pecan shell AE on macroscopical changes in the urinary bladder of rats treated with CP (values are expressed as median±quartile, n=7).
0 (normal) and 4 (severe changes). *Indicates a significant difference from C group (P<0.05); $^{+}$Indicates a significant difference from AE plus CP group (P<0.05).

Table 2. Effects of pecan shell AE on histological changes in the urinary bladder of rats treated with CP.
+++Severe, ++Moderate, +Mild, ND = not demonstrated.

Figure 1:

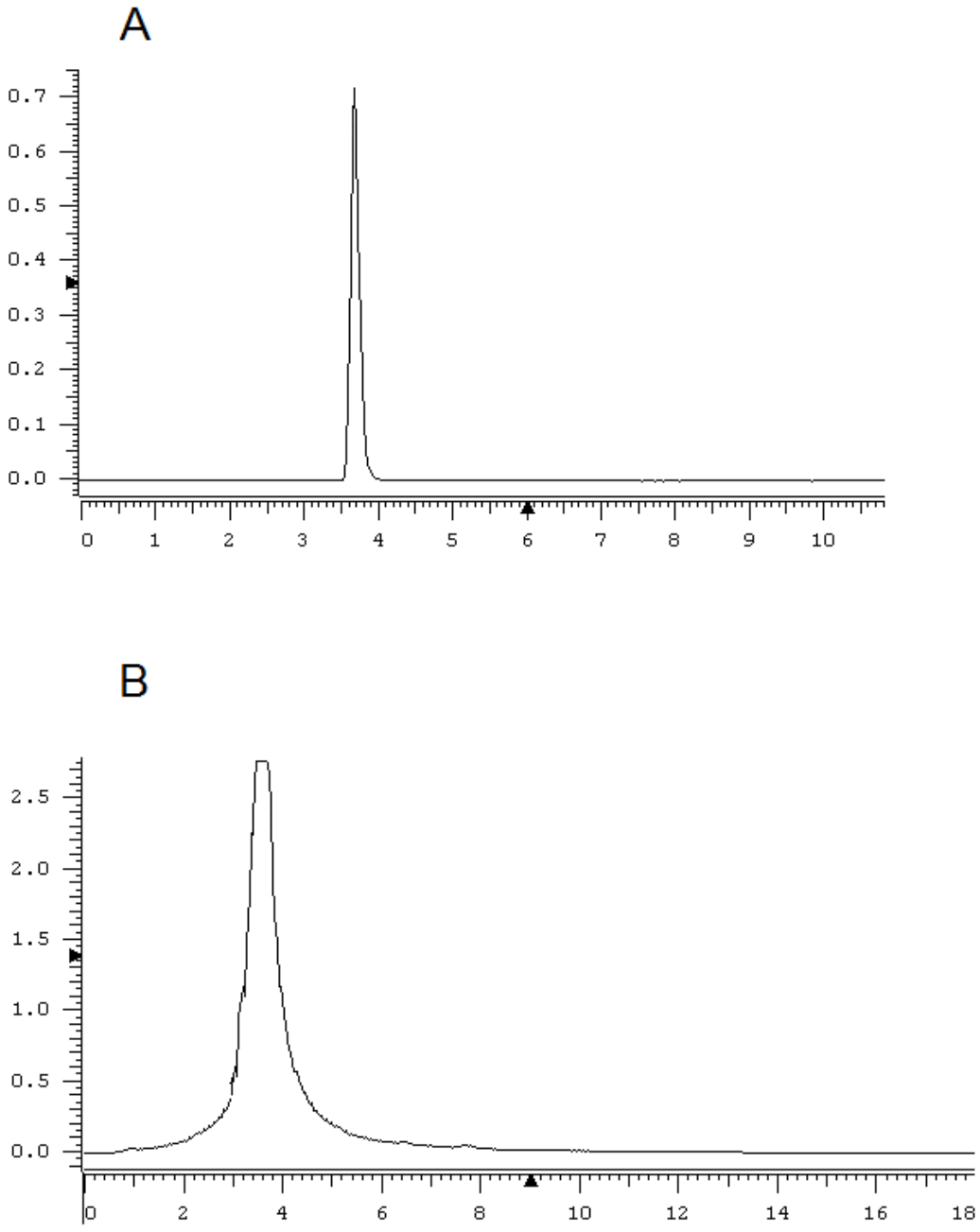
A
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 1 2 3 4 5 6 7 8 9 10
B
2.5
2.0
1.5
1.0
0.5
0.0
0 2 4 6 8 10 12 14 16 18

Figure 2:

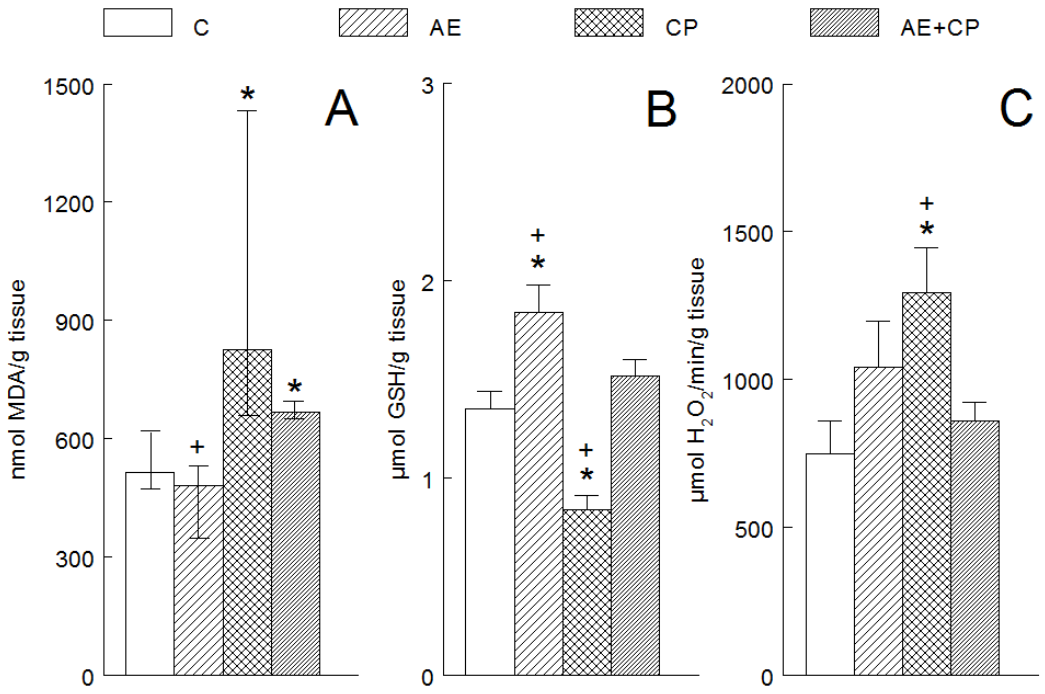
C
AE
CP
AE+CP
A
B
C
nmol MDA/g tissue
µmol GSH/g tissue
µmol H_2O_2/min/g tissue
0
300
600
900
1200
1500
1
2
3
500
1000
1500
2000
+
*

Figure 3:

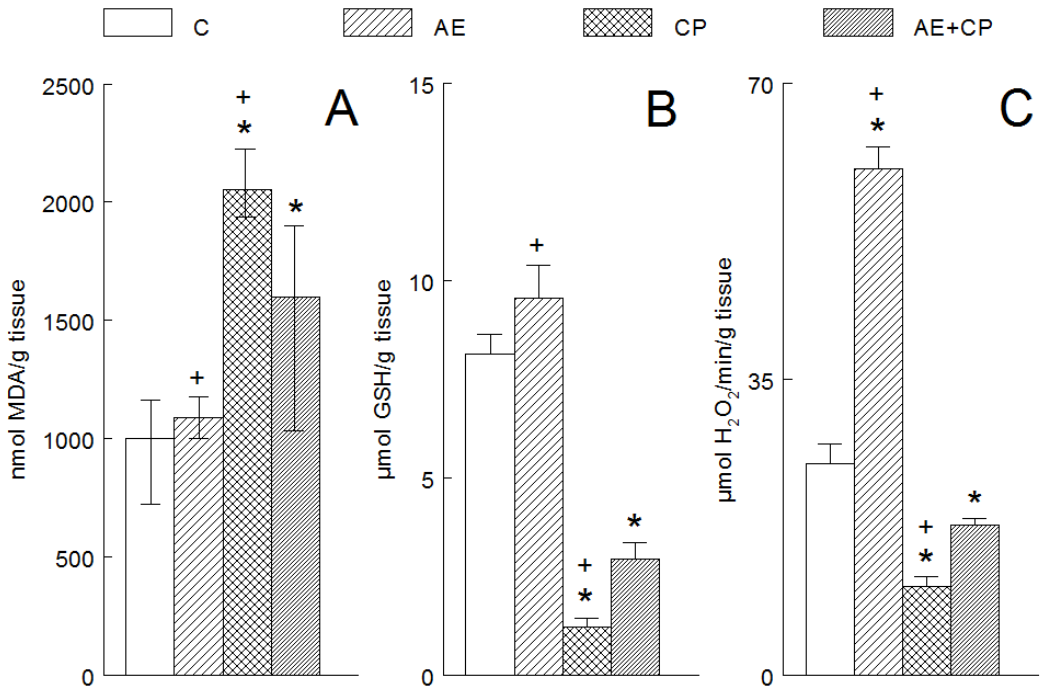
C
AE
CP
AE+CP
A
B
C
nmol MDA/g tissue
µmol GSH/g tissue
µmol H2O2/min/g tissue
2500
2000
1500
1000
500
0
15
10
5
0
70
35
0
+
*

Figure 4:

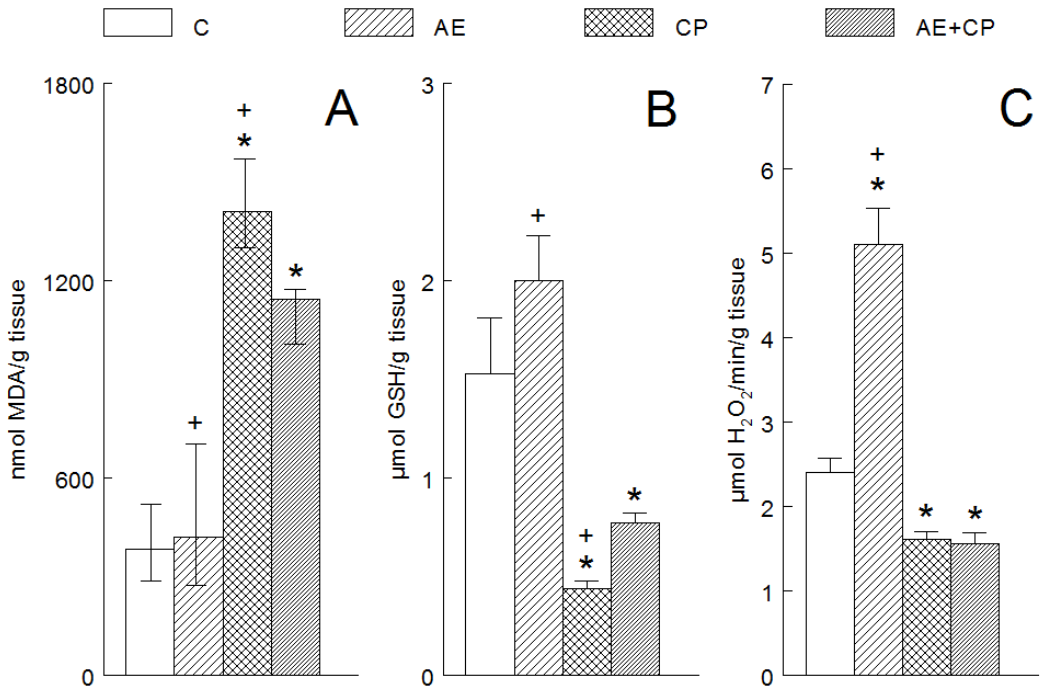
C
AE
CP
AE+CP
A
B
C
nmol MDA/g tissue
µmol GSH/g tissue
µmol H2O2/min/g tissue
1800
1200
600
0
3
2
1
0
7
6
5
4
3
2
1
0
+
*

Figure 5:

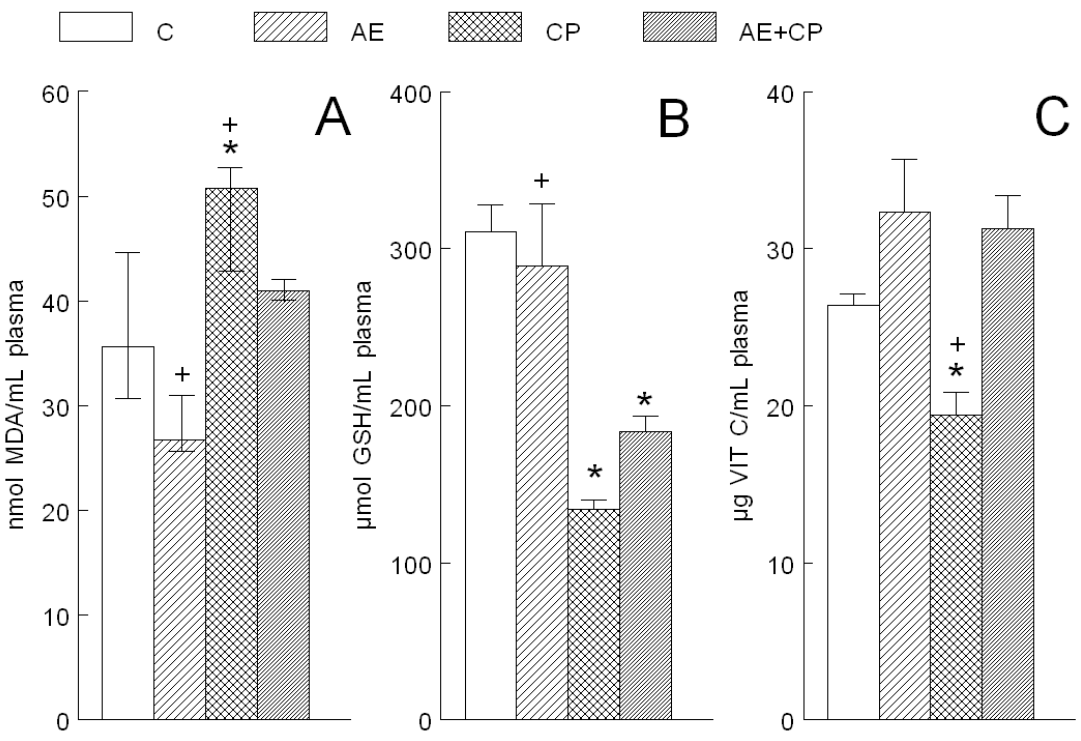
C
AE
CP
AE+CP
A
B
C
nmol MDA/mL plasma
µmol GSH/mL plasma
µg VIT C/mL plasma
0
10
20
30
40
50
60
100
200
300
400
+
*

Figure 6:

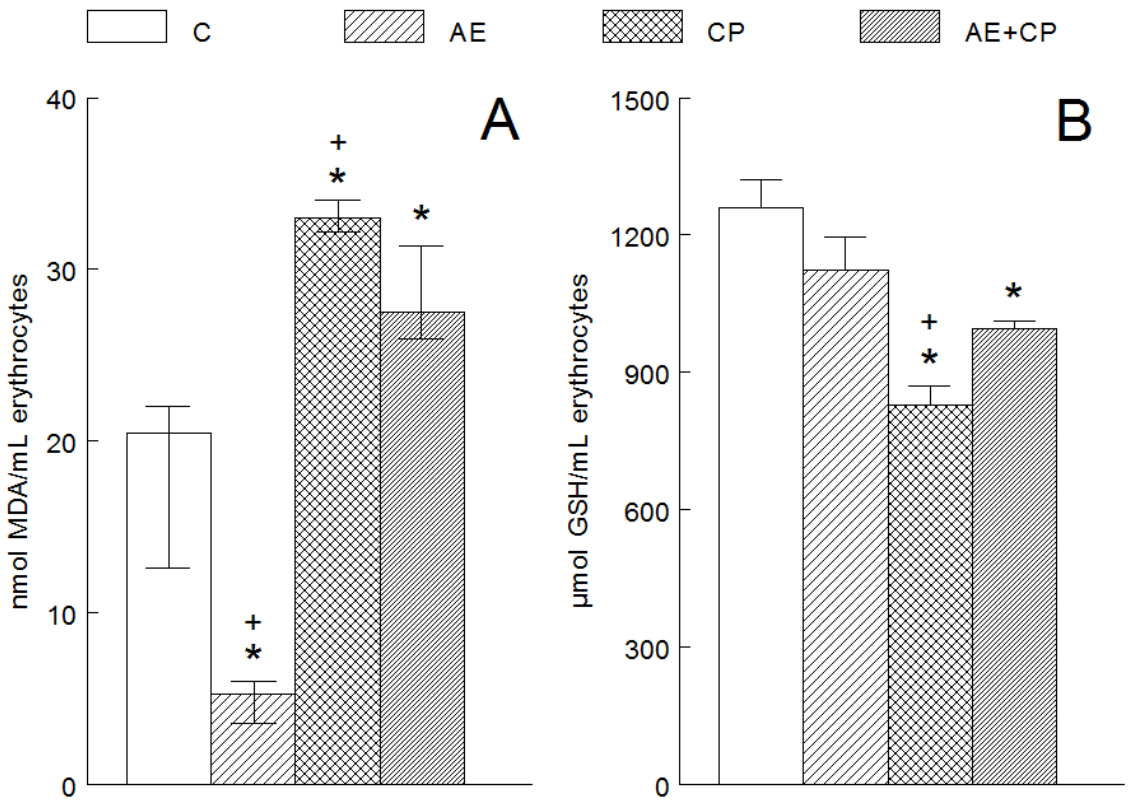
C
AE
CP
AE+CP
A
B
nmol MDA/mL erythrocytes
µmol GSH/mL erythrocytes
0
10
20
30
40
300
600
900
1200
1500
+
*

Figure 7:

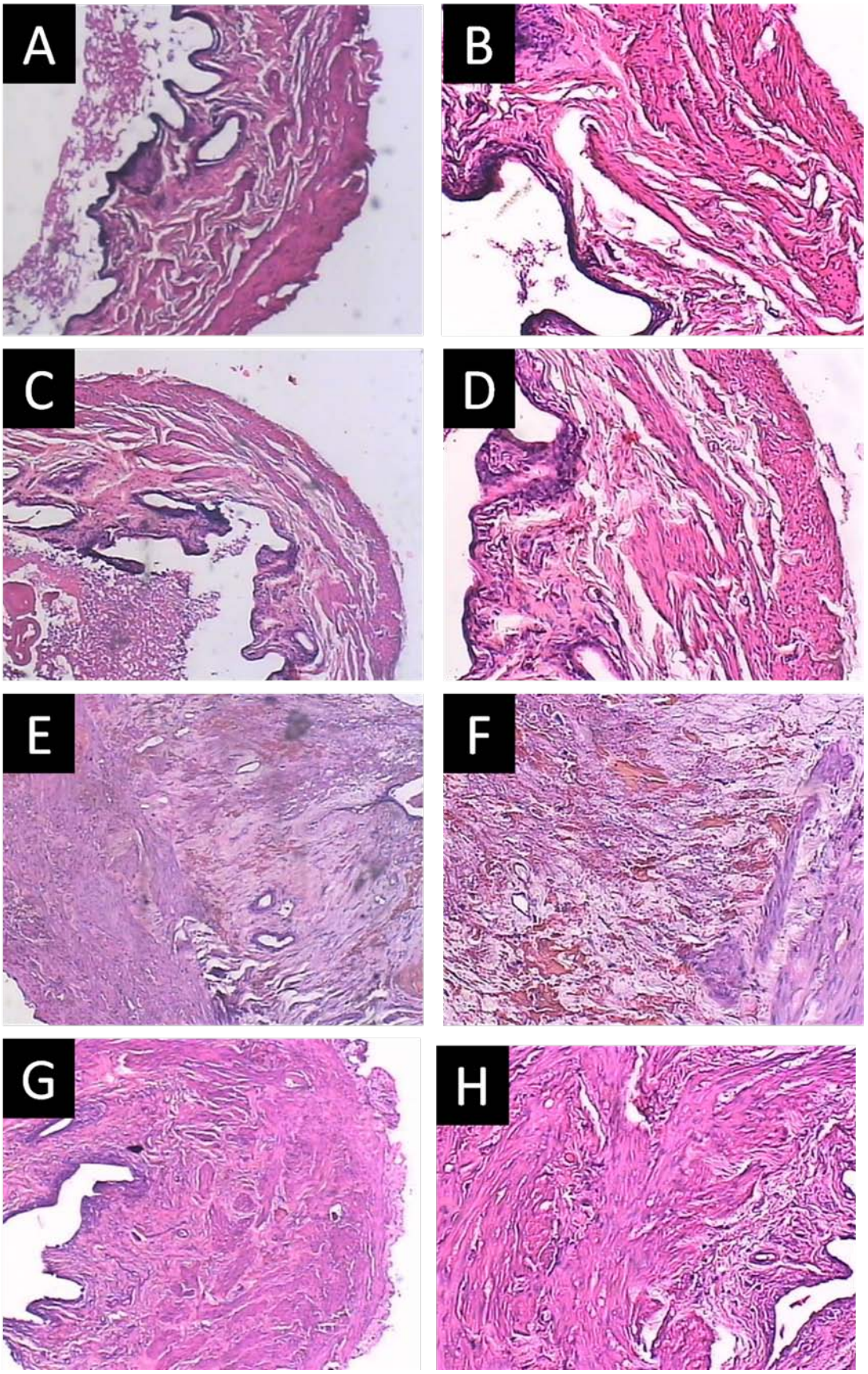

Table 1:

Groups	Score
C	0 (0-0)
AE	0 (0-0)
CP	4 (4-4) *
AE+CP	0 (0-1)

Table 2:

Groups	Hemorrhage	Edema	Leukocyte infiltration	Vascular proliferation	Thickening of muscle layer	Thickening of lamina propria
C	ND	ND	ND	ND	ND	ND
AE	ND	ND	ND	ND	ND	ND
CP	+++	+++	++	++	++	+++
AE+CP	+	ND	+	ND	++	+

5 DISCUSSÃO

Os dados obtidos no presente estudo servem para confirmar que a administração de doses terapêuticas altas do quimioterápico CP em ratos é capaz de gerar toxicidade em tecidos vitais. Além disso, foi demonstrado que o antioxidante avaliado neste estudo, o EAB da casca da noz pecã, é eficaz na prevenção do aparecimento de efeitos colaterais indesejáveis induzidos por este fármaco.

CP é um agente alquilante com potentes propriedades antineoplásica e imunossupressora, sendo possivelmente o fármaco antitumoral mais utilizado (GERSHWIN et al., 1974; TEW et al., 1996). Apesar de sua seletividade tumoral, a CP também apresenta um amplo espectro de toxicidades (FRAISER et al., 1991), ou seja, acaba lesando os tecidos sadios, causando uma gama de sintomas indesejáveis aos pacientes que necessitam o uso desta medicação. O mecanismo exato desta toxicidade ainda não está totalmente elucidado, entretanto diferentes evidências apontam para o EO gerado pelas EROs como uma das principais causas (HAQUE et al., 2001; DAS et al., 2002; GHOSH et al., 2002). O presente estudo está de acordo com essas evidências, principalmente pelo fato de que diferentes marcadores indicaram a ocorrência de EO em órgãos vitais de ratos tratados com CP, mostrando dessa forma uma provável causa para sua patogênese.

Já está bem documentado que a LPO determinada através do método de TBARS constitui uma importante ferramenta para observação do EO (LIU et al., 1997). Neste trabalho, o tratamento com CP foi capaz de aumentar os níveis de TBARS em todos os tecidos analisados. Juntamente ao aumento da LPO causada por um excesso de EROs pode ocorrer a diminuição dos estoques de GSH, importante defesa contra xenobióticos (GUL et al., 2000). Novamente, o tratamento com CP mostrou uma redução nesse parâmetro em todos os tecidos avaliados. Estes achados estão de acordo com outros dados da literatura que apontam um aumento da LPO e uma diminuição do GSH em diferentes estruturas corporais de ratos tratados com CP (DAVIS & KUTAN, 2000; PREMKUMAR et al., 2001; MYTHILI et al. 2004; SELVAKUMAR et al. 2004; 2005a; 2005b; SUGUMAR & ABRAHAM, 2007).

As enzimas antioxidantes SOD e CAT trabalham em cooperação para manter estáveis os níveis de EROs. Dessa forma, alterações no funcionamento dessas

enzimas podem indicar um desequilíbrio da produção de EROS, podendo levar ao EO (PIGEOLET et al., 1990). A atividade dessas duas enzimas foi analisada nos testículos dos ratos tratados com CP e mostrou uma elevação na atividade da SOD e diminuição na atividade da CAT. Dados da literatura geralmente mostram uma diminuição na atividade de ambas as enzimas (SELVAKUMAR et al., 2004; 2005). No entanto, aqui nesse estudo, o período que sucede ao tratamento com CP é menor do que nos demais estudos realizados. Assim, inicialmente poderia haver um aumento na atividade da SOD tentando compensar a produção de radical superóxido. No coração, fígado e rim dos mesmos ratos, a atividade da enzima CAT foi avaliada e também estava reduzida, exceto no coração, cuja atividade estava aumentada. Dados mostrados por Stankiewicz & Skrzydlewska (2003) e Selvakumar et al., (2005b) confirmam a diminuição na atividade dessa enzima no rim e fígado, respectivamente. Já no coração, novamente há a possibilidade de um mecanismo compensatório, exigindo maiores estudos.

A VIT C tem um papel fundamental na detoxificação de compartimentos corporais aquosos (PACKER et al., 1979). Assim, sua determinação no plasma dos ratos tratados com CP foi de grande valia. Os resultados mostraram uma diminuição dos níveis plasmáticos deste composto antioxidante. Outros estudos também mostraram que doses altas de CP são capazes de reduzir os níveis plasmáticos de VIT C (MYTHILY et al., 2004; SENTHILKUMAR et al., 2006).

A enzima LDH testicular é útil como um indicador para diagnóstico da toxicidade testicular (READER et al., 1991). Quando os níveis desta enzima encontram-se reduzidos, ocorrem alterações na espermatogênese, bem como depleção e prejuízo na função das células germinativas (ABD-ALLAH et al., 2000). Os resultados aqui obtidos monstraram uma redução nos níveis dessa enzima em ratos tratados com CP, demostrando toxicidade testicular induzida por este agente quimioterápico. Outros estudos também obtiveram resultados semelhantes aos observados aqui, confirmado estes efeitos da CP sobre a função testicular (SELVAKUMAR et al., 2004; 2006).

Já está bem descrito que o efeito mais sério gerado pela CP é a toxicidade urinária, demonstrada por meio da cistite hemorrágica. Vários estudos indicam uma alteração tanto macroscópica, quanto microscópica da bexiga de ratos após o tratamento com CP. Esses dados incluem alterações na coloração e modificações histológicas como: espessamento das camadas teciduais, hemorragia, edema,

proliferação leucocitária e vascular (ABD-ALLAH et al., 2004; OZCAN et al., 2005; BHATIA et al., 2008). Com o objetivo de focar melhor esses danos físicos induzidos pela CP, foi realizada a análise histológica da bexiga dos animais, que resultou na presença de todos os sinais descritos acima, em maior ou menor grau.

Estudos clínicos múltiplos demonstraram que pacientes que fizeram o uso de antioxidantes em combinação com a quimioterapia ou a radioterapia padrão toleraram melhor o tratamento e tiveram um tempo de sobrevivência prolongado em relação aos pacientes que não receberam suplementação com antioxidantes (JAAKKOLA et al., 1992; LAMM et al., 1994; WAGDI et al., 1996; WHELAN et al., 1999). Exceto por três interações específicas (flavonóides com tamoxifeno, N-acetilcisteína com doxorrubicina e β-caroteno com 5-fluoruracil) não há evidências de que antioxidantes interfiram *in vivo* com as terapias convencionais para o câncer (LAMSON et al., 1999). Além disso, alguns trabalhos demonstraram que antioxidantes aliados ao tratamento quimioterápico, podem aumentar a efetividade do tratamento (CONKLIN, 2004). No presente estudo não houve óbitos no tempo de tratamento realizado, entretanto, observou-se que os animais tratados com CP em associação com o antioxidante em estudo (casca da noz pecã) apresentaram um aspecto físico de maior tolerabilidade ao tratamento quimioterápico do que os animais tratados apenas com o quimioterápico.

A suplementação prévia com o chá da casca da noz pecã foi capaz de prevenir total ou parcialmente os efeitos tóxicos da CP em diferentes tecidos vitais. Isso pode ser demonstrado pela prevenção do aumento da LPO em todos os tecidos estudados, exceto no coração, bem como por impedir a redução nos estoques de GSH em todos os tecidos, exceto no plasma. Ainda, foi capaz de manter estáveis os níveis da enzima LDH e a atividade das enzimas SOD e CAT nos testículos, bem como impedir alterações na atividade desta última no coração e fígado. A administração do extrato ainda impediu a redução dos níveis plasmáticos de VIT C. Por último, o achado mais importante, foi que o antioxidante utilizado preveniu a indução da cistite hemorrágica, efeito adverso mais preocupante da CP.

Estudos maiores precisam ser desenvolvidos para a determinação do principal ou principais compostos envolvidos na atividade antioxidante observada aqui. Até o momento podemos sugerir que o EAB da casca da noz pecã contém compostos polifenólicos e taninos condensados, com elevadas propriedades antioxidantes, os quais precisam ser quimicamente determinados. Neste trabalho, foi

realizado um estudo cromatográfico, o qual mostrou elevado teor de ácido gálico. Recentemente, foi demonstrado que o ácido gálico exerce efeitos benéficos devido à sua propriedade de sequestrar EROs, preservando a viabilidade celular. E a sua atividade antioxidante está relacionada à presença de três grupos hidroxilas (-OH) ligados ao anel aromático, principalmente ao que está na posição *para* do anel (LU et al., 2006).

De modo geral pode-se concluir que o EAB da casca da noz pecã apresentou potencial antioxidante evidenciado pela prevenção dos efeitos colaterais induzidos pelo quimioterápico CP em ratos. Esses resultados mostram que o uso de produtos naturais não se restringe à etnofarmacologia e também podem ser terapeuticamente úteis junto ao tratamento com xenobióticos, como a quimioterapia com CP.

6 CONCLUSÕES

Através dos resultados experimentais obtidos podemos chegar às seguintes conclusões:

1. A CP foi capaz de aumentar os níveis de LPO, determinados através do método de TBARS em todos os tecidos avaliados. O co-tratamento com EAB preveniu este efeito em todos os tecidos, em maior ou menor grau, exceto no coração.

2. Os níveis de GSH encontraram-se reduzidos após tratamento com CP em todos os tecidos avaliados. Com exceção do plasma, o co-tratamento com EAB preveniu esses efeitos em todos os tecidos, em maior ou menor grau.

3. A atividade da enzima CAT apresentou-se aumentada no coração e diminuída no fígado, rim e testículos dos ratos tratados com CP. O co-tratamento com EAB foi capaz de prevenir os efeitos da CP no coração, no fígado e nos testículos.

4. A atividade da enzima SOD testicular apresentou-se aumentada após o tratamento com CP e o co-tratamento com EAB foi capaz de prevenir este efeito.

5. O tratamento com CP reduziu os níveis plasmáticos de VIT C, ao passo que o co-tratamento com EAB preveniu este efeito.

6. Os níveis da enzima LDH testicular foram reduzidos pelo tratamento com CP. O co-tratamento com EAB contribuiu com a prevenção deste efeito.

7. A bexiga dos ratos tratados com CP mostrou escores de danos aumentados. Os animais submetidos ao co-tratamento com EAB mostraram esta pontuação mais baixa.

8. As análises histológicas das bexigas dos ratos tratados com CP evidenciaram um aumento da lâmina própria com elevado grau de hemorragia e edema, além de moderada infiltração leucocitária e proliferação vascular. Ainda, houve um

espessamento da camada muscular. O co-tratamento com EAB apresentou um leve espessamento da lâmina própria, ausência de edema e proliferação vascular e um baixo grau de hemorragia e infiltração leucocitária, porém, a camada muscular se manteve semelhante ao grupo tratado com CP.

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