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ESTUDO DE FRUTAS DO CERRADO BRASILEIRO PARA AVALIAÇÃO DE PROPRIEDADE FUNCIONAL COM FOCO NA ATIVIDADE ANTIOXIDANTE

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Quando uma criatura humana desperta para um grande sonho
e sobre ele lança toda a força de sua alma...
Todo o universo conspira a seu favor!"
(Goethe)

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SUMÁRIO

RESUMO

Annona crassiflora (araticum), *Solanum lycocarpum* (lobeira), *Eugenia dysenterica* (cagaita), *Caryocar brasiliense* (pequi) e *Swartzia langsdorfii* (banha de galinha) são frutas do 2º maior bioma do Brasil (Cerrado). Atualmente, o cerrado enfrenta duas realidades diferentes: a grande possibilidade de produção de alimentos, sendo considerado o maior celeiro do mundo e por outro lado, a riquíssima biodiversidade que está sendo recentemente descoberta e conhecida.

A importância da pesquisa por antioxidantes naturais tem aumentado muito nos últimos anos uma vez que o *stress* oxidativo tem sido associado ao desenvolvimento de muitas doenças crônicas e degenerativas, incluindo o câncer, doenças cardíacas, doenças degenerativas como Alzheimer, bem como está envolvido no processo de envelhecimento.

Neste trabalho foi estudada a atividade antioxidante (AAO) de diferentes extratos de frutas nativas do cerrado, a fim de avaliar o potencial desses extratos como antioxidante natural para possíveis aplicações nos setores farmacêutico, cosmético e alimentício.

A capacidade de seqüestrar radicais livres em relação ao radical estável 2,2 difenil-1-picril hidrazil (DPPH) foi inicialmente avaliada por se tratar de uma metodologia simples, rápida e sensitiva. O IC_{50} (quantidade de extrato necessária para inibir a oxidação do radical DPPH em 50%) obtido para os extratos etanólicos de semente e casca de araticum, casca de pequi e semente de cagaita está na faixa de 14 – 50 $\mu g\ mL^{-1}$. Compostos mundialmente reconhecidos como antioxidantes (ácido gálico, ácido cafeico, ácido ascórbico, ácido ferrúlico, ácido clorogênico e rutina) foram avaliados e os resultados obtidos estão em concordância com a literatura (IC_{50} 1,4 – 10 $\mu g\ mL^{-1}$). Por meio desse ensaio, as frutas de pequi, araticum e cagaita revelaram-se excelentes fontes de compostos antioxidantes.

O potencial dos extratos em inibir a peroxidação lipídica foi avaliado por meio da indução química da peroxidação lipídica dos microssomas de fígado de rato e quantificação dos malonaldeídos (MDA). Os resultados obtidos para os extratos encontram-se na faixa de 0,7 to 4,5 $\mu g\ mL^{-1}$. Esse ensaio confirmou a alta AAO proporcionada pelos extratos das frutas pequi, araticum e cagaita previamente obtidos pelo radical DPPH.

A alta quantidade de compostos fenólicos determinada por Folin-Ciocalteau, e principalmente, a identificação desses compostos por espectrofotometria de massa com fonte de ionização por "electrospray" (ESI-MS) comprovaram que substâncias bioativas amplamente reconhecidas como antioxidantes naturais estão presentes nos extratos etanólicos de sementes e cascas dos frutos do cerrado, indicando portanto que a AAO desses extratos está diretamente relacionada a compostos fenólicos como ácido cafeico e seus derivados como cafeoil e dicafeoiltartárico e cafeoil glucose; ácido quínico, ácido ferrúlico, rutina, xantoxilina, ácido gálico; quercetina e quercetina 3-O-arabinose.

Em função de sua excelente AAO, os extratos etanólicos de casca e semente de araticum foram escolhidos para a avaliação "in vivo" com ratos. A atividade das enzimas hepáticas da fase 1 e 2, responsáveis pela detoxificação de espécies reativas de oxigênio (ROS) e peróxidos de lipídios, foram monitoradas em grupos de ratos com e sem intoxicação induzida por tetracloreto de carbono (CCl_4), a fim de avaliar o efeito protetor dos extratos em condições normais e contra compostos tóxicos. Em concordância com os dados de literatura, o tratamento com CCl_4 induziu significativamente a peroxidação lipídica, aumentou o nível de equivalentes de glutationa e o conteúdo de Cb5, paralelamente, houve redução das atividades das enzimas catalase (CAT), superoxido dismutase (SOD) e conteúdo de citocromo (CP450). O tratamento com os extratos de sementes e casca de araticum inibiu a peroxidação lipídica nos ratos saudáveis quando comparados ao controle em 27 e 22% respectivamente, bem como preservou o conteúdo de CP450. Em relação aos animais que receberam CCl_4, os extratos de araticum também preveniram a peroxidação lipídica, a reduçao na atividade da CAT e a indução dos equivalentes de glutationa. Entretanto, os extratos de araticum não impediram os danos causados por CCl_4 na atividade de CP450, b5 e SOD. Por meio desse estudo, conclui-se que os extratos etanólicos de semente e casca de araticum apresenta AAO "in vivo", contribuindo significativamente no controle da peroxidação lipídica promovida por stress oxidativo bem como verificou-se que esses extratos não interferem no conteúdo de CP450, reduzindo a probabilidade de interações medicamentosas entre terapias a base de plantas e medicamentos convencionais.

Por último, foi possível estimar o potencial tóxico dos melhores extratos por meio da avaliação citotoxicológica e fototoxicológica "in vitro" utilizando células 3T3 (linhagem de fibroblastos da pele de camundongo Balb/C).

SUMMARY

Annona crassiflora (araticum), *Eugenia dysenterica* (cagaita), *Solanum lycocarpum* (lobeira), *Caryocar brasiliense* (pequi) e *Swartzia langsdorfii* (banha de galinha) are fruits of the second biggest biome of Brazil called Cerrado. Nowadays, the Brazilian cerrado biome is challenged by two different realities: the great possibility of food production and the extremely rich Biodiversity. Thus it is estimated that 40% of the cerrado biome has already been deforested. The aim of this study was evaluate extracts of cerrado native fruits in order to estimate their potential as natural antioxidant to food, cosmetic and pharmaceutical applications. Oxidative stress has been associated with the development of many chronic and degenerative diseases, including cancer, heart disease, and neuronal degeneration such as Alzheimer's, as well as being involved in the process of aging. Therefore, the importance of the search for and exploitation of natural antioxidants, especially of plant origin, has greatly increased in recent years.

The radical scavenging activity toward the stable radical 2,2-diphenyl-1-picryl hydrazyl (DPPH) was firstly used since it is a very simple, sensitive and rapid method. The best results were found for ethanolic extracts of pequi peel, cagaita seeds, araticum seeds and araticum peel and the IC_{50} (concentration of dried extracts required to decrease the initial DPPH concentration by 50%) results were in the range of 14 – 50 $\mu g\ mL^{-1}$. The IC_{50} of widely known antioxidants such as caffeic acid, gallic acid, ascorbic acid, ferrulic acid, clorogenic acid and rutin were also evaluated and the results were in the range of 1.4 – 10 $\mu g\ mL^{-1}$, in accordance with previous reports.

The validation of the highly efficient antioxidant activity of the extracts was done using the biological relevant method of chemically induced lipid peroxidation using rat liver microsome as oxidative substrate. The IC_{50} results were in the range of 0.7 to 4.5 $\mu g\ mL^{-1}$.

The high quantity of total phenol and the investigating by direct infusion electrospray ionization mass spectrometry (ESI-MS) revealed the presence of important bioactive molecules in the cerrado fruits extracts such as ascorbic acid, caffeic acid, quinic acid, ferulic acid, gallic acid, xanthoxylin, rutin caffeoyltartaric and dicaffeoyltartaric acid, caffeoyl glucose, quercetin and quercetin 3-O-arabinose probably responsible for their antioxidant activity.

The araticum peel and seed extracts were selected to continue the studies by "in vivo" model using albino rats of the Wistar strain in order to evaluated the detoxifying enzymes (Phase I and Phase II) such as catalase (CAT), superoxide dismuase (SOD), cytochrome P450, gluthatione peroxidase (GPx), glutathione redutase (GRed), glutathione transferase (GST), lipid peroxidation and glutathione equivalents content. The hepatoprotective effect of cerrado fruits against CCl_4-Induced liver damage will be also evaluated. Treatment with CCl_4 significantly induced lipid peroxidation by 44%, increased the level of GSH equivalents by 140% and the content of cytochrome b5 by 32%; and concomitant reduced the activities of CAT, SOD and CP450 by 23, 34 and 39% respectively. Treatment with *A. crassiflora* seeds and peel extracts alone inhibited lipid peroxidation by 27 and 22% and preserved CP450 content. Pretreatment with the extract from *A. crassiflora* prevented the lipid peroxidation, the reduction in CAT activity and the induction of GSH equivalents content caused by CCl_4, but had no effect on CCl_4-mediated changes in the activities of CP450 and b5 and SOD. These results showed that *A. crassiflora* seeds and peel contain antioxidant activity "in vivo" mainly preventing lipid peroxidation induced by oxidative stress. The components responsible for this activity could be of potential therapeutic use.

Besides, the cytotoxicity and fototoxicity of the best extracts were explored in order to estimate their toxicity by using the "in vitro" 3T3 mouse fibroblasts neutral red uptake (NRU) methods.

INTRODUÇÃO GERAL

Annona crassiflora (araticum), *Solanum lycocarpum* (lobeira), *Eugenia dysenterica* (cagaita), *Caryocar brasilense* (pequi) e *Swartzia langsdorfii* (banha de galinha) são frutas do segundo maior bioma do Brasil (Cerrado) conhecidas e consumidas principalmente por populações nativas dessa região.

Atualmente, o cerrado brasileiro enfrenta duas realidades diferentes: a grande possibilidade de produção de alimentos, sendo considerado o maior celeiro do mundo e por outro lado, a riquíssima biodiversidade que está sendo recentemente descoberta e conhecida. Porem, nos últimos 30 anos, a progressiva mecanização da lavoura e a facilidade de limpar e adubar a terra tem contribuindo para uma devastação acelerada de vegetação nativa e estima-se que cerca de 40% do bioma já tenha sido desmatado.

O Brasil possui forte tradição na utilização de plantas para manutenção da saúde e tratamento de uma grande variedade de sintomas como febre, inflamações, dores, etc. Entretanto, embora a tradição popular seja muito forte, há pouquíssimos estudos científicos que comprovam a relação entre a atividade biológica e o uso etnobotanicos de forma a validá-los. Adicionalmente, há pouca ou nenhuma informação que possa assegurar que essas plantas não são tóxicas.

Dentro desse contexto, ou seja, utilização de plantas e comprovação de benefícios, a importância de compostos antioxidantes naturais vem despertando grande interesse entre cientistas e consumidores, apresentando uma forte tendência mundial em relação aos alimentos funcionais com beneficio específico de saúde.

A oxidação é um processo metabólico que leva à produção de energia necessária para as atividades essenciais das células. Entretanto, o metabolismo do oxigênio nas células vivas também leva à produção de radicais livres de oxigênio (ROS), comumente denominados de espécies reativas de oxigênio. Na ausência de defesa antioxidante endógena adequada, a propagação dos radicais livres pode levar a co-oxidação de constituintes celulares nucleofílicos bem como produtos secundários da oxidação lipídica podem reagir com macromoléculas nucleofílicas como constituintes de membranas, enzimas e DNA. Ainda, o excesso de radicais livres circulando no corpo oxida as proteínas

de baixa densidade (LDL), tornado-as potencialmente letais, acelera o processo de envelhecimento e tem sido relacionado a uma série de patologias crônicas e degenerativas como derrame cerebral, diabetes mellitus, artrite, Parknson, Alzheimer e câncer. Estudos clínicos e epidemiológicos têm mostrado evidências de que antioxidantes fenólicos de cereais, frutas e vegetais são os principais fatores que contribuem para a baixa e significativa redução da incidência de doenças crônicas e degenerativas encontradas em populações cujas dietas são altas na ingestão desses alimentos. Compostos típicos que possuem AAO incluem a classe de fenóis, ácidos fenólicos e seus derivados, flavonóides, tocoferóis, fosfolipidios, aminoácidos, ácido fítico, ácido ascórbico, pigmentos e esteróis.

Neste estudo foi avaliada a atividade biológica “in vitro” e “in vivo” dos compostos do metabolismo secundário de diferentes extratos de frutas nativas do cerrado, com ênfase na AAO e toxicidade, a fim de avaliar o potencial desses extratos como antioxidante natural para possíveis aplicações nos setores farmacêutico, cosmético e alimentício.

Espera-se que os resultados obtidos por meio desse estudo apresentem uma aplicação economicamente viável e ambientalmente correta dos recursos desse bioma que vem sendo rapidamente devastado para criação de áreas de pastagens ou plantio de oleaginosas como a soja.

REVISÃO BIBLIOGRÁFICA

Cerrado Brasileiro

A região do cerrado abrange aproximadamente uma área de 204 milhões de hectares distribuída principalmente nos estados de Minas Gerais, Goiás, Mato Grosso, Mato Grosso do Sul, Tocantins, Bahia, Piauí, Maranhão e Distrito Federal (Silva & Tassara, 2001). O Cerrado é o segundo maior bioma da América do Sul, perdendo em tamanho somente para a Floresta Amazônica. Ocupa 25% do Brasil e é também o mais brasileiro dos biomas sul-americanos, pois, excetuando algumas pequenas áreas na Bolívia e no Paraguai, está totalmente inserido no território Nacional (Proença et al., 2000).

O cerrado vive uma perpetua oscilação entre a época das chuvas e época da seca. Durante a estação da seca, que é no inverno pode ficar de dois a seis meses sem chover, dependendo do ano e região (Dias, 1992). A vegetação sofre, mas rebrota rapidamente, pois as plantas do cerrado têm muitas adaptações para suportar as secas e as queimadas com as quais tem evoluído por milhões de anos. As freqüentes chuvas do verão também oferecem condições ideais para a germinação e o estabelecimento das pequeninas plântulas, que precisam ser profundamente enraizadas para enfrentar a seca que não demora a chegar (Oliveira, 1998).

Sua flora riquíssima só agora começa a ser conhecida, existindo cerca de 1000 espécies de árvores, 3000 espécies de ervas ou arbustos e quase 500 trepadeiras (Mendonça et al., 1988). Nos últimos 30 anos, a progressiva mecanização da lavoura e a facilidade de limpar e adubar a terra tem contribuindo para uma devastação acelerada de vegetação nativa e estima-se que cerca de 40% do bioma já tenha sido desmatado (Ratter et al., 1997). O plantio em larga escala de soja, milho, arroz e o desmatamento para extração de carvão são os principais inimigos do cerrado, e infelizmente, somente 1,5% é preservado na forma de Reservas (Dias, 1992). Assim, o cerrado brasileiro enfrenta duas realidades diferentes: a grande possibilidade de produção de alimentos, sendo considerado o maior celeiro do mundo e por outro lado, a riquíssima biodiversidade que está sendo recentemente descoberta e conhecida.

3.2. Frutas do Cerrado Brasileiro

Muitos frutos das plantas nativas do cerrado, provenientes de atividade extrativista e muitas vezes predatória, são comercializadas e consumidas in natura ou beneficiados por indústrias caseiras na forma de sorvetes, sucos, licores, geléias com grande aceitação popular. A maioria desses frutos possui elevados teores de açúcar, proteína, vitaminas e sais minerais, além de sabor muito característico (Silva et al., 1994).

O Brasil possui forte tradição na utilização de plantas para manutenção da saúde e tratamento de uma grande variedade de sintomas como febre, inflamações, dores, etc. Entretanto, embora a tradição popular seja muito forte, há pouquíssimos estudos científicos que comprovam a relação entre a atividade biológica e o uso etnobotânicos de forma a validá-los. Adicionalmente, há pouca ou nenhuma informação de forma a assegurar que partes dessas plantas não são potencialmente tóxicas ou podem causar interações medicamentosas.

O critério para escolha de plantas para investigação de atividade biológica como a AAO é um exercício importante uma vez que todas as plantas elaboram metabólitos secundários que podem apresentar potencial de uso medicinal. Segundo Williamson et al., 1996 os seguintes aspectos são importantes para essa seleção de plantas:

1. Seleção baseada no uso tradicional: essa estratégia pode ser escolhida principalmente em sociedades onde a medicina tradicional é utilizada como uma forma de tratamento de doenças.
2. Plantas tóxicas: a busca por componentes potentes e altamente específicos que possam ser relacionados a uma determinada atividade biológica é mais produtiva entre plantas tóxicas do que em plantas utilizadas regularmente na medicina tradicional.
3. Composição química: essa escolha é realizada principalmente em função de facilidades de equipamentos de laboratório, onde as plantas sofrem uma extração direcionada para classes de compostos específicos e faz se seleção por vários modelos.

4. Seleção de atividade biológica específica: utilizando ou não um banco de dados, muitas plantas escolhidas por critérios distintos são estudados para determinada ação biológica por diferentes modelos. Essa seleção aleatória não é menos produtiva que a seleção de plantas por uso tradicional.
5. Combinação de critérios: utilização de maior quantidade de critérios possíveis, uma vez que essa busca é muito dispendiosa em termos de materiais e expertise.

As frutas do cerrado enfrentam a estação da seca, permanecendo de dois a seis meses sem chuvas. Desta forma essas plantas possuem muitas adaptações para resistir ao sol, as secas e as conseqüentes queimadas e por esses motivos essas plantas têm evoluído por milhões de anos. Estudos afirmam que plantas tropicais que recebem luz em excesso sofrem foto-oxidação. Sob alta quantidade de luz e calor, a síntese e atividade de enzimas antioxidantes tendem a aumentar nessas plantas a fim de seqüestrar radicais livres que são tipicamente produzidos nessas condições e que são tóxicos as membranas (Demming-Adams and Adams, 1992; Chow, 1994). Assim, as plantas do cerrado apresentam-se como uma alternativa potencial para avaliação de atividade antioxidante. Adicionalmente, as plantas do cerrado são muito utilizadas por populações nativas para o tratamento de doenças diversas, conforme apresentado na Tabela 1.

Tabela 1. Dados etnobotânicos das Frutas dos Cerrado

Nome Científico	Família	Parte usada	Preparo	Nome popular	Utilização popular
Annona crassiflora; Annona rodriguesii	Annonaceae	semente		araticum, marolo, cabeça de negro	picada de cobra (1)
		sementes, folhas	água		antidiarréico, indutor de menstruaçao (2,3)
		sementes	óleo		afecções do couro cabeludo (2)
Eugenia dysenterica; Stenocalyx dysentericus	Myrtaceae	frutas		cagaita	efeito purgativo (2)
		folhas			antidiarreico (2)
Caryocar brasilense	Caryocaraceae	amêndoas	óleo	pequi, piqui, piquia	asma, bronquite, coqueluche (2,3)
		sementes	óleo		asma, bronquite, coqueluche e gripe (2,3)
		sementes	etanol		afrodisiaco, tonico (3)
		folhas	água		regulador fluxo menstrual (2)
Solanum lycocarpum	Solanaceae	folhas	água	lobeira, fruta do lobo	emoliente, anti-reumática (3)
		frutas e flores	água		asma, gripes e resfriados (2,3)
		raízes	água		hepatite (2)
		frutos verdes			diabetes (2)
Swartzia langsdorfii	Leguminosae			banha de galinha	

(1) Weinberg et. al., 1993
(2) Almeida et. al., 1998
(3) Rodrigues et. al., 2001

3.2.1. Araticum

Os frutos do araticum (*Annona crassiflora)* são também conhecidos como marolo, pertencem à família das Annonaceae e são coletados entre fevereiro e março. Os frutos são utilizados na alimentação e são muito apreciados por sua polpa doce, amarelada e de aroma bastante forte. A árvore do araticum é útil para plantio em reflorestamentos mistos de áreas degradadas de preservação permanente, tanto pela sua adaptação a solos pobres como pela produção de frutos apreciados pela fauna. As sementes possuem ação contra afecções parasitárias do couro cabeludo, depois de pulverizadas, são misturadas ao óleo e faz-se massagem no cabelo. Na medicina popular, a infusão das folhas e das sementes pulverizadas serve para combater a diarréia e induzir a menstruação (Almeida et al., 1994; Lorenzi, 1988; Silva et al., 1994).

A família de annonáceas possui uma grande variedade de frutos exóticos, aparentemente rústicos e com forma típica como a fruta do conde *(Annona squamosa*), graviola (*Annona muricata*) e o araticum. (Silva & Tassana 2001). Nos últimos 15 anos, estudos fitoquímicos e farmacológicos das annonáceas tem se intensificado em função da descoberta de uma classe de compostos denominados acetogeninas tetrahidrofurânicas ou acetogeninas annonáceas com uma grande variedade de atividade biológica como

antitumor, antimalarial, antiprotozoários e com atividades pesticidas e contra picadas de cobra (Weinberg et al., 1993; Kim et al, 1998; Alali et al, 1999; Galvis-Betancur et al., 1999).

3.2.2. Banha de Galinha

A Banha de galinha, também conhecida por banana de papagaio e pacová-de-macaco, é da família das leguminosae (*Swartzia langsdorfii Radlk.*) e seus frutos são coletados de agosto a outubro. A árvore de banha de galinha é pequena e notável pelos frutos muito grandes (tamanho de uma manga comum) com arilos polposos, cor de laranja, de sabor e cheiro repugnantes ao homem, porem muito apreciado pelas antas (Braga,1976; Silva et al., 1994).

A atividade contra moluscos, principalmente no controle de esquistossomose, foi reportada na Tanzânia por um fruto da família das Swartzia (Borel et al. 1987). Recentemente, a Swartzia langsdorfii também demonstrou atividade contra B. glabrata, importante intermediário hospedeiro no do esquistossomo (Magalhães et al., 2003).

3.2.3. Cagaita

A cagaita (*Eugenia dysenterica*) ocorre no cerrado ou cerradão e frutifica entre outubro e dezembro (Silva et al., 1994). O uso alimentar é bastante difundido na região, sendo consumido ao natural, devendo apenas ser tomadas algumas precauções em relação à quantidade ingerida, uma vez que pode tornar-se laxante, principalmente quando fermentados ao sol. Quanto ao seu uso medicinal, além do efeito purgativo dos frutos, a garrafada das folhas produz efeitos contrários, sendo antidiarréico e também utilizada para combater problemas cardíacos (Almeida et al., 1994).

Recentemente, a atividade antifúngica tem sido estudada nos constituintes voláteis do óleo das folhas da cagaita, sendo que atividade antimicrobiana já foi relatada no óleo essencial e no suco do gênero Eugênia (Costa et al, 2000) .

3.2.4. Lobeira

A lobeira (*Solanum lycocarpum*) também conhecida como fruto do lobo ocorre no cerrado, cerradão e campo sujo, sendo seus frutos produzidos de julho a janeiro (Silva et al., 1994). Os frutos da lobeira são comestíveis e reputados como medicinais. A polpa é enjoativa, possui cheiro muito ativo e penetrante e contém alcalóides de natureza pouco conhecida. A infusão da raiz da loberia é usada contra hepatite e o xarope dos frutos contra asma. Um pó branco extraído do fruto verde é também utilizado para combater diabetes. Os frutos verdes contêm solasodina, substância química precursora de esteróides . Esteróides obtidos a partir da transformação da solasodina são utilizados para fabricação de anticoncepcionais, anabolizantes e antiinflamatórios (Almeida et al., 1994; Lorenzi, 1988). Recentemente, foi demonstrado que o alcalóide presente no extrato etanólico de *Solanum lycocarpum* pode possuir efeitos anti-inflamatórios (Vieira et. al., 2003). Adicionalmente, muitos estudos tem sido realizados para demonstrar a ação hipoglicêmica no combate ao diabetes e para avaliar os efeitos tóxicos do consumo crônico da planta (Maruo et. al., 2003; Dall'Agnol et. al., 2000; Oliveira et. al., 2003)

3.2.5. Pequi ou Piqui

O pequi (*Caryocar brasiliense*) assim como o piquiá (*Caryocar villosum*) ocorre no cerrado, cerradão e mata calcária, sendo seus frutos produzidos de outubro a março (Silva et al, 1994). A polpa do pequi contém uma boa quantidade de óleo comestível e é rico em vitamina A e proteínas, transformando-se em importante complemento alimentar. A amêndoa do pequi, pela alta quantidade de óleo que contém e por suas características químicas, pode ser também utilizada com vantagem na indústria de cosmética para a produção de sabonetes e cremes. Ambos os frutos de pequi e piquiá possuem a mesma característica, sendo que a grande e notável diferença entre as duas espécies reside no tamanho da planta como um todo (Silva & Tassara, 2001). Em relação a seu uso medicinal, o óleo da polpa tem efeito tonificante, sendo usado contra bronquites, gripes e resfriados e no controle de tumores. O chá das folhas é tido como regulador do fluxo menstrual. Da

casca e das folhas extraem-se corantes amarelos de ótima qualidade, empregados pelos tecelões em tinturaria caseira, contendo alto teor de tanino (Almeida et. al., 1994).

Recentemente, a cera epicuticular e óleo das sementes dessa planta têm sido avaliados quanto a atividade antifúngica (Passos et. al., 2002).

3.3 Oxidação e Saúde

A oxidação é um processo metabólico que leva a produção de energia necessária para as atividades essenciais das células. Entretanto, o metabolismo do oxigênio nas células vivas também leva a produção de radicais (Adegoke, et. al., 1998; Mccord, J. M., 1994). Oxidantes são compostos produzidos pelo metabolismo normal do corpo e, se não controlados, podem provocar danos extensivos. Na ausência de defesa antioxidante endógena adequada, a propagação dos radicais livres pode levar a co-oxidaçao de constituintes celulares nucleofilicos bem como produtos secundários da oxidação lipídica podem reagir com macromoléculas nucleofilicas como constituintes de membranas, enzimas e DNA. Ainda, o excesso de radicais livres circulando no organismo oxida as proteínas de baixa densidade (LDL), tornando-as potencialmente letais, acelera o processo de envelhecimento e tem sido relacionado a uma série de patologias crônicas e degenerativas como derrame cerebral, diabetes mellitus, artrite, Parknson, Alzheimer e câncer. (Ames, et. al., 1995; Ames, et. al., 1993; Christen, Y., 2000; Diaz, et. al., 1997; Lang & Lozano, 1998; Shahidi, F., 1996). O balanço entre o stress oxidativo e as funções antioxidantes dos organismos vivos parece ter um papel relevante na carcinogênese (Weisburger 1999; Wettasinghe, et. al., 2002). Estudos clínicos e epidemiológicos têm mostrado evidências que antioxidantes fenólicos de cereais, frutas e vegetais são os principais fatores que contribuem para a baixa e significativa redução da incidência de doenças crônicas e degenerativas encontradas em populações cujas dietas são altas na ingestão desses alimentos (Shahidi, F., 1996). Compostos típicos que possuem AAO inclui a classe de fenóis, ácidos fenólicos e seus derivados, flavonóides, tocoferóis, fosfolipídios, aminoácidos, ácido fítico, ácido ascórbico, pigmentos e esteróis. Antioxidantes fenólicos são antioxidantes primários que agem como terminais para os radicais livres (Xing & White, 1996).

Desta forma, a importância da pesquisa por antioxidantes naturais tem aumentado muito nos últimos anos (Jayaprakasha & Jaganmohan, 2000).

3.3.1 Oxidação de lipídios

A oxidação lipídica não é apenas um problema de óleos comestíveis e de alimentos, mas também do corpo humano. Excesso de radicais livres de oxigênio, particularmente radicais hidroxilas, pode afetar membranas celulares lipídicas de forma a produzir hidroperóxidos e espécies reativas de oxigênio (ROS) que são relacionadas a uma variedade de doenças bem como ao processo de envelhecimento (Shahidi, F., 1996). A peroxidação lipídica é importante "in vivo" principalmente porque contribui para o desenvolvimento de aterosclerose (Esterbauer, et. al., 1991; Esterbauer, et. al., 1992). Enquanto os hidroperóxidos, compostos primários da oxidação lipídica, são incolores e inodoros, eles rapidamente produzem uma série de compostos secundários com alcanos, álcoois, aldeídos e ácidos. Adicionalmente, polímeros e outros compostos indesejáveis são produzidos, sendo que muitos desses produtos da oxidação secundária são altamente reativos, como por exemplo, o malonaldeido (MDA) e 4-hidroxi-2-nonenal (HNE) (Benedetti, et al., 1980). MDA E 4-HN são conhecidos por interagir com componentes biológicos como proteínas, aminoácidos, aminas e DNA, sendo que essas reações implicam em envelhecimento, mutagênese e carcinogênese das células do corpo (Fujimoto, et. al., 1984). MDA e HNE são formado largamente durante a peroxidação de ácidos graxos poliinsaturados (PUFAs) com mais de duas duplas ligações, como ácido linolênico, araquidônico e docosahexaenoico . Grandes quantidades de MDA são também formadas durante a peroxidação de microssomas hepáticos na presença de ferro. MDA é oxidado a semialdeido, acido dicarboxilico de ácido málico, que por sua vez é descaboxilado a acetadeído, substrato de oxidação das enzimas aldeídos desidrogenases a acetato. Em relação ao HNE, as células podem reduzir o aldeído livre em álcool pela aldose redutase ou oxidá-lo a ácido caboxílico pela aldeído desidrogenase. Adicionalmente, HNE pode formar conjugados com glutationa de forma não enzimática ou enzimaticamente por meio da glutationa transferase (Halliwell & Gutteridge, 1999).

3.3.2 Oxidação de Proteínas

Os danos oxidativos para proteínas podem ser de particular importância "in vivo" pois afeta a função de receptores, enzimas e proteínas transportadoras, podendo gerar antígenos que provoquem respostas imunes, bem como pode contribuir para os danos secundários a biomoléculas como inativação das proteínas reparadoras do DNA e perda de fidelidade das DNA polimerases na replicação do DNA (Halliwell, B., 1978; Wiseman & Halliwell, B., 1996).

Os danos às proteínas podem ocorrer por ataque direto das espécies reativas de oxigênio e nitrogênio (ROS/RNS) ou por danos secundários envolvendo o ataque das proteínas por produtos secundários da peroxidação lipídica como MDA e HNE. Proteínas modificadas podem surgir do metabolismo "in vivo" mas também por meio da dieta alimentar, como por exemplo, carnes cozidas. Quando uma proteína e danificada "in vivo" por ROS/RNS, elas são geralmente marcadas para degradação proteolítica e removidas, aumentando a eliminaçao de proteínas em celulas com strees oxidativo. Entretanto, proteínas fortemente degradadas e/ou agregados de proteínas podem resistir ao ataque proteolítico e acumular nas células. A habilidade das células em degradar proteínas anormais decresce em função da idade (Halliwell & Gutteridge, 1999).

3.3.3 Oxidação do DNA

Há crescentes evidencias que ROS/RNS estão involvidos no desenvolvimento de câncer, não apenas por efeitos diretos ao DNA mas também por afetar sinais de tradução e transcrição, proliferação celular, morte celular e comunicação intercelular (Halliwell & Gutteridge, 1999). A química dos danos causados ao DNA pelos radicais ROS/RNS (espécies reativas de oxigênio e nitrogênio) são bem caracterizados "in vivo" (Aruoma, et. al., 1989; Spencer et. Al., 1996). O oxido Nítrico e seus derivados NO_2', HNO_2, ONOO'e N_2O_3 podem causar nitrosação e desaminação dos grupos aminas das bases do DNA levando a pontos de mutação. O O_2' e H_2O_2 a nível patológico e fisiológico parece não reagir com as bases do DNA. Entretanto, o radical OH' gera uma multiplicidade de produtos das quatro bases (Aruoma, et. al., 1989; Halliwell, B., 1978). Uma fonte de produção de radical OH' pode ser a radiação, mas a radiação gera OH' por toda a célula e

apenas uma pequena quantidade atinge o DNA (Nackerdien, et al., 1992). Outra potencial fonte de OH' e espécies de OH' é a decomposição do radical ONOO' e a reação de O_2' com HOCl (Candeias, et. al., 1993). O correto funcionamento das enzimas de reparo do DNA é essencial à sobrevivência dos organismos aeróbicos sem excessivas taxas de mutação. Entretanto, o fato de DNA isolados de organismos aeróbicos conterem baixos níveis de produtos alterados (inclusive humanos) sugere que o reparo realizado pelas enzimas não conseguem remover completamente os danos das bases (Halliwell & Gutteridge, 1999).

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3.4 Antioxidantes Naturais de Plantas

Assim como nas células aeróbicas em que o metabolismo do oxigênio, responsável pela produção de energia, leva também a produção de radicais livres, os produtos secundários da fotossíntese podem também produzir altos níveis de espécies reativas de oxigênio. Portanto, as plantas usam uma variedade de compostos antioxidantes para lidar com esses ROS a fim de sobreviver. Muitos desses compostos possuem estruturas básicas similares nas quais todos têm pelo menos um anel aromático e um grupo hidroxila. Esses compostos incluem os ácidos fenólicos, flavonóides e isoflavonas, éster galatos (taninos hidrolizados), ligninas, cumarinas, estilbenes, flavononas e proantocianidinas oligoméricas. Juntos, esses compostos produzem uma variedade de antioxidantes que podem agir por diferentes mecanismos, conferindo um sistema de defesa eficiente contra o ataque de radicais livres (Shahid, F., 1996).

Desta forma, as plantas proporcionam uma rica fonte de antioxidantes naturais (Caragay, A. B. 1992) nos quais estão incluídos os tocoferóis, vitamina C, carotenóides e compostos fenólicos. Os compostos fenólicos de plantas são considerados protetores contra danos nos tecidos pois se oxidam e combinam-se com proteínas e outros componentes. Adicionalmente, compostos fenólicos de plantas podem funcionar como sistema de defesa contra herbívoros (Harbone, J. B., 1994).

Tabela 2. Fontes Naturais de Antioxidantes

Vitamina E (Tocoferóis e tocotrienóis)	Oleaginosas, óleo de palma, amêndoas, ovos, produtos lácteos, grãos integrais, vegetais, cereais
Vitamina C	Frutas e vegetais, frutas cítricas, amêndoas, pimentas, "berries", etc
Carotenóides	Folhagens verde escuras, cenouras, tomates, frutas cítricas, óleo de palma, etc
Flavonóides/Isoflavonas	Frutas e vegetais, frutas cítricas, amêndoas, pimentas, cebola, vegetais crucíferos, etc
Ácidos Fenólicos/Derivados	Cereais, grãos integrais, algumas oleaginosas e óleos
Catequinas	Chá verde, "berries", algumas oleaginosas

Fonte: Shahid, F. Natural Antioxidants: An Overview "in" Natural Antioxidants Chemistry, Health Effects, and Applications. AOCS Press: Champaign, Illinois, 1996, p. 1-11.

3.5 Compostos Fenólicos de Plantas

Compostos fenóis e polifenólicos de plantas são componentes largamente consumidos na dieta humana e encontrados nos vegetais, frutas, cereais, chá, café, ervas e pimenta. Os compostos fenólicos, além de proporcionar AAO que prolongam a vida de prateleira de produtos, têm demonstrado importante atividade "in vivo" e podem ser benéficos no controle de diversas doenças relacionadas à formação excessiva de radicais livres que excedem a capacidade de defesa antioxidante do corpo humano como aterosclerose, cataratas e câncer. Certos processos patológicos são conseqüências da ação de ROS, por exemplo, a aterosclerose é relacionada à peroxidação lipídica, o envelhecimento e a formação de cataratas estão relacionados à oxidação de proteínas e alguns tipos de câncer são resultantes da modificação do DNA induzida por radicais. Antioxidantes polifenólicos têm efeitos positivos que podem contra reagir muita dessas reações adversas porque possuem a habilidade de terminar reações de radicais em cadeia, seqüestrar RSO e prender eletrófilos (Offord et. al., 1997).

Um composto fenólico possui pelo menos um anel benzeno ligado a um grupo –OH. Esses compostos exercem poderosa AAO "in vitro" pois inibem a peroxidação lipídica por meio da ação sequestrante sobre os radicais peroxil. Os compostos fenólicos podem ainda seqüestrar os ROS tais como radicais OH, ONOOH e HOCl. Compostos fenólicos com dois grupos –OH adjacentes podem também ligar metais de transição como ferro e cobre

formando compostos pobremente reativos na promoção das reações dos radicais livres (Halliwell & Gutteridge, 1999).

Recentemente, duas observações chamaram atenção para o papel antioxidante dos compostos fenólicos "in vivo". Pela constatação "in vitro" de que os fenóis presente no vinho tinto inibiam a oxidação de lipoproteínas de baixa densidade (LDL) , sugeriu-se uma explicação para o Paradoxo Francês, ou seja, embora haja grande prevalência de fatores que contribuem para a doença cardiovascular em determinadas áreas da França como cigarro e o alto consumo de gorduras, nas mesmas áreas há baixíssima incidência de ataques cardíacos (Frankel, EN, 1995). Outra observação resultou no estudo epidemiológico realizado na Holanda (Zuthen Study) o qual sugeriu a relação inversa entre a incidência de doenças coronárias e derrame em homens idosos com a ingestão de flavonóides (especialmente quercetina), provenientes de chá, vegetais (cebolas) e frutas (maçã) na população examinada (Keli et. Al., 1996).

3.6 Antioxidantes Endógenos

A evolução da tolerância ao O_2 tem permitido aos organismos aeróbicos o uso de O_2 para transformações metabólicas (enzimas oxidases e hidrolases) e para aumentar a eficiência da produção energética (80% do ATP necessário para as células aeróbicas são gerados por meio do transporte de elétrons pela mitocôndria). Entretanto, o metabolismo do oxigênio nas células vivas também leva a produção de radicais.

Alguns antioxidantes são sintetizados pelo organismo humano como superoxido dismutase, catalase, glutationa peroxidase, urato, ubiquinol. Há muitas evidências que sugerem que antioxidantes endógenos não previnem completamente os danos causados por ROS e RNS (Chance, et al., 1976; Rubbo, et. al., 1994).

Enzimas envolvidas na detoxificação de ROS e peróxidos de lipídios incluem superoxide dismutase (Cu/Zn-citoplasma) e (Mn-mitocôndria), catalase (CAT: peroxisomas), enzimas glutationa do ciclo redox: selênio-dependente como glutationa peroxidase (GSH-Px) e glutationa redutase (GSSG-Red; citoplasma e mitocôndria) e finalmente as não selênio dependentes glutationa-S-transferases (citoplasma). Antioxidantes celulares não enzimáticos ativos "in vivo" incluem ∂-tocoferol, β-caroteno e

ascorbil palmitato que estão presentes nas membranas, assim com os tripeptídios de glutationa (GSH) presente no citoplasma. Antioxidantes extracelulares incluem componentes do plasma como ácido úrico, bilirubina e carnosina.

Na ausência de defesa adequada dos antioxidantes endógenos, a propagação dos radicais livres pode levar a co-oxidação de constituintes celulares nucleofílicos assim com reações de produtos secundários da oxidação lipídica com macromoléculas nucleofílicas, como constituintes da membrana, enzimas ou DNA (Fraga et. al., 1989; Reed, D. J. 1989). Esses eventos podem levar a disrupturas das membranas celulares e a morte da célula (citotoxicidade), levando a danos no tecido. A significância patológica dos peróxidos de lipídios e danos causados por radicais livres continuam sendo investigada e demonstrada. Por exemplo, o malonaldeído (OHC-CH2-CHO), produto secundário da autoxidação de ácidos graxos poliinsaturados (PUFA), é capaz de se ligar com proteínas, inativando enzimas e interagir com o DNA celular. Danos ao DNA causados pelos radicais OH', reações com lipídios livres ou aberrações cromossômicas podem estar envolvidos na inicialização da carcinogênese. A relação entre envelhecimento e o desenvolvimento de doenças crônicas como câncer e arteriosclerose é refletido pela progressão desses resultados biológicos durante muitos anos. Fatores nutricionais e hábitos alimentares têm um efeito significativo na regulação da atividade de enzimas antioxidantes assim como na concentração em vivo de vários antioxidantes não enzimáticos (Yuan, & Kitts, 1996).

Um mecanismo específico para quimio-prevenção de câncer é a habilidade de induzir enzimas detoxificantes (enzimas da fase 1 e fase 2) no fígado e em outros órgãos (Talay et al., 1995). Enzimas do metabolismo xenobiótico pertencem a duas famílias. Enzimas da fase 1 (citocromos P-450) que funcionalizam compostos, geralmente por oxidação ou redução. Embora o papel primário das enzimas de fase 1 seja detoxificar xenobióticos, vários citocromos P-450 podem ativar pró-carcinógenos à carcinógenos altamento reativos (Miller & Miller, 1985). Enzimas da fase 2 (catalase, superoxido dismutase, glutationa do ciclo redox) conjugam produtos funcionalizados com ligantes endógenous como glutationa e ácido glucurônico, possuindo um papel primário de detoxificação (Jakoby & Ziegler, 1990). Uma vez que o mecanismo regulador da neoplasia é o balanço entre as enzimas da fase 1 que ativam carcinógenos e enzimas da fase 2 que detoxificam-os, os indutores monofuncionais, aqueles que induzem as enzimas da fase 2 mas não as da fase 1, são

preferidas aos bifuncionais (Prochaska & Santamaría, 1988; Talalay et al., 1987; Talalay P. 1989). Várias evidências promovem convincente suporte para a proposição de indutores de enzimas de metabolismo xenobiótico, particularmente as enzimas da fase 2, como protetores contra os efeitos tóxicos e tumorais de carcinógenos (Talalay et. al., 1987; Talalay P. 1989).

Muitos compostos aparentemente não relacionados (incluindo antioxidantes fenólicos, coumarinas, cinamatos, isotiocianatos, lactonas) podem proteger roedores contra carcinógenos em condições que invariavelmente evocam a indução de enzimas da fase 2 em muitos tecidos. Adicionalmente, novos anti-carcinógenos tem sido isolados e identificados somente com base na sua habilidade em induzir enzimas da fase 2 (Lam, et. al., 1982; Miller & Miller, 1985). Tais anti-carcinógenos alteram o metabolismo de carcinogênese e diminuem a formação de metabólicos mutagênicos (Kensler, et. al., 1987; Wattenberg, et. al., 1976). Os indutores de enzimas anti-carcinogênicas protegem contra uma vasta variedade de carcinógenos com estrutura não similar, sugerindo que o mecanismo envolvido não pode ser estruturalmente meticuloso, como o metabolismo xenobiótico. Enzimas como glutationas transferases, quinona redutase QR; NAD(P)H: (aceptor de quinona) oxidoredutase, EC 1.6.99.2, UDP-glucoronosiltransferases quando encontradas em quantidades elevadas protegem contra a toxicidade dos carcinógenos. Células em que as glutationas transferases são induzidas por meio do desenvolvimento de resistência a agentes alcalinos quimioterápicos ou por transferência com enzimas clonadas, mostram diminuição na susceptibilidade à toxicidade de carcinógenos e reduzem a formação de adutos de DNA (Black, et. al., 1990; Puchalsk & Fahl, 1990; Waxman, D. J. 1990).

Desta forma, muitos modelos experimentais recentes avaliam a habilidade de extratos vegetais com AAO ”in vitro” em induzir enzimas da fase 1 e 2 em roedores. Adicionalmente, modelos nos quais se provoca uma indução química de hepato-toxicidade em ratos ou camundongos por meio da administração de CCl_4/paracetamol têm sido amplamente utilizados para avaliar o mecanismo celular de danos causados pela oxidação (Basu, 2003). O CCl_4 é ativado pelo citocromo P450 (CYP) 2E1, 2B1/B2 e possivelmente pela isoforma 3A formando radicais triclorometil (CCl_3*) e triclorometil peroxido (CCl_3OO*) que causam peroxidação lipídica e subseqüentes danos aos tecidos (Weber and

Boo, 2003). O aumento da peroxidação lipídica e a redução de antioxidantes endógenos é uma característica de ratos tratados com CCl_4 (Sipes et al., 1977). Níveis tóxicos de CCl_4 produzem acumulo de gordura no fígado e sua estrutura torna-se distorcida, reduzindo a síntese protéica e a atividade de enzimas como glucose-6-fosfatase e citrocromos P450. Assim, elevado número de estudos induzem a hepatotoxicidade em ratos a fim de estudar "in vivo" o efeito protetor de extratos vegetais diversos na manutenção de níveis normais de antioxidantes endógenos como citocromo P450, superoxide dismutase (SOD), catalase (CAT), enzimas glutationa do ciclo redox: selênio-dependente como glutationa peroxidase (GSH-Px) e glutationa redutase (GSSG-Red) e finalmente as não selênio dependentes glutationa-S-transferases (citoplasma) (Jayakumar, et al., 2006, Srinivasan et al., 2005; Singab et al., 2005 e Chidambara Murthy et al., 2002; Zhu et al., 1999). Outra forma de avaliar os danos de hepatotoxicidade no fígado é por meio da avaliação de enzimas do sangue dos animais como as aminotransferases: aspartato aminotransferase (AsAT) e alanina aminotransferase (ALAT) (Williamson, Okpako & Evans, 1996).

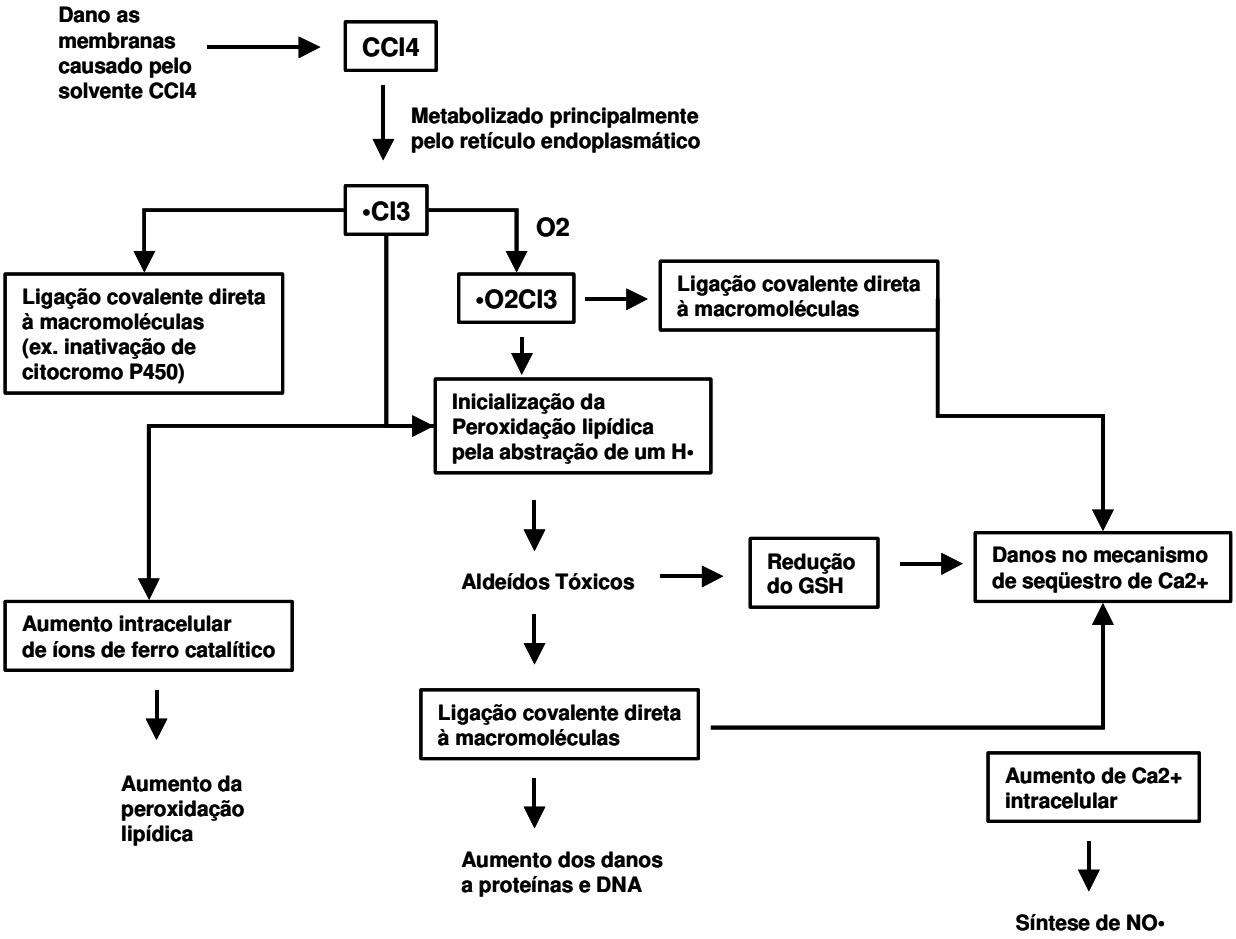

Figura 1. Mecanismos de hepatotoxicidade do CCl_4

Tabela 3. Antioxidantes Endógenos

Não Enzimáticos	Localização	Função
Albumina	plasma	Ligar íons de ferro e cobre
Ácido Ascórbico	plasma	Seqüestrar O_2'; regenerar α - tocoferil
β - Caroteno	plasma e membranas celulares	Doador de elétrons e seqüestrar O_2'
Glutationa	plasma, citosol intracelular e mitocôndria	Seqüestrar radicais livres, manter potencial redox do grupo tiol
α - Tocoferol e isômeros	plasma e membranas celulares	Doador de elétrons e H^+, seqüestrar O_2'; seqüestrar radicais livres,
Ubiquinona	plasma	
Enzimáticos	**Localização**	**Função**
Catalase (CAT)	intracelular	Metabolizar H_2O_2 em H_20 e O_2
Superoxide dismutase (SOD)	plasma, leite e intracelular (citosol e mitocôndrias)	Converter O_2' em H_20
Glutationa Peroxidase (GSH-Px)	plasma e intracelular	Inativar hidroperóxidos utilizando equivalentes reduzidos de GSH
Glutationa Redutase (GSSG-Red)	intracelular	Regenerar GSH do GSSG
Glutationa S-Transferase (GST)	intracelular	Conjugar xenobióticos e agentes alcalinos com GSH para excreção

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3.6.1. Super Oxide Dismutase (SOD)

O radical superoxido (O_2') resultante da reação intracelular de radicais livres com dioxigênio ou gerado pelo metabolismo oxidativo pode ser desativado por meio da enzima superoxide dismutase - SOD (Fridovich I., 1975; Mccord & Fridovich, 1969). A SOD possui duas principais formas localizadas no citosol e na mitocôndria. As duas formas Cu/Zn-SOD (citosol) e Mn- SOD (mitocôndria) catalisam a conversão do radical (O_2') para peróxido de hidrogênio e oxigênio, conforme equações abaixo:

$$2\ O_2' + 2H^+ \rightarrow H_2O_2 + O_2$$

$$HO_2' + HO_2' \rightarrow H_2O_2 + O_2$$

A interação entre o metabolismo dos ROS e a atividade da SOD parece tem um papel importante na detoxificação de várias classes de radicais peroxils (Winterbourn, C. C., 1993). O conceito do radical superoxido agindo como um "sink" bioquímico para o metabolismo de radicais livres é baseado na produção de (O_2') pelos radicais livres com poder de redução, diretamente reagindo com oxigênio molecular conforme reação abaixo:

$$RH' + O_2 \rightarrow R + O_2' + H^+$$

Na presença da atividade da SOD, o equilíbrio da reação se desloca para a direita permitindo a desativação do radical livre, isso ocorre "in vivo" devido principalmente ao metabolismo do radical super oxido (Yuan & Kitts, 1996).

3.6.1. Catalase

A catalase (CAT) é localizada intracelularmente na mitocôndria e perioxomas. A atividade da CAT varia em função dos tecidos, sendo que o fígado e os rins possuem maiores quantidades, e os tecidos conectivos menores. A CAT decompõe H_2O_2 em água e oxigênio e também converte hidroperóxidos nos seus correspondentes álcoois na presença de um doador de hidrogênio (Aebi, H., 1974)

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$
$$ROOH + AH_2 \rightarrow H_2O + ROH + A$$

O H_2O_2 sozinho não é um composto altamente reativo em relação as moléculas biológicas, entretanto, é considerado um precursor de oxidantes mais reativos como radical hidroxila (HO)'.

3.6.2. Equivalentes de Glutationa ou "Glutationa total"

O tripepitídio de glutationa (y-glutamil cisteina glicina) é o principal triol livre nas células do organismo vivo e participa de diversos processos biológicos como detoxicação de xenobióticos, remoção de hidroperóxidos, proteção contra os efeitos da radiação ionizante entre outras.

O termo glutationa total engloba a glutationa (GSH) e a glutationa disulfito (GSSG), forma oxidada da glutationa (Theodorus et. Al., 1991)

O principal papel da GSH "in vivo" é como agente primário envolvido na desativação de radicais livres eletrofílicos.

$$2\ GSH + O_2 \rightarrow GSSG + H_2O_2$$

$$O_2' + GSH + H^+ \rightarrow GS' + H_2O_2$$

A oxidação da GSH como antioxidante "in vivo" ocorre com a produção de GSSG (glutationa oxidada) e peróxido de hidrogênio. A reação da GSH com o radical superoxido leva a formação do glutationil radical GS' que representa um risco potencial para outras reações de radicais livres caso não seja reduzido, como por exemplo oxidação de membranas biológicas. Desta forma, a GSH é um seqüestrador eficiente de radicais livres quando o radical GS' é removido ou quando o potencial de reação com ácidos graxos poliinsaturados é baixo (Yuan,& Kitts, 1996).

3.6.3. Glutationa Peroxidase

A Glutationa peroxidase (GSH-Px) é uma enzima que assim como a catalase, metaboliza o H_2O_2. As enzimas que são encontradas em tecidos animais são reconhecidas como sistema de proteção contra a peroxidação lipídica endógena e exógena por meio do poder de redução da glutationa (GSH). Essa enzima possui concentrações estequiométricas de selênio e reagem com uma variedade de hidroperóxidos orgânicos assim como com peróxido de hidrogênio derivados de lipídios insaturados (Wendel, A. 1981).

$H_2O_2 + 2GSH \rightarrow 2\ H_2O + GSSG$

$ROOH + 2GSH \rightarrow H_2O + ROH + GSSG$

3.6.4. Glutationa Redutase

A glutationa oxidada retorna a sua forma nativa por meio da ativiadade da enzima GSSH – Red e nicotinamida adenina dinucleotídio fosfato, conforme reação abaixo (Yuan,& Kitts, 1996):

$GSSH + NADPH \rightarrow 2GSH + NADP$

3.6.5. Glutationa Transferase

A enzima glutationa S-transferase (GTS) está envolvida na biotransformação de xenobióticos (detoxificação). Esse grupo de enzimas detoxifica principalmente agentes alquil. Essa enzima também metaboliza os reativos intermediários da metabolização de xenobióticos pela GSH (Yuan,& Kitts, 1996).

Tabela 4. Influência de Fatores Nutricionais na Atividade Antioxidante das Enzimas

Enzima	Tecido	Efeito na Atividade	Fator Nutricional
Catalase (CAT)	coração, fígado	aumenta	dieta restrita em energia
	pulmão	diminui	deficiência de ferro
	fígado, pulmão	aumenta	baixa ingestão de proteína
Glutationa Peroxidase (GSH-Px)	coração, fígado, rins	aumenta	dieta restrita em energia
		aumenta	baixa ingestão de proteína
	aorta	aumenta	alta quantidade de lipídios no plasma
	todos tecidos	diminui	deficiência de selênio
	fígado	diminui	obesidade
Glutationa Redutase (GSSG-Red)	aorta	aumenta	alta quantidade de lipídios no plasma
	todos tecidos	diminui	deficiência em riboflavina
	fígado	diminui	obesidade
Glutationa S-Transferase (GST)	fígado	diminui	ácidos graxos saturados e esteres de ácidos graxos
Cu/Zn - SOD	cérebro, fígado e rins	aumenta	dieta restrita em energia
	aorta, eritrócitos	diminui	deficiência de cobre
Mn - SOD	fígado	aumenta	baixa ingestão de proteína
	coração, fígado	diminui	consumo de óleo de peixe

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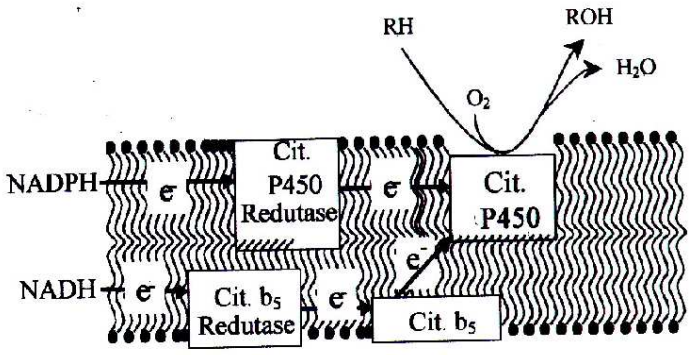
3.6.6. Citocromo P450 e b5

O citocromo P450 é uma hemoproteína que recebe este nome por exibir um pico em 450nm proeminente em espectros ópticos na região visível, quando reduzido por ditionito de sódio (DTN) e ligado ao monóxido de carbono (MO) (Omura e Sato, 1964). É uma enzima protagonista do sistema monooxigenase (SMO) de hepatócitos que possui um papel preponderante na biotransformação de xenobióticos (de Mateis, 1978). Conforme figura 3, o seu funcionamento consiste primariamente na quebra de um oxigênio molecular, para a inserção de um átomo na molécula representada por um radical R, e o outro átomo destinado à formação de uma molécula de água. Isto torna o R uma molécula mais polar, consequentemente mais solúvel, podendo ser transportado pelo sangue. Com isso, "compostos insolúveis em água que se acumulariam no organismo até atingirem níveis tóxicos, tornam-se suficientemente solúveis para que possam deixar a célula e serem excretados na urina (Alberts et al, 1997). O citocromo P450 representa uma vasta família de isoenzimas codificadas por 221 genes divididos em 26 sub-famílias (Nelson et al., 1993).

Frente a essa diversidade, diferentes isoformas de P450 são responsáveis por catalisar diferentes reações como dealquilação, carboxilação e hidroxilação (Omura e Sato, 1978). Em muitos casos, estas reações despertam interesse farmacológico e/ou toxicológico por promover a ativação de substâncias inertes ao organismo para compostos tóxicos (Bresnick, E. 1993)

Figura 4: Sistema monooxigenase do retículo endoplasmático de hepatócitos



O citocromo b5 também é uma hemoproteína e possui papel transportador de elétrons entre o citocromo b5 redutase e o citocromo P450, elétrons estes oriundos do NADH (Omura e Sato, 1978). Destaca-se em espectros visíveis de microssomos, reduzidos por NADH, em um pico preeminente em 424 nm.

Determinação de atividade antioxidante (AAO) - Considerações

A palavra antioxidante é muito utilizada mas raramente definida. Antioxidantes são substâncias que, quando presente em pequenas quantidades, comparados com o substrato a ser oxidado, significativamente retardam ou previnem a oxidação desse substrato. O termo substrato oxidável inclui qualquer tipo de molécula encontrada “in vivo”. Essa definição enfatiza a importância do substrato estudado “in vivo” e as fontes de ROS/RNS. Quando os ROS/RNS são formados “in vivo”, muitas substâncias antioxidantes desempenham uma ação protetora. A importância de cada antioxidante depende de qual ROS/RNS foi gerado, como e onde esses radicais foram gerados e a quantidade de substrato danificado (Halliwell & Gutteridge, 1999).

Os antioxidantes podem exercer as propriedades de proteção por mecanismos diferentes no processo de oxidação. Há dois tipos principais de antioxidantes, os denominados primários (quebra a cadeia de oxidação, seqüestra radicais livres) e os secundários (desativa metais, inibe a cadeia reativa dos hidroperoxidos, regenera os antioxidantes primários, seqüestra oxigênio singlet, etc) (Gordon, 1990).

Um grande número de métodos tem sido desenvolvido para avaliar a atividade antioxidante. Entretanto, a comparação entre esses métodos torna-se muito difícil uma vez que os métodos e os substratos utilizados são muito diferentes. Adicionalmente, formas de acelerar a oxidação do diversos substratos são empregadas a fim de aumentar a velocidade dos estudos. Desta forma, torna-se impossível expressar a AAO como um valor absoluto, sendo muito recomendável a utilização de padrões comparativos não importa o método escolhido. A melhor estratégia é utilizar padrões com natureza química e propriedades físico-químicas semelhantes às amostras estudadas. Da mesma forma, a fim de se obter um resultado significativo, o ambiente de estudo deve ser similar a situação real, ou seja, a AAO depende do substrato a ser oxidado e da associação entre o antioxidante e os componentes do substrato. Em função da grande complexidade entre os processos oxidantes e antioxidantes, nenhum método sozinho é capaz de promover uma interpretação da amostra antioxidante testada. Multi métodos devem ser empregados para avaliar a AAO de uma amostra. A combinação de variáveis como sensibilidade, rapidez, aplicabilidade, quantidade de amostra, disponibilidade de equipamentos, complementação entre resultados provenientes propriedades antioxidantes primarias e secundarias, correlação dos resultados com outros métodos e principalmente com o sistema real de oxidação devem ser avaliadas antes da escolha dos métodos mais apropriados (Koleva et al., 2002).

As diferenças entre mecanismos e sistemas de avaliação dos ensaios devem ser exploradas de forma a entender a reatividade dos compostos e os mecanismos de interação entre os solventes, os substratos e as concentrações de amostras.

De uma forma bastante resumida, é possível classificar em quatro categorias os métodos recentemente mais empregados na avaliação de AAO proporcionada por extratos vegetais.

1- Métodos que empregam modelos de radicais relativamente estáveis como o DPPH, ABTS, 2-desoxi-D-ribose, nos quais é avaliada a capacidade do extrato vegetal em doar hidrogênio a fim de formar não radicais, ou seja, seqüestrar os radicais livres (OH') formados durante a oxidação. Esses métodos costumam ser rápidos, simples e muito convenientes para o "screening" de grande numero de amostras.

2- Modelos contendo lipídios ou emulsões lipícas como β - Caroteno Bleanching Test, muito utilizado para avaliar amostras menos polares uma vez que o substrato empregado é uma emulsão lipídica.

3- Testes "in vitro" utilizando como substratos sistemas biológicos como células de cortex cerebral de rato/boi, lipoproteína de baixa densidade humana (LDL), cultura de células hepáticas de camundongos e microssomas de fígado de ratos; nos quais a reação de oxidação é iniciada por meio de um agente agressor como H_2O_2, nitriloacetato (NTA) ou cloreto férrico ($FeCl_3$). Modelos que empregam células são particularmente úteis na determinação dos mecanismos de reação e dose-resposta de um determinado composto, ou seja, é possível estudar a quantidade de amostra necessária para induzir uma ação e alterar uma resposta bem como observar a variedade de respostas em função de desafios diversos impostos as células.

4- Testes "in vivo" utilizando animais para avaliar os efeitos de proteção proporcionados pela ingestão de extratos vegetais, com ou sem intoxicação por substâncias como o tetracloreto de carbono CCl_4. Nesses estudos, a indução ou decréscimo no conteúdo e atividade das enzimas detoxificantes da fase 1 e 2 é monitorada por métodos bioquímicos bem como os danos às células e tecidos são avaliados por meio de estudos histopatológicos. Somente nesses modelos é possível avaliar a absorção, distribuição, metabolismo e excreção dos antioxidantes bem como a real contribuição desses compostos.

Desta forma, a interpretação dos resultados deve ser muito cuidadosa e a extrapolação dos resultados não pode ser realizada de forma indiscriminada. O ensaio químico "in vitro"

é o primeiro passo para avaliação de antioxidantes visto que se assume que a capacidade em seqüestrar radicais livres é a única ação dos antioxidantes. Modelos "in vitro" utilizando células ajudam a entender o metabolismo de absorção do composto com atividade antioxidante. Entretanto, somente os modelos "in vivo" fornecerão dados mais precisos e completos sobre o metabolismo do antioxidante, avaliando a redução do stress oxidativo e a ativação ou inativação de enzimas, complexação de metais, etc; bem como os outros efeitos da natureza também indesejável como toxicidade.

Por último, deve ser ressaltado o isolamento e concentração de um composto ativo de um extrato vegetal natural nem sempre aumenta sua efetividade. Extratos vegetais muitas vezes contêm uma variedade de antioxidantes com diferentes solubilidades e reatividades. Desta forma, componentes com efeitos sinérgicos podem aumentar a solubilidade e promover mecanismos de reações complementares tornando-o mais efetivo que um composto puro isolado, principalmente no desenvolvimento de aplicações complexas como alimentos.

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CAPÍTULO 1: Atividade Antioxidante de Frutas do Cerrado

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RESUMO

Annona crassiflora (araticum), *Solanum lycocarpum* (lobeira), *Eugenia dysenterica (*cagaita), *Caryocar brasilense* (pequi) e *Swartzia langsdorfii* (banha de galinha) são frutas do bioma cerrado, conhecidas e consumidas principalmente por populações nativas dessa região. Nesse estudo, as diferentes frações dos frutos acima descritos (polpa, semente e casca) foram avaliadas por meio de extratos aquosos e etanólicos. Alguns extratos mostraram altíssimos conteúdos de compostos fenólicos e foram escolhidos para avaliação do potencial em seqüestrar radicais livres por meio do modelo 2,2- difenil-1-picril hidrazil (DPPH). Os melhores resultados foram: extrato aquoso e etanólico de casca de pequi (IC_{50} igual a 9,44 e 17,98 μg/mL respectivamente), extrato etanólico de sementes de cagaita (IC_{50} igual a 14,15 μg/mL), extrato etanólico de sementes e casca de araticum (IC_{50} igual a 30,97 e 49,18 μg/mL, respectivamente). Este é o primeiro estudo que avalia o potencial em seqüestrar radicais livres de frações de frutas do cerrado. Os resultados indicam que os extratos possuem grande potencial antioxidante e estudos adicionais são necessários para avaliar essa propriedade dos extratos como uma aplicação sustentável dos recursos do cerrado nos setores farmacêuticos, cosméticos e nutricionais.

Palavras-Chave: frutas tropicais, cerrado, atividade antioxidante, DPPH, fenóis

SUMMARY

Annona crassiflora (araticum), *Solanum lycocarpum* (lobeira), *Eugenia dysenterica (*cagaita), *Caryocar brasilense* (pequi) e *Swartzia langsdorfii* (banha de galinha) *are* exotic tropical fruits consumed mainly by native people of the Brazilian Cerrado (second biggest biome of Brazil). In this study, pulp, seed and peel of the fruits were extracted using ethanol and water. Some of the extracts showed high content of total phenols and were screening for their potential as antioxidants using the in vitro model 2,2- diphenyl-1-picryl hydrazyl (DPPH). The best results were found for aqueous and ethanolic extracts of pequi peel (IC_{50} of 9.44 and 17.98 μg/mL respectively), etanolic extract of cagaita seeds (IC_{50} of 14.15 μg/mL), etanolic extract of araticum seeds and peel (IC_{50} of 30.97 and 49.18 μg/mL respectively). This is the first report on the antioxidant properties of the extracts of cerrado fruits fractions. Owing to these properties, the studies can be further extending to exploit them for their possible application as natural antioxidant for cosmetic, supplements and functional ingredients for food products as well as could represent a sustainable application of the natural sources of the Brazilian bioma cerrado which has been destroyed in the last 30 years.

KEYWORDS: tropical fruits, cerrado, antioxidant activity, DPPH, phenols

INTRODUÇÃO

A oxidação é um processo metabólico que leva à produção de energia necessária para as atividades essenciais das células. Entretanto, o metabolismo do oxigênio nas células vivas também leva à produção de radicais [1,27]. Oxidantes são compostos produzidos pelo metabolismo normal do corpo e, se não controlados, podem provocar danos extensivos. O *stress* oxidativo tem sido associado ao desenvolvimento de muitas doenças crônicas e degenerativas, incluindo o câncer, doenças cardíacas, doenças degenerativas como Alzheimer , bem como está envolvido no processo de envelhecimento [4, 5, 12, 14, 22, 35]. O balanço entre o *stress* oxidativo e as funções antioxidantes dos organismos vivos parece ter um papel na carcinogênese [43,44]. Estudos clínicos e epidemiológicos têm mostrado evidências de que antioxidantes fenólicos de cereais, frutas e vegetais são os principais fatores que contribuem para a baixa e significativa redução da incidência de doenças crônicas e degenerativas encontradas em populações cujas dietas são altas na ingestão desses alimentos [36]. Desta forma, a importância da pesquisa por antioxidantes naturais tem aumentado muito nos últimos anos [17]. Compostos típicos que possuem AAO incluem a classe de fenóis, ácidos fenólicos e seus derivados, flavonóides, tocoferóis, fosfolipídios, aminoácidos, ácido fítico, ácido ascórbico, pigmentos e esteróis. Antioxidantes fenólicos são antioxidantes primários que agem como terminais para os radicais livres [42]. O Cerrado que ocupa 25% do território brasileiro é o segundo maior bioma da América do Sul, perdendo em tamanho somente para a Floresta Amazônica [29]. Sua flora riquíssima só agora começa a ser conhecida, existindo cerca de 1000 espécies de árvores, 3000 espécies de ervas ou arbustos e quase 500 trepadeiras [28]. Nos últimos 30 anos, a progressiva mecanização da lavoura e a facilidade de limpar e adubar a terra tem

contribuindo para uma devastação acelerada de vegetação nativa e estima-se que cerca de 40% do bioma já tenha sido desmatado [31].

O primeiro grupo de frutas escolhido para avaliação da AAOengloba cinco frutas típicas do cerrado: *Annona crassiflora* (araticum), *Solanum lycocarpum* (lobeira), *Eugenia dysenterica (*cagaita), *Caryocar brasilense* (pequi) e *Swartzia langsdorfii* (banha de galinha).

Os frutos do araticum, também conhecidos como marolo, pertencem à família das Annonaceae e são coletados entre fevereiro e março. Os frutos são utilizados na alimentação e são muito apreciados por sua polpa doce, amarelada e de aroma bastante forte. As sementes possuem ação contra afecções parasitárias do couro cabeludo. Na medicina popular, a infusão das folhas e das sementes pulverizadas serve para combater a diarréia e induzir a menstruação [2,23,37].

A banha de galinha, também conhecida por banana de papagaio e pacová-de-macaco, é da família das leguminosae (*Swartzia langsdorfii Radlk.)* e seus frutos são coletados de agosto a outubro. A árvore de banha de galinha é pequena e notável pelos frutos muito grandes (tamanho de uma manga comum) com arilos polposos, cor de laranja, de sabor e cheiro repugnantes ao homem, porém muito apreciados pelas antas [10,37]. Apesar de a polpa deste fruto ser tradicionalmente consumida por populações tradicionais, poucos relatos são encontrados sobre essa fruta visualmente muito intrigante. Somente a atividade contra moluscos, principalmente no controle de esquistossomose, foi reportada na Tanzânia por um fruto da família das Swartzia [9]. Recentemente, a *Swartzia langsdorfii* também demonstrou atividade contra B. glabrata, importante intermediário hospedeiro do esquistossomo [24].

A cagaita (*Eugenia dysenterica*) ocorre no cerrado ou cerradão e frutifica entre outubro e dezembro [37]. O uso alimentar é bastante difundido na região, sendo consumido ao natural, apenas com algumas precações em relação à quantidade ingerida, uma vez que pode tornar-se laxante, principalmente quando fermentados ao sol. Quanto ao seu uso medicinal, além do efeito purgativo dos frutos, a garrafada das folhas produz efeitos contrários, antidiarréicos, é também utilizada para combater problemas cardíacos [2].

A lobeira (*Solanum lycocarpum*) também conhecida como fruto do lobo ocorre no cerrado, cerradão e campo sujo, sendo seus frutos produzidos de julho a janeiro [37]. Os frutos da lobeira são comestíveis e reputados como medicinais. A polpa é enjoativa, possui cheiro muito ativo e penetrante e contém alcalóides de natureza pouco conhecida. A infusão da raiz da lobeira é usada contra hepatite e o xarope dos frutos, contra asma. Um pó branco extraído do fruto verde é também utilizado para combater diabetes. Os frutos verdes contêm solasodina, substância química precursora de esteróides [2,23].

O pequi (*Caryocar brasilense*) assim como o piquiá *(Caryocar villosum)* ocorre no cerrado, cerradão e mata calcária, sendo seus frutos produzidos de outubro a março [37]. A polpa do pequi contém uma boa quantidade de óleo comestível e é rico em vitamina A e proteínas, transformando-se em importante complemento alimentar. A amêndoa do pequi, pela alta quantidade de óleo que contém e por suas características químicas, pode ser também utilizada com vantagem na indústria de cosmética para a produção de sabonetes e cremes. Ambos os frutos de pequi e piquiá possuem a mesma característica, sendo que a grande e notável diferença entre as duas espécies reside no tamanho da planta como um todo [38]. O pequi é bastante apreciado nas regiões onde ocorre e o uso alimentar é bastante difundido. O arroz, feijão e a galinha cozida com pequi são pratos fortes da culinária regional; o licor

de pequi tem fama nacional; e há também boa variedade de receitas de doces aromatizados com seu sabor [2]. Como uso medicinal, o óleo da polpa tem efeito tonificante, sendo usado contra bronquites, gripes e resfriados e no controle de tumores. O chá das folhas é tido como regulador do fluxo menstrual [2].

O objetivo do presente trabalho foi selecionar um grupo de frutas típicas do cerrado (araticum, banha de galinha, cagaita, lobeira e pequi), preparar extratos aquosos e etanólicos das diferentes frações das frutas (polpa, semente e casca), quantificar o total de compostos fenólicos e avaliar a capacidade de seqüestrar radicais livres, ou seja, o potencial antioxidante por meio de modelo "in vitro" 2,2 difenil-1-picril hidrazil radical (DPPH). Espera-se que os resultados do trabalho em questão proporcionem o desenvolvimento social, econômico e ambiental do cerrado por meio da valorização de frutas nativas da região, gerando renda para as populações locais e a proteção ambiental por meio da redução de áreas destinadas a pastagens e plantio de oleaginosas.

MATERIAIS E MÉTODOS

Matérias-Primas.

As frutas foram obtidas da Fazenda Erlow, Km 07 Br 070, Goiânia, Brasil, transportadas para a Universidade Estadual de Campinas e armazenadas a 5°C até o preparo dos extratos, período não superior a 02 meses.

Extração Aquosa.

As frutas foram separadas em sementes, polpas e cascas e homogeneizadas por aproximadamente 20 minutos com água destilada na proporção de 1:3 (m/m) fruta:água. O material foi filtrado em gazes e o resíduo foi reextraído com água nas mesmas condições. O

material filtrado, bem como o material retido no filtro, foi liofilizado a –18°C a 13,3 Pa. Os extratos liofilizados foram armazenados em frascos âmbar a -18°C até sua utilização.

Extração Etanólica.

As frutas foram separadas em sementes, polpas e cascas e o material resultante foi utilizado para extração com solução aquosa de etanol (5:95, v/v, água:etanol) na proporção de 1:3 (m/m) fruta: solução de etanol. O material foi filtrado em gazes e o resíduo foi reextraído nas mesmas condições. Os materiais obtidos, resíduo e extrato etanólico, foram colocados em rota-evaporador a 40°C, sendo que o extrato concentrado e o resíduo obtidos foram liofilizados a –18°C a 13,3 Pa e armazenado em frascos âmbar a -18°C até sua utilização.

Determinação dos Macronutrientes.

Os conteúdos de água, cinzas, proteínas, açúcares totais e lipídios foram determinados segundo a Associação Analítica de Química (AOAC): Official Methods of Analysis. Washington (USA) 1998.

Ensaio de Determinação de Fenóis Totais.

A quantificação de compostos fenólicos foi realizada por Folin-Ciocalteau, método que envolve a redução do reagente pelos compostos fenólicos das amostras com concomitante formação de um complexo azul cuja intensidade aumenta linearmente a 760 nm, conforme descrito por Swain and Hillis, 1959. Os extratos aquosos e etanólicos foram dissolvidos em metanol, a fim de se obter uma concentração de 0,5mg sólidos/mL, e analisados utilizando-se o reagente de Folin-Ciocalteu. A quantidade total de fenóis de cada extrato foi quantificada por meio de uma curva padrão preparada com ácido gálico e expresso como equivalentes de ácido gálico (GAE). Para a reação calorimétrica, uma alíquota de 0,5mL da solução metanólica de extrato (concentração 0,5mg sólidos/mL) foi adicionada de 2,5mL de solução aquosa do reativo Folin-Ciocalteau a 10% e 2,0mL de carbonato de sódio a

7,5%. A mistura foi incubada por 5 minutos em banho-maria a 50°C e, posteriormente, a absorbância foi medida usando-se branco como referência. A quantificação dos compostos fenólicos nos extratos de frutas foi realizada em triplicata.

Determinação da Capacidade de Seqüestrar Radicais Livres.

O radical estável 2,2-difenil-1-picril hidrazil (DPPH') tem sido amplamente utilizado para avaliar a capacidade de antioxidantes naturais em seqüestrar radicais livres [8,11,34]. A partir dos extratos aquosos e etanólicos, soluções etanólicas com diferentes concentrações foram preparadas pela adição de 1000 μL de DPPH (0,004% m/v), e o volume final foi ajustado para 1200 μL com etanol. A concentração final dos extratos nas cubetas foi de 1,0 a 2000,0 μg/mL. Cada amostra foi incubada 30 minutos à temperatura ambiente no escuro. O mesmo procedimento foi adotado para o ácido gálico e para o extrato comercial de alecrim, para efeito comparativo. O controle foi preparado conforme procedimento acima, sem adição de extrato, e etanol foi utilizado para correção da linha de base. A solução de DPPH' foi preparada diariamente e estocada em frascos cobertos com folhas de alumínio, mantidas no escuro a 4°C até o momento das determinações. O percentual de decréscimo na absorbância foi medido para cada concentração e a capacidade de seqüestrar radicais livres foi calculada com base no decréscimo da absorbância observada. Mudanças na absorbância da amostra foram acompanhadas a 517 nm. A capacidade de seqüestrar radical livre foi expressa como percentual de inibição de oxidação do radical e calculado conforme fórmula abaixo [47]:

% Inibição = $((A_{DPPH} - A_{Extr})/A_{DPPH})*100$

onde A_{DPPH} é a absorbância da solução de DPPH' e A_{Extr} é a absorbância da amostra em solução. A_{Extr} foi calculado com base na diferença da absorbância da solução de amostra em

teste com seu branco. O valor de IC_{50} é definido com a concentração final em ug/mL do extrato seco presente na cubeta, requerido para decrescer a concentração inicial de DPPH em 50%.

RESULTADOS E DISCUSSÕES

Determinação do percentual das frações (semente, polpa e casca) e dos macronutrientes.

A determinação centesimal das diferentes frações das frutas e dos principais macronutrientes segue na Tabela 1. Os dados obtidos permitem afirmar que as polpas e cascas das frutas estudadas são basicamente constituídas de açúcares e água, enquanto as sementes são ricas em nutrientes como proteínas e lipídios. Em relação aos constituintes nutricionais, destacam-se as sementes de araticum que representam 13% (m/m) do fruto e possuem alto teor de óleo, 15,1% (m/m), e proteínas, 10,4% (m/m). Estudos previamente realizados sugerem o uso do óleo de sementes de araticum como óleo de fritura, por conter alta quantidade de ácidos graxos insaturados, 84,0%, sendo 50% de ácido oléico e 34% de ácido linoléico [48]. Essa fração do araticum é usualmente descartada durante o consumo, sendo utilizada apenas a polpa, que representa aproximadamente 53% (m/m). As sementes do araticum são descritas pela medicina tradicional como agentes contra diarréias e para afecções do couro cabeludo. Destacam-se, ainda, as sementes de banha de galinha, que representam 11% (m/m) do fruto e possuem 7,7% (m/m) de proteínas; e as sementes de lobeira, que constituem apenas 3% (m/m) do fruto, com 13,4% (m/m) de proteínas e 3,7% de lipídios (m/m). Em relação ao pequi, a fração mais importante (polpa e semente) representa 24,8% do fruto. Há uma vasta quantidade de dados referentes a esse fruto em

relação a sua composição centesimal, composição graxa dos lipídios encontrados na polpa e amêndoa, bem como de vitaminas, minerais e componentes voláteis presentes no óleo essencial da polpa, conforme descrito por Marx et al., 1997 e Rodriguez-Amaya, 1999.

O mesocarpo (polpa) contém aproximadamente 76% de óleo na matéria seca, 3% de proteínas, 14% de fibras e 11% de outros carboidratos [26]. O endocarpo (semente), por sua vez, contém aproximadamente 6,8% de carboidratos, 1,0% de proteínas e 10% de lipídios [37]. Os dados acima confirmam a grande tradição da utilização desse fruto para consumo alimentar e para extração de óleo com fins industriais.

Quanto à acidez, destaca-se a fração polpa + casca da cagaita (26,4%) e suas sementes (12,1%) com pH de 2,8 e 4,3, respectivamente. A cagaita é descrita como uma fruta bastante susceptível à fermentação, quando exposta ao sol.

Tabela 1: Composição Centesimal, pH e acidez das frações (semente, polpa e casca) das frutas do Cerrado.

		Fração	Proteínas (%m/m)	Lipídios (%m/m)	Cinzas (%m/m)	Umidade (%m/m)	Açúcares Totais (%m/m)	pH	Acidez (%m/m)
Banha	Polpa	15,8%	3,77 ± 0,23	0,46 ± 0,15	0,54 ± 0,10	76,2170	34,04 ± 2,77	6,0	3,6 ± 0,14
	Casca	73,4%	7,72 ± 0,23	0,29 ± 0,02	0,62 ± 0,29	82,9489	17,91 ± 3,31	5,4	1,9 ± 0,07
	Semente	10,9%	2,67 ± 0,47	0,45 ± 0,05	0,93 ± 0,03	45,5832	18,41 ± 2,09	6,5	2,7 ± 0,10
Cagaita	Polpa + Casca	75,6%	2,09 ± 0,48	0,32 ± 0,07	0,23 ± 0,04	89,7127	20,47 ± 0,59	2,8	26,4 ± 0,20
	Semente	24,4%	4,42 ± 1,36	0,49 ± 0,11	0,75 ± 0,12	51,1452	17,84 ± 1,84	4,3	12,2 ± 0,14
Araticum	Polpa	55,7%	1,80 ± 0,06	3,22 ± 0,73	0,77 ± 0,01	67,85 ± 0,59	19,05 ± 1,50	4,8	4,66 ± 0,22
	Casca	31,8%	2,14 ± 0,40	0,75 ± 0,14	0,61 ± 0,02	57,88 ± 0,19	19,23 ± 1,11	4,7	2,96 ± 0,11
	Semente	12,5%	9,61 ± 0,62	15,91 ± 0,04	1,14 ± 0,13	30,97 ± 1,67	20,14 ± 1,29	5,7	3,56 ± 0,10
Lobeira	Polpa	68,3%	1,79 ± 0,44	0,40 ± 0,05	0,58 ± 0,23	74,7047	24,17 ± 1,41	4,2	8,5 ± 0,43
	Casca	29,1%	2,51 ± 0,50	0,55 ± 0,12	0,61 ± 0,24	70,8425	30,40 ± 1,01	4,1	4,03 ± 0,43
	Semente	2,6%	13,41 ± 0,50	3,73 ± 0,26	1,80 ± 1,01	36,1926	n.d.	5,7	5,4 ± 0,21
Pequi	Casca	75,2%							
	Semente + polpa	24,8%				n.d.(1)			

Cada valor foi obtido por meio da média ± desvio padrão de pelo menos três replicatas. n.d. = não determinado. (1) = priorização do material em função da baixa quantidade de amostra disponível para testes e alta quantidade de dados científicos já publicados.

Determinação de fenóis totais.

O método de Folin-Ciocalteau permite quantificar flavonóides, antocianinas e compostos fenólicos presentes nas amostras. A Tabela 2 demonstra a quantidade total de fenóis dos extratos provenientes de cada fração das diferentes frutas obtidas por extração aquosa e etanólica, bem como dos resíduos obtidos após a etapa de filtração e reextração. Os extratos que contêm maiores conteúdos de fenóis totais em ordem decrescente foram: extratos etanólico e aquoso de casca de pequi (209,37 e 208,42 g GAE/kg, respectivamente), extrato etanólico de semente de araticum (136,99g GAE/kg), extrato etanólico de semente de cagaita (136,96g GAE/kg), extrato etanólico de casca de banha de galinha (99,18 g GAE/kg) e extrato etanólico de casca de araticum (90,72g GAE/kg). Com exceção do

extrato aquoso de casca de pequi, a extração etanólica foi a mais eficiente para remoção dos compostos fenólicos das diferentes frações das diversas frutas estudadas. As frações que apresentaram maior conteúdo de compostos fenólicos foram as sementes e as cascas, frações normalmente desprezadas durante o consumo in natura ou nas formulações caseiras de compotas, sorvetes e outras. Os extratos com menores concentrações de fenóis foram aqueles obtidos das polpas, principalmente, quando utilizada a extração aquosa, como por exemplo, o extrato aquoso de polpa de banha de galinha com apenas 1,59g GAE/kg. E. O processo de extração não apresentou performance máxima para alguns extratos, como por exemplo, para o extrato etanólico de casca de pequi e extrato etanólico de casca de araticum, cujos resíduos, mesmo após reextração, apresentaram alto conteúdo de fenóis totais, 161,77 e 79,64g GAE/kg, respectivamente. O aproveitamento total dos compostos fenólicos desses extratos deverá ser estudado por meio de estudos adicionais dos parâmetros empregados no processo de extração como razão solvente: massa, tempo de extração, número de reextrações, etc. Um extrato comercial de *Rosmarinus officinalis* (alecrim), usualmente empregado como antioxidante em formulações alimentares e cosméticas, foi empregado para efeito comparativo para a análise do potencial antioxidante dos extratos obtidos de frações das frutas do cerrado. Considerando o alto conteúdo de fenóis totais dos extratos de algumas frações das frutas do cerrado, comparado com estudos de extratos de frutas recentemente publicados, em que a AAO foi correlacionada, principalmente, com alto conteúdo de fenóis totais, como, por exemplo, extratos obtidos de romã com 18% de fenóis totais como ácido tânico equivalente (massa seca) [39], extrato de maçã com 2866 mg/100g, extrato de ameixa com 2643 mg/100g, extrato de pêra com 1194 mg/100g de fenóis totais como catequina equivalente (massa seca) [16], extrato de casca e

polpa de goiaba com 58,7 g/kg e 26,3 g/kg, respectivamente, de fenóis totais como ácido gálico equivalente (massa seca) [19] e "berries" diversas com valores entre 177,5 e 690,2 mg/100g de fenóis totais como ácido gálico equivalente (frutos frescos) [7], a capacidade de seqüestrar radicais livres dos extratos produzidos por diferentes frações de frutas do cerrado foi determinada utilizando-se o modelo "in vitro" 2,2-difenil-1-picril hidrazil (DPPH).

Tabela 2: Teor de fenóis totais expressos com ácido gálico equivalente (GAE)[a]

		FT (g GAE/kg ms)			
		Extração		Resíduo	
Fruta	**Parte Utilizada**	**Etanólica**	**Aquosa**	**Etanólica**	**Aquosa**
Banha de Galinha	casca	99,18±3,935	19,55±1,046	3,18±0,492	4,73±2,608
	semente	7,38±0,425	4,53±0,296	n.d.	n.d.
	polpa	4,68±0,574	1,59±0,502	2,75±0,350	n.d.
Cagaita	casca + polpa	18,38±0,817	16,23±1,363	15,22±0,465	n.d.
	semente	136,96±6,215	38,18±1,887	11,90±0,731	22,78±1,036
Pequi	casca	209,37±3,573	208,42±1,349	161,77±1,145	n.d.
	semente + polpa	27,19±1,248	20,88±3,451	15,03±2,846	20,59±0,641
Araticum	casca	90,72±4,999	48,86±3,059	79,64±3,539	43,23±1,783
	semente	136,99±7,565	29,07±1,403	27,17±0,846	45,17±0,844
	polpa	20,31±3,525	16,91±0,810	n.d.	17,39±1,454
Lobeira	casca	35,15±19,267	15,09±0,541	n.d.	n.d.
	semente + polpa	35,58±19,726	25,81±2,227	28,84±15,798	55,67±32,394
Rosmarinus officinalis	extrato comercial	35,68±0,950 *	n.d.	n.d.	n.d.

[a] Cada valor foi obtido por meio da média ± desvio padrão de pelo menos três replicatas. FT = fenóis totais. n.d. = não determinado. ms = massa seca. GAE = ácido gálico equivalente. * Fenóis totais por kg de extrato comercial.

Determinação da capacidade de seqüestrar radicais livres.

A capacidade de seqüestrar radicais livres em relação ao radical estável 2,2-difenil-1-picril hidrazil (DPPH) foi inicialmente escolhida por se tratar de uma metodologia simples, rápida e sensitiva, muito conveniente para realização de "screening" de um grande número de amostras com diferentes polaridades [21]. O potencial dos diferentes extratos de frutas

do cerrado em seqüestrar radicais livres foi expresso como concentração final do extrato necessária para inibir a oxidação do radical DPPH em 50%, e os resultados são descritos na Tabela 3 e Figuras 1, 2, 3, 4, 5 e 6.

Tabela 3: Determinação da capacidade de seqüestrar radicais livres (DPPH).

		IC 50 (ug/mL) (m/v)			
		Extração		Resíduo	
	Parte Utilizada	Etanólica	Aquosa	Etanólica	Aquosa
Banha Galinha	casca	37,42±1,54	n.d	n.d	n.d
Cagaita	casca + polpa	387,47±8,70	879,33±11,70	1038,17±5,21	n.d
	semente	14,15±0,18	247,93±0,29	775,99±8,21	548,97±10,50
Pequi	casca	9,44±0,30	17,98±0,35	28,49±0,45	n.d.
	semente + polpa	298,75±3,80	534,43±7,32	974,55±1,29	847,23±34,83
Araticum	casca	49,18±3,13	198,28±8,24	423,99±31,96	404,52±1,09
	semente	30,97±0,99	417,54±11,06	590,33±13,11	222,90±1,67
	polpa	148,82±0,98	1321,93±20,77	n.d.	1853,49±44,33
Lobeira	casca	182,16±9,58	1328,98±9,42	n.d.	n.d.
	semente + polpa	162,97±2,05	199,34±2,75	209,18±5,03	299,6±6,51
Rosmarinus officinalis		80,84±4,53	n.d.	n.d.	n.d.
Galic Acid		1,38±0,01	n.d.	n.d.	n.d.

IC50 é definido como a concentração final em ug/mL do extrato seco presente na cubeta, requerido para decrescer a concentração inicial de DPPH em 50%.
O valor IC_{50} foi obtido por meio de três replicadas de pelo menos seis diferentes concentrações de extratos, abrangendo a faixa de baixa inibição até alta inibição da oxidação do radical DPPH. n.d. = não determinado.

As substâncias antioxidantes presentes nos extratos reagem com o DPPH que é um radical estável, e converte-o em 2,2-difenil-1-picril hidrazina. O grau de descoloração indica o potencial antioxidante do extrato. Um extrato que apresenta alto potencial em seqüestrar radicais livres possui baixo valor de IC_{50}. Desta forma, uma pequena quantidade de extrato é capaz de decrescer a concentração inicial do radical DPPH em 50%, ou seja, inibir a oxidação do radical em 50%. Os menores valores de IC_{50} foram obtidos pelo ácido gálico (1,38 μg/mL), extrato etanólico e aquoso de casca de pequi (9,44 e 17,98 μg/mL, respectivamente), extrato etanólico de semente de cagaita (14,15 μg/mL), extrato etanólico

de semente e casca de araticum (30,97 e 49,18 μg/mL, respectivamente). O IC_{50} para o extrato comercial de alecrim foi de 80,84 μg/mL. Conforme relatado na determinação de compostos fenólicos, o resíduo do extrato etanólico de casca de pequi apresentou alto conteúdo de fenóis e conseqüentemente bom potencial para seqüestrar radicais livres (IC_{50} de 28,49 μg/mL). É importante ressaltar que esse extrato poderá ser ainda mais efetivo como antioxidante por meio de aprimoramentos no processo de extração dos compostos fenólicos. Os extratos aquosos apresentaram alto IC_{50} para todas as frações de frutas estudas, com exceção do extrato aquoso de casca de pequi. A performance do extrato etanólico e aquoso de casca de pequi, extrato etanólico de semente de cagaita, extrato etanólico de semente e casca de araticum e extrato etanólico de casca de banha de galinha foi excelente, sendo que a AAO dos extratos pode ser atribuída à habilidade de seqüestrar radicais livres por meio da doação de hidrogênio, visto que os extratos mencionados apresentam altíssimo conteúdo de compostos fenólicos. Desta forma, os estudos realizados indicam a presença de compostos com alto potencial antioxidante nos extratos das frações das frutas citadas acima. A relação entre concentração de fenóis totais e a capacidade de seqüestrar radicais livres dos extratos de frações das frutas do cerrado parece ser bastante significativa, visto que os extratos com maior concentração de fenóis totais são justamente os extratos com maior AAO (extrato de casca de pequi etanólico e aquoso, extrato de semente de cagaita etanólico e extrato de semente de araticum etanólico). Os extratos de polpas que, por sua vez, possuem baixa quantidade de fenóis totais não apresentaram atividade antioxidante, independente do solvente utilizado na extração. Essa relação sugere que a contribuição dos compostos fenólicos nesse modelo é relevante. Outros compostos como o ácido ascórbico e carotenóides não foram mensurados, mas podem estar presentes

nos extratos estudados e contribuírem para o potencial antioxidante dos extratos. Embora o método de Folin-Ciocalteau seja o método mais utilizado para quantificação de compostos fenólicos, o reagente Folin-Ciocalteau pode interagir com outros compostos não fenólicos o que pode levar a resultados superestimados de fenóis totais [40]. Desta forma, alguns autores sugerem um passo adicional na análise de fenóis totais, ou seja, a quantificação de ácido ascórbico ou sua destruição por calor ou condições ácidas [46]. Entretanto, esse composto apresenta menor potencial antioxidante no modelo DPPH que os compostos fenólicos, conforme relatado por quantidade extensiva de artigos [18,30,33,34]. O coeficiente de correlação relatado entre DPPH e vitamina C para frutas tropicais diversas como carambola, goiaba, papaia e manga é de 0,20, enquanto o coeficiente de correlação para DPPH e fenóis totais é de 0,92, segundo estudos da USDA-ARS [25]. Resultados similares foram obtidos para quatro diferentes "berries", ou seja, o coeficiente de correlação entre DPPH e vitamina C para "berries" estudadas conjuntamente foi de 0,47, enquanto que o coeficiente de correlação para DPPH e fenóis totais foi de 0,992 [7]. De um modo geral, a correlação entre fenóis totais e AAO relatada na literatura é contraditória. Enquanto alguns autores observaram uma alta correlação [7,19,20]; outros não observam correlação direta [15,16,45]. Estudos indicam que a correlação entre fenóis totais e a capacidade antioxidante pode depender do método escolhido e também das características hidrofóbicas ou hidrofílicas do sistema teste e dos antioxidantes testados. Por meio da análise de regressão linear entre a concentração de fenóis e a capacidade do extrato em seqüestrar radicais livres, obtiveram-se os seguintes coeficientes angulares em ordem decrescente: 3,1054; 2,6289; 2,4844; 1,5561; 1,0289 e 0,962, para o extrato etanólico de semente de cagaita, extrato aquoso de casca de pequi, extrato etanólico de casca de pequi, extrato etanólico de

semente de araticum, extrato etanólico de casca de banha de galinha e extrato etanólico de casca de araticum, conforme Figuras 1, 2, 3, 4, 5 e 6.

Os resultados do presente trabalho indicam a presença de compostos com excelente capacidade antioxidante provenientes de frações diversas de frutas do cerrado brasileiro, sendo os extratos etanólicos de cascas e sementes as melhores fontes de compostos antioxidantes.

Verifica-se que a extração etanólica resulta em extratos com maiores conteúdos de compostos fenólicos e, conseqüentemente, com maior capacidade de seqüestrar radicais livres, ou seja, maior atividade antioxidante, com exceção para a casca de pequi, que apresentou alta capacidade antioxidante para o extrato etanólico e também para o extrato aquoso. Desta forma, os resultados indicam que, para a extração seletiva de antioxidantes naturais, é de grande importância e necessário um estudo sobre o solvente mais apropriado.

Figura 1: Percentual da atividade antioxidante em função da concentração do extrato etanólico de semente de cagaita

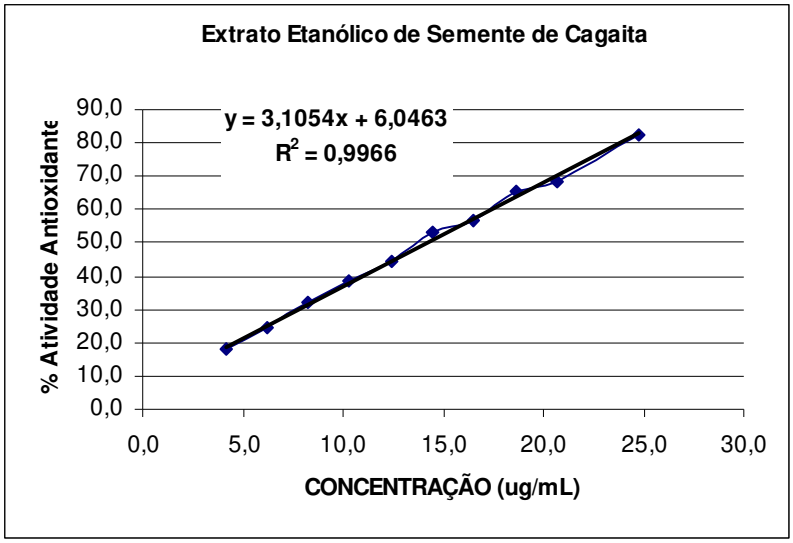


Figura 2: Percentual da atividade antioxidante em função da concentração do extrato aquoso de casca de pequi

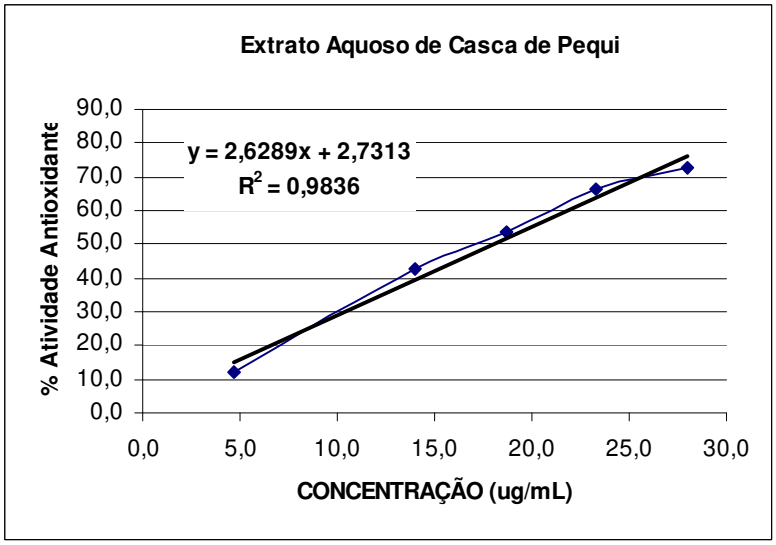


Figura 3: Percentual da atividade antioxidante em função da concentração do extrato etanólico de casca de pequi

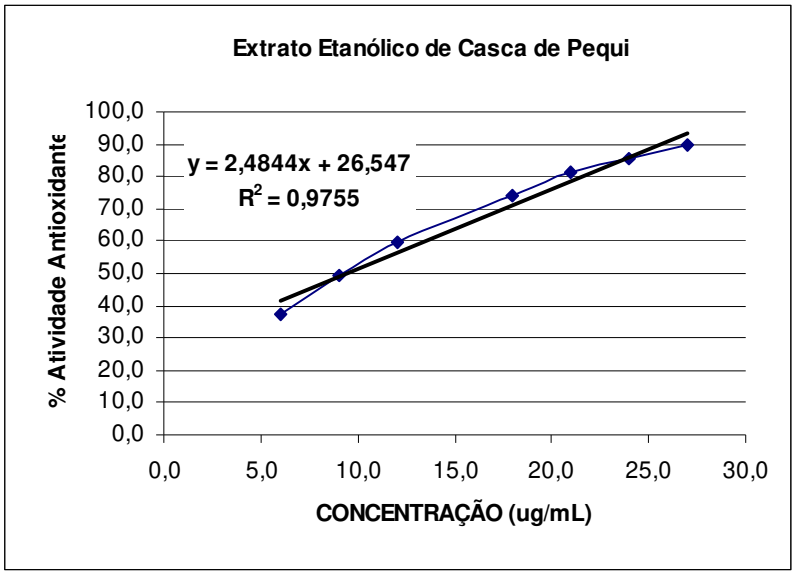


Figura 4: Percentual da atividade antioxidante em função da concentração do extrato etanólico de semente de araticum

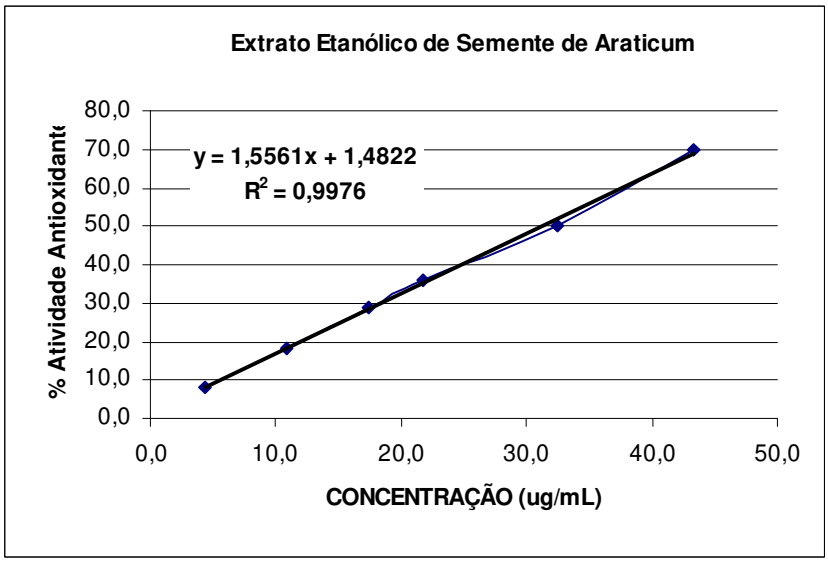


Figura 5: Percentual da atividade antioxidante em função da concentração do extrato etanólico de casca de banha de galinha

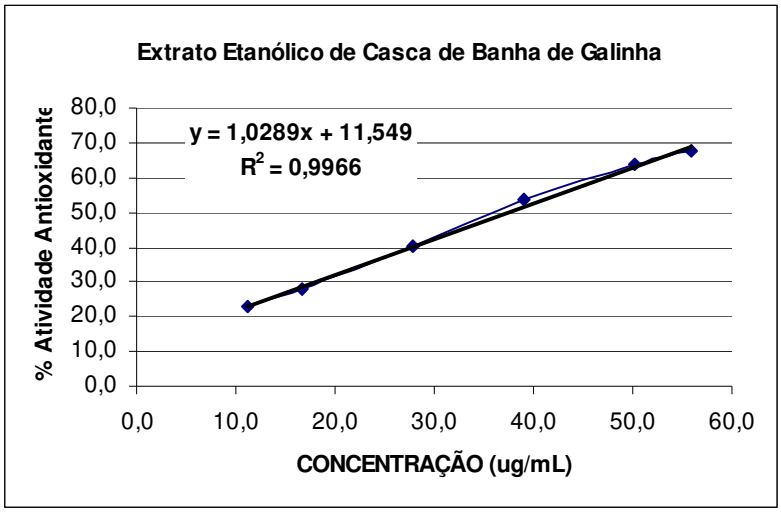
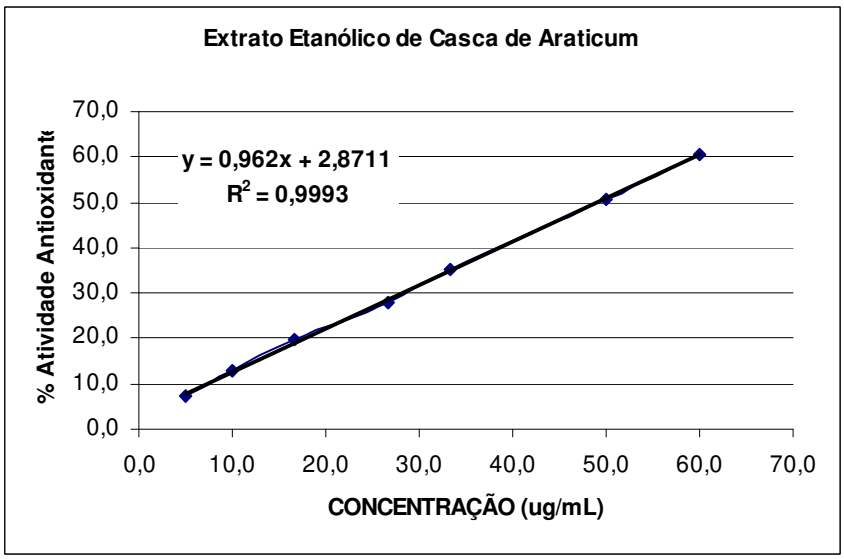


Figura 6: Percentual da atividade antioxidante em função da concentração do extrato etanólico de casca de araticum



CONCLUSÕES

Este é o primeiro estudo que relata a AAO das diferentes frações de diversas frutas do cerrado brasileiro. Por meio dos resultados obtidos, conclui-se que os extratos etanólico e aquoso de casca de pequi, extrato etanólico de semente de cagaita, extrato etanólico de semente e casca de araticum e extrato etanólico de casca de banha de galinha possuem excelente capacidade de seqüestrar radicais livres, ou seja, atividade antioxidante. Estudos adicionais serão necessários para as etapas de isolamento, caracterização dos compostos fenólicos responsáveis pela AAO e, finalmente, para elucidação do mecanismo de ação desses compostos e possível sinergismo entre os compostos encontrados. É importante salientar que as frutas avaliadas neste estudo atualmente são utilizadas apenas pelas populações regionais e apresentam pouco ou nenhum valor comercial. Em função da baixa valorização econômica desses recursos naturais, o bioma cerrado vem sendo rapidamente devastado para criação de áreas de pastagens ou plantio de oleaginosas como a soja. Os resultados obtidos por meio desse estudo apresentam uma aplicação economicamente viável e ambientalmente correta dos recursos desse bioma, uma vez que a busca por antioxidantes naturais tem aumentado muito nos últimos anos, principalmente para aplicação nos setores farmacêuticos, cosméticos e nutricionais.

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CAPÍTULO 2 - Evaluation of the Antioxidant Properties of the Brazilian Cerrado Fruit Annona crassiflora (Araticum)

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ABSTRACT

Annona crassiflora known commonly as araticum is an exotic tropical fruit consumed mainly by native people of the Brazilian Cerrado (second biggest biome of Brazil). In this study, pulp, seed and peel of slight ripe and overripe fruits were extracted using ethanol and water. The extracts showed high content of total phenols and were screening for their potential as antioxidants using the "in vitro" model 2,2-diphenyl-1-picrylhydrazyl (DPPH). The ethanol extracts of peel and seeds showed IC_{50} of 48.82 μg mL^{-1} and 31.14 μg mL^{-1} respectively for the slightly ripe fruits. As the ethanolic fractions of araticum showed the highest antioxidant activity, they were selected for testing of its effect on lipid peroxidation. The ethanolic extracts of slightly ripe fruits showed IC_{50} of 4.44 μg mL^{-1}, 1.72 μg mL^{-1} and 8.62 μg mL^{-1} for the peel, seed and pulp respectively. This is the first report on the antioxidant properties of the extracts of araticum. Owing to these properties, the studies can be further extending to exploit them for their possible application as natural antioxidant for cosmetic, supplements and functional ingredients for food products.

KEYWORDS: araticum, antioxidant activity, DPPH, phenols, TBA

INTRODUCTION

Oxidation is a metabolic process that leads to energy production necessary for essential cell activities. However, metabolism of oxygen in living cells also leads to the unavoidable production of oxygen-derived free radicals, commonly known as reactive oxygen species (ROS) (Mccord 1994, Adegoke and others 1998). Oxidants are by-products of normal body metabolism and, if not controlled, may cause extensive damage. Oxidative stress has been associated with the development of many chronic and degenerative diseases, including cancer, heart disease, and neuronal degeneration such as Alzheimer's , as well as being involved in the process of aging (Ames and others 1993, Ames and others 1995, Diaz and others 1997, Lang and Lozano 1998, Christen 2000). Lipid peroxidation is important "in vivo" for several reasons, in particular because it contributes to the development of atherosclerosis (Esterbauer and others 1991, Esterbauer and others 1992).

The balance between oxidative stress and antioxidant function in living organisms appears to have a role in carcinogesis (Weisburger 1999, Wettasinghe and others 2002). Both epidemiological and clinical studies have provided evidence that phenolic antioxidants present in cereals, fruits, and vegetables are principal contributing factors in accounting for the significant reduced incidences of chronic and degenerative diseases encountered by populations whose diet is high in the intake of these foods (Shahidi 1996). Therefore, the importance of the search for and exploitation of natural antioxidants, especially of plant origin, has greatly increased in recent years (Jayaprakasha and Jaganmohan 2000). Typical compounds that possess antioxidant activity include phenols, phenolic acids and their derivatives, flavonoids, tocols, phospholipids, amino acids and peptides, phytic acid, ascorbic acid, pigments, and sterols. Phenolic antioxidants are primary antioxidants which

act as free-radical terminators (Xing and White 1996). The Cerrado is the second largest biome in South America, losing only to the Amazon rainforest. It occupies almost 25% of Brazil (Proença and others 2000). Its very rich flora is just beginning to become known, ant it is estimated there are about 1000 species of trees, 3000 species of herbs and shrubs, and about 500 of climbers (Mendonça and others 1988). In the last 30 years, progressive mechanization with improved techniques for clearing and fertilizing the land have contributed to an accelerated destruction of the natural vegetation, and it is estimated that 40% of the cerrado biome has already been deforested (Ratter and others 1997). The *Anonnaceas* family has a great variety of exotics fruits which are apparently rustic and have typical form such as conde fruit (*Annona squamosa*), graviola fruit (*Annona muricata*) and araticum of cerrado or marolo (*Annona crassiflora*) (Silva and Tassara 2001). Many members of annonaceae are used in folk medicine for antiparasitic or antitumoral treatment of intestinal diseases. In recent years, many interesting compounds have been reported and have gained organic chemist's and biochemist's attention because of their novel structure and wide –range of bioactivities termed tetrahydrofuranic acetogenins (or annonaceous acetogenins) (Araya 2004). Araticum (*Annona crassiflora)* has not been studied and nothing has been found about the possible biological activities which could validate the ethno botanical uses by traditional herbal medicine. *Annona crassiflora* is a tree that bears a typical fruit known as araticum of cerrado or cerradão. Its fruits are highly consumed "in natura" by native people or can be used to prepared juice, ice-cream or jelly. The fruits are sold in regional markets and have no commercial value in Brazil. The seeds in oil are used against scalp infections and in the folk medicine, the leaves and seeds infusion are used against diarrhea and as antitumoral (Lorenzi 1988, Almeida and others 1994). The aim of

the present work was to prepare extracts from pulp, seed and peel of overripe and slight ripe araticum fruits using ethanol and water and evaluate their antioxidant activity by using "in vitro" models. This report also describes the total phenols and their correlation to antioxidant activity as well as the centesimal composition of the fractions of the araticum. Searching for a economically viable and environmentally correct application of Brazilian cerrado natural resources is highly important and equally urgent in order to avoid the total destruction of natural vegetation and Cerrado Biodiversity.

MATERIAL AND METHODS

Reagents and standards.

Methanol and ethanol were purchased from Merck. All other solvents and reagents were purchased from Sigma Aldrich Chemical Co.

Plant Material.

Araticum fruits *(Annona crassiflora)* were obtained from Erlow Farm, Km 07 Br 070 Goiânia Brazil, in the center west of Brazil. Fruit harvests were conduced twice and special care was taken to avoid damaged fruits. Fruits was transported to the Univ. of Campinas (UNICAMP) and stored at 5°C until use which was usually no longer than 2 months.

Aqueous extraction.

Undamaged fruits were selected and peel, pulp and seeds were manually separated. About 100g of each part of the fruit (pulp, peel and seeds) was cut in small parts, mixed and extracted twice with 300 mL of fresh water using a household mixer for 20 minutes. The extracts were filtered through cotton membrane and the residue was re-extracted under the same conditions. The residues were kept for further evaluations. The pooled filtrates were lyophilized and stored at – 18°C in amber glass bottles until used

Ethanolic extraction.

Undamaged fruits were selected and peel, pulp and seeds were manually separated. About 100g of each part of the fruit (pulp, peel and seeds) was cut in small parts, mixed and extracted twice with 300 mL of aqueous ethanol (5:95, v/v, water: ethanol) using a household mixer for 20 minutes. The extracts were filtered through cotton membrane and the residue was re-extracted under the same conditions. The residues were kept for further evaluations. The resultant material was subjected to vacuum rotary evaporation at 40°C to remove ethanol. The concentrated ethanolic extracts were lyophilized and stored at – 18°C in amber glass bottles until used.

Centesimal content.

The contend of water, ash, protein and lipids was determined according to Association of Official Analytical Chemists (AOAC): Official Methods of Analysis. Washington (USA) 1998.

Determination of total polyphenols assay.

Estimation of the global polyphenol content in the extracts was performed by the Folin-Ciocalteu method that involves reduction of the reagent by phenolic compounds, with concomitant formation for a blue complex, its intensity at 760 nm increases linearly with the concentration of phenols in the reaction medium as described by Swain and Hillis 1959. Aqueous and ethanolic extracts were dissolved in methanol to obtain a concentration of 0.5 mg mL^{-1}. Samples (0.5 mL) were passed through a 0.45 μm membrane filter and mixed with 2.5 mL of 10 fold diluted Folin-Ciocalteu reagent and 2.0 mL of 7.5% sodium carbonate solution. After a mixture had been allowed to stand for 5 minutes at 50°C, the absorbance was measured at 760 nm. In this study, galic acid was used as spectrophotometric standards and the total phenolic content of the fruit extracts were

express as galic acid equivalents (GAE/100g). Estimation of the phenolic compounds was carried out in triplicate and averaged.

Determination of radical scavenging activity.

2,2-diphenyl-1-picrylhydrazyl (DPPH) has been widely used to evaluate the free radical scavenging of natural antioxidants (Brand-Williams and others 1995, Bondet and others 1997, Sanchez-Moreno and others 1998). From aqueous and ethanolic extracts, ethanolic solutions in different concentrations were prepared by adding 1000 µL of DPPH (0.004% w/v) and the final volume was brought to 1200 µL with ethanol. Final concentration of test materials in the cuvettes were between 1.0 to 2000 µg mL^{-1} Each tube was incubating for 30 minutes at room temperature in the dark. The same procedure was taken for galic acid and commercial rosmarinus extract for comparative purpose. The control was prepared as above without any extract, and ethanol was used for the baseline correction. The DPPH solution was freshly prepared daily, stored in a flask covered with aluminum foil, and kept in the dark at 4°C between measurements. The percent decrease in absorbance was recorded for each concentration and percent quenching of DPPH was calculated on the basis of the observed decrease in absorbance of the radical. Changes in the absorbance of the samples were measured at 517 nm. Radical scavenging was expressed as the inhibition percentage and was calculated using the following formula (Yen and Duh 1994):

$\%\ \text{Inhibition} = ((A_{DPPH} - A_{Extr})/A_{DPPH})*100$

where A_{DPPH} is the absorbance value of the DPPH blank sample and A_{Extr} is the absorbance value of the test solution. A_{Extr} was evaluated as the difference between the absorbance value of the test solution and the absorbance value of its blank. The IC_{50} values are reported

as final concentration of extract in the cuvettes defined as μg mL^{-1} of dried extracts required to decrease the initial DPPH concentration by 50%.

Preparation of rat liver microsomes.

Male Wistar Rats receiving normal diets and weighing 200 – 300 g were killed by decapitation after overnight starvation. Livers were removed and homogenized (1:2 w/v) in ice-cold phosphate buffer (100 mM, pH 7.4) containing 1 mM EDTA and 1.15% KCl. The homogenate was centrifuged (10000g, 20 min, 4°C), and the supernatant was collected and centrifuged (105000g, 60 min, 4°C). To be stored, the pellet was suspended (0.5mg liver mL^{-1}) in ice-cold phosphate buffer (100 mM, pH 7.4) containing 0.1 mM EDTA and 20% glycerol (Omura and Sato 1964). Microsomal protein concentrations were determined by the Lowry method (Lowry and others 1951) with bovine serum albumin used as standard.

Antioxidant Activity.

Thiobarbituric acid reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm (Van der Sluis and others 2000). It was optimized to be able to use microtiter plates and ELISA reader, which makes it possible to analyze large numbers of samples in run. Microsomes were thawed on ice and were diluted with Tris-HCl buffer (50mM, pH 7.4) containing 150 mM KCl to 1mg mL^{-1} protein and centrifuged (100000g, 60 min, 4°C). The pellet was ressuspended with 1mL of the Tris buffer and diluted to the concentration needed (final concentration 0.5 mg mL^{-1} protein unless otherwise stated) (Omura and Sato 1964, Van der Sluis and others 2000, Singh and others 2002).

The microsomes (aliquots of 240 μL) were pre incubated in a 48-well plate for 5 min at 37°C. Samples of 30 mL of different concentration of extracts dissolved in ethanol or blank (corresponding with the solvent ethanol) were added. Lipid peroxidation (LPO) was

induced by adding 15 µL of ascorbic acid (4mM) and 15 µL of $FeCl_3$ (0.2 mM). After incubation for 60 min at 37°C the reaction was stopped by addition of 0.5 mL of 0.83% thiobarbituric acid dissolved in TCA-HCl (16.8% w/v trichloroacetic acid in 0.125 N HCl). LPO was assessed by measuring thiobarbituric acid reactive species (TBARS) after the plates were heated for 15 min at 80°C and subsequent centrifugation (2500 rpm, 15 min). A 250 µL sample of each incubation was transferred to 96-well plates, and absorption was read at 540 nm (color) vs 620 nm (turbidity correction) by ELISA reader (Van der Sluis and others 2000, Singh and others 2002). Final concentration of test materials in the cuvettes were between 1.0 to 50.0 µg mL^{-1}.

Calculations. The percentage of inhibition produced by a sample at a given concentration can be calculated from the absorbance readings. The percentage of inhibition is expressed as the inhibition of lipid peroxidation of that sample compared to the lipid peroxidation in a blank.

$$\% \text{ Inhibition} = ((A_{blank} - A_{sample})/ A_{blank})*100$$

where A_{blank} = absorbance of the blank ($A_{540} - A_{620}$), and A_{sample} = absorbance of the sample ($A_{540} - A_{620}$).

RESULTS AND DISCUSSION

Centesimal determination.

The centesimal composition of the araticum pell, pulp and seed is show in Table 1. As mention before, the part often consumed by native people is the pulp which represents approximately 53% (w/w) of the fruit. It is composed basically by water (72% w/w) and total sugar (21% w/w) and has a very intense and exotic accent. The use of the seed is reported as a medicine against diarrhea and scalp infections by natives. It represents approximately 13% (w/w) of the fruit and has good quantities of lipids 15.1% (w/w) and proteins 10.4% (w/w). Previous studies suggested the use of the araticum seeds as a source of vegetable oil for frying once it has high quantity of unsaturated fatty acids (84.0%) composed by 50% of oleic acid and 34% of linoleic acid (Zuppa and Antoniosi 2001). Regards the peel, no reference has been found about traditional use by natives. It represents around 34% (w/w) of the fruit and has high amount of water (25.5% w/w) and total sugar (12.2% w/w). The results showed that the overripe parts of the fruits have higher pH and less acidity than the ripe fruits. Besides, the quantity of protein and total sugars in the ripe fruits are bigger than overripe which indicated a possible transformation of these compounds in other such as flavors.

Table 1. Centesimal composition of araticum pulp, peel and seed.

		Fraction	Protein (%w/w)	Lipids (%w/w)	Ash (%w/w)	Water (%w/w)	Total Sugar (%w/w)	pH	Acidity (%w/w)
Sample 1	Pulp	49.5%	2.31 ± 0.23	2.34 ± 0.16	1.24 ± 0.07	75.83 ± 0.57	15.49 ± 0.17	5.23	2.90 ± 0.29
	Seed	13.8%	11.15 ± 0.23	14.23 ± 0.21	1.16 ± 0.09	41.26 ± 0.57	8.43 ± 0.78	6.41	2.65 ± 0.33
	Peel	36.7%	3.34 ± 0.47	1.36 ± 0.03	1.09 ± 0.05	47.08 ± 0.58	10.26 ± 0.37	4.89	2.14 ± 0.21
Sample 2	Pulp	55.7%	1.80 ± 0.06	3.22 ± 0.73	0.77 ± 0.01	67.85 ± 0.59	27.20 ± 4.18	4.83	4.66 ± 0.22
	Seed	12.5%	9.61 ± 0.62	15.91 ± 0.04	1.14 ± 0.13	30.97 ± 1.67	11.88 ± 0.21	5.7	3.56 ± 0.10
	Peel	31.8%	2.14 ± 0.40	0.75 ± 0.14	0.61 ± 0.02	57.88 ± 0.19	14.12 ± 0.28	4.73	2.96 ± 0.11

Each value is the mean ± standard deviation of at least three replicate experiments.

Determination of total polyphenols assay.

Folin-Ciocalteau's method allows the estimation of all flavonoids, anthocyanins and nonflavonoid phenolic compounds, that is, of all the phenolics present in the sample. Table 2 reports the amounts of total polyphenols quantified in each part of the araticum fruit obtained by aqueous and ethanolic extraction. The parts of the fruit containing the highest value of total phenols were the peel and seed as show in Table 2. The highest amount was 111.42 g kg^{-1} dm for peel ethanolic extract and 136.98 g kg^{-1} dm for the seed ethanolic extract. Overall, the lowest concentration was found in the aqueous seed extract at 16.36 g kg^{-1} dm. The phenolic content of the EtOH extracts was maximum while the phenolic content was very low in the water extracts. The ethanolic extraction was not fully completed specially for the peel which respective residues still gave high content of total phenolic at 81.40 g kg^{-1} dm. The total phenol content could probably be improved by further studies such as increasing the solvent quantity, the time of extraction and the number of re-extractions from the residue. The aqueous extraction of phenolic compounds was not efficient to any part of the fruit compared to the ethanolic extraction in the conditions used. A commercial *Rosmarinus officinalis* extract often used as a antioxidant in food and cosmetic industry was also measured in this study in order to be compared with

the araticum extracts. Considering the high total phenol content of araticum extracts compared to recent results of fruits extracts published in the literatures such as pomegranate (Singh and others 2002), apple, plum and pear (Imeh and Khokhar 2002), guava (Jimenez and others 2001) and berries (Benvenuti and others 2004), the antioxidant activity of araticum extracts was determined by two different methods.

Table 2. Total Phenol Content Expressed as Galic Acid Equivalents (GAE)[a].

		TP (g GAE kg-1 dm)	
		Ethanolic Extracts	**Aqueous Extracts**
***Anonna crassiflora* Peel**	Extract 1	111.42 ± 8.57	35.89 ± 1.61
	Extract 2	90.72 ± 4.99	48.85 ± 3.05
	Residue 1	81.40 ± 13.33	37.65 ± 1.69
	Residue 2	79.63 ± 3.53	43.23 ± 1.78
***Anonna crassiflora* Seed**	Extract 1	110.48 ± 6.05	16.36 ± 1.52
	Extract 2	136.98 ± 7.56	29.06 ± 1.40
	Residue 1	12.69 ± 0.38	17.38 ± 0.52
	Residue 2	27.16 ± 0.84	45.16 ± 0.84
***Anonna crassiflora* Pulp**	Extract 1	31.08 ± 1.23	17.01 ± 1.87
	Extract 2	20.30 ± 3.52	16.91 ± 0.80
	Residue 1	n. m.	49.20 ± 0.91
	Residue 2	n. m.	17.39 ± 1.45
Rosmarinus officinalis	Extract	35.68 ± 0.95*	n.m.

[a] Each value is the mean ± standard deviation of at least three replicate experiments. TP = total phenols. n.m. = not measure. dm = dry matter. GAE = galic acid equivalents. Extract 1 = extract obtained from overripe fruits. Extract 2 = extract obtained from slightly ripe fruits. Residue 1 = residue obtained from the filtration step of extract 1. Residue 2 = residue obtained from the filtration step of extract 2. * Total phenols per kg of commercial extract.

Determination of radical scavenging activity.

A radical scavenging activity toward the stable radical 2,2-diphenyl-1-picryl hydrazyl (DPPH) was firstly used since it is a very simple, sensitive and rapid method which was very convenient for the screening of large numbers of samples of different polarity (Koleva

and others 2002). Free radical scavenging potentials of araticum peel, pulp and seed extracts at different concentrations were tested by the DPPH method, and the results expressed as final concentration of test materials are shown in Table 3 and Figures 1, 2 and 3 respectively. Antioxidant reacts with DPPH, which is a stable free radical, convert it to 2,2-diphenyl-1-picryl hydrazine. The degree of discoloration indicates the scavenging potentials of antioxidants extracts. All the different ethanolic extracts of araticum tested had exceptionally high scavenging activity. The seed and peel presented the lower IC_{50}. A stronger radical quenching agent generally resulted in a lower IC_{50} value. The lowest IC_{50} was achieved by the gallic acid (1.38 μg mL^{-1}), seed ethanolic araticum extract (31.14 μg mL^{-1}) and peel ethanolic araticum extract (48.82 μg mL^{-1}), both from slightly ripe fruit. The IC_{50} value for commercial rosemary extract was 80.82 μg mL^{-1}. Even the residues left from the extract preparation of araticum showed a good scavenging activity which could be used to enhance the extracts activity by improvements in the extraction methods. The water extracts gave higher IC_{50} in all the extracts tested. Regarding the ripening, the IC_{50} values was lower for the slightly ripe fruits than to the overripe fruit extracts. The extracts obtained by overripe fruits were identified by the nomenclature Extract 1 in Table 3. All of them had higher IC_{50} which may suggest that the compounds responsible for the scavenging activity could be depleted during ripening. Further studies should be done to conclude the relationship between the ripening and scavenging activity for araticum fruits. For comparative purposes, the galic acid and rosemary commercial extract often used as antioxidant for food and cosmetics were also tested. The performance of araticum extracts, particularly ethanolic seed and peel extracts are excellent and the activity of the extracts is attributed to their hydrogen donating ability. Regression analyses of the scavenging activity

of the slightly ripe extracts showed a high linear dependence of the concentration as illustrated by the Figures 1, 2, and 3. The slopes for seed, peel and pulp was 1.5144; 0.9096 e 0.2849 respectively. Comparison of the slopes of linear regression showed scavenging activity in the order: seed > peel > pulp. The same order was obtained for total phenol content and the correlation analysis between total phenol content and scavenging activity showed a remarkable correlation (R = 0.821).

Table 3. Free radical DPPH˙ scavenging activity of araticum extracts

		IC 50 (ug mL-1)	
		Ethanolic Extracts	**Aqueous Extracts**
***Annona crassiflora* Peel**	Extract 1	54.64 ± 1.041	1111.66 ± 32.150
	Extract 2	48.82 ± 2.933	198.28 ± 8.235
	Residue 1	359.83 ± 2.457	656.22 ± 41.311
	Residue 2	423.99 ± 31.963	404.52 ± 1.091
***Annona crassiflora* Seed**	Extract 1	62.40 ± 1.116	1678.66 ± 25.863
	Extract 2	31.14 ± 0.856	417.54 ± 11.056
	Residue 1	1287.87 ± 6.775	n.m.
	Residue 2	590.33 ± 13.11	222.90 ± 1.675
***Annona crassiflora* Pulp**	Extract 1	1204.22 ± 27.432	n.m.
	Extract 2	148.82 ± 0.978	1321.93 ± 20.774
	Residue 1	n.m.	n.m.
	Residue 2	n.m.	1853.49 ± 44.328
Rosmarinus officinalis	-	80.84 ± 4.534*	n.m.
Gallic Acid	-	1.38 ± 0.007	n.m.

Radical scavenging activity expressed as IC_{50} (final concentration of extract in the cuvettes defined as ug/mL of dried extracts required to decrease the initial DPPH concentration by 50%). Each IC_{50} value was based on triplicate determination of at least six antioxidants concentrations covering a range from no inhibition to full inhibition. dm = dry matter. nm = not measured. * Commercial rosemary extract. Extract 1 = extract obtained from overripe fruits. Extract 2 = extract obtained from slightly ripe fruits. Residue 1 = residue obtained from the filtration step of extract 1. Residue 2 = residue obtained from the filtration step of extract 2.

Figure 1. Radical scavenging activity of slightly ripe araticum peel extract

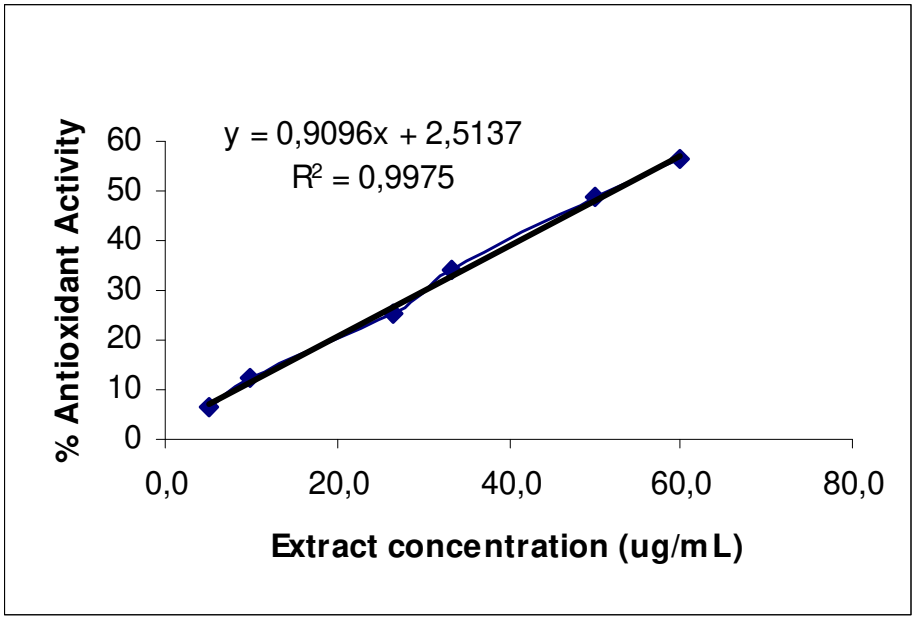


Figure 2. Radical scavenging activity of slightly ripe araticum seed extract

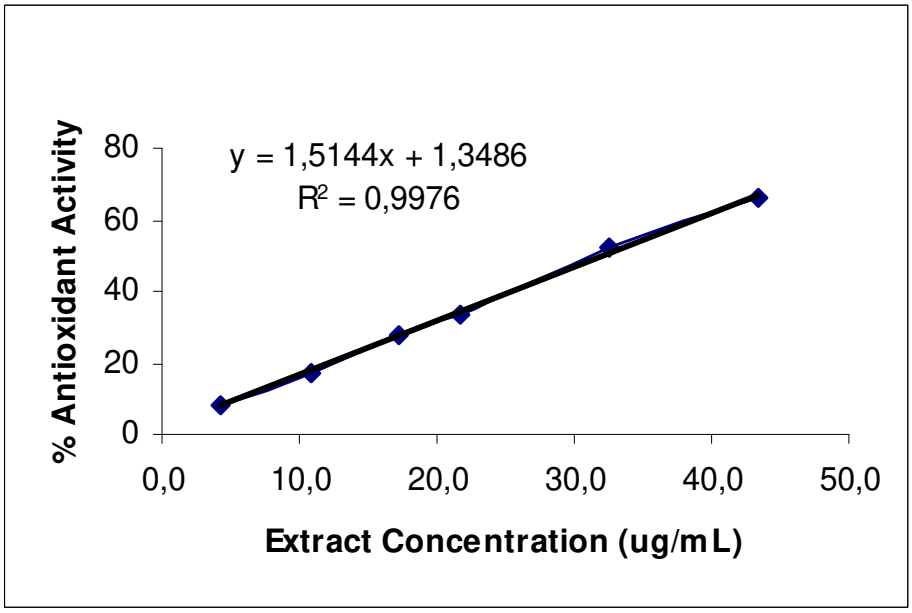
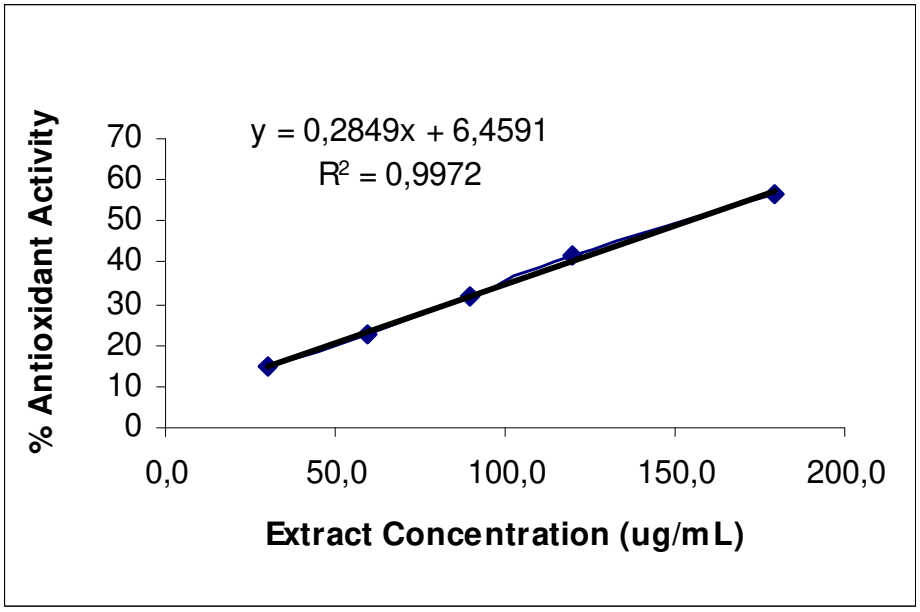


Figure 3. Radical scavenging activity of slightly ripe araticum pulp extract



Inhibition of Lipid Peroxidation.

The rat liver microsomes was chosen as an oxidative system because it is close to the "in vivo" situation where both an aqueous phase and a lipid phase are present. The extent of lipid peroxidation after chemical induction by radical formation is monitored by the thiobarbituric acid (TBA) test. The results of the effect of ethanolic extracts of araticum fractions and standards to prevent lipid peroxidation are shown in Table 4 and Figures 4, 5 and 6. Fifty percent inhibition of lipid peroxidation of microsomes to TBARS by hydroxyl radicals, generated by $FeCl_3$ system, requires 1.72 µg mL^{-1} of seed ethanolic extract, 4.44 µg mL^{-1} of peel ethanolic extract and 8.62 µg mL^{-1} of pulp etanolic extract, all of them obtained from the slightly ripe fruits. Similarly to the results shown in Table 3, the overripe fruits produced extracts not so efficient to inhibit the lipid peroxidation of microsomes as those obtained by slightly ripe fruits. Once more, it suggests that the compounds responsible for the antioxidant activity of the araticum extracts probably are lost during the

ripening. For comparative purposes, the IC_{50} for gallic acid and catechin were also measured. The IC_{50} achieved for gallic acid and was 1.01 μg mL^{-1} or 5.94 μM and for catechin was 1.96 μg mL^{-1} or 6.76 μM. Van der Sluis and others 2000 reported about the same order of antioxidant potency for catechin using the same method. Reports on antioxidant activity of other plant extracts such as *Emblica officinalis* (Jose and Kuttan 1995), *Phoenix dactylifera* (Vayalil 2002) and *Punica granatum* (Singh and others 2002) require an IC_{50} of 3.4 mg mL^{-1}, 2.2 mg mL^{-1} and around 100 ppm respectively of extracts solution. Expressing the antioxidant activity of the extract solution instead of the final cuvettes concentration as mentioning before, the IC_{50} for seed, peel and pulp was 0.043 mg mL^{-1} (43 ppm), 0.12 mg mL^{-1} or 115 ppm and 0.243 mg mL^{-1} or 243 ppm. This further indicates the presence of compounds with potent free-radical-scavenging activity in araticum fruit with so high activity as others known antioxidant plant products. Correlation analysis showed only a weak correlation between total polyphenols and inhibition of lipid peroxidation activity (R^2 = 0.497). However, there was noticeable correlation between free radical $DPPH^{\cdot}$ scavenging activity and total polyphenols (R^2 = 0.821). Literature reports on the relationship between total phenols and antioxidant activities are contradictory; while some authors have observed a high correlation (Jimenez and others 2001, Benvenuti and others 2004, Kahkonen and others 1999); others found no direct correlation (Imeh 2002, Eberhardt and others 2001, Wu and others 2004). The results of this study indicates that the correlation may depend on the method chosen which could be also explained by the hydrophilic or lipophilic properties of the different test systems and the polarity of the antioxidant itself. Thus, measure the total phenols alone may not be a good indicator of antioxidant activity. In addition, the hydrophilic or lipophilic properties of the test systems

and the polarity of the antioxidant present in the extracts could be also the cause of the differences observed between the IC_{50} obtained for the extracts in the methods chosen. Therefore it is very important to test the standard compounds of interest and the extracts in which they are present in one and the same antioxidant test system.

Table 4. Inhibition of lipid peroxidation by slightly ripe araticum extracts

	Ethanolic Extract	IC 50 (ug mL-1)
***Annona crassiflora* Peel**	overripe	9.96 ± 0.306
	slightly ripe	4.44 ± 0.499
***Annona crassiflora* Seed**	overripe	2.96 ± 0.120
	slightly ripe	1.72 ± 0.227
***Annona crassiflora* Pulp**	overripe	26.01 ± 2.227
	slightly ripe	8.62 ± 0.711
Gallic Acid	-	1.01 ± 0.056
Catechin	-	1.96 ± 0.291

TBARs formation monitored by an ELISA reader (A540-A620). Inhibition of Lipid Peroxidation activity expressed as IC_{50} (final concentration of extract in the cuvettes defined as ug/mL of dried extracts required to inhibit the lipid peroxidation by 50%). Each IC_{50} value was based on triplicate determination of at least six antioxidants concentrations covering a range from no inhibition to full inhibition. dm = dry matter. nm = not measured.

Figure 4. Inhibition of lipid peroxidation by slightly ripe araticum peel extract

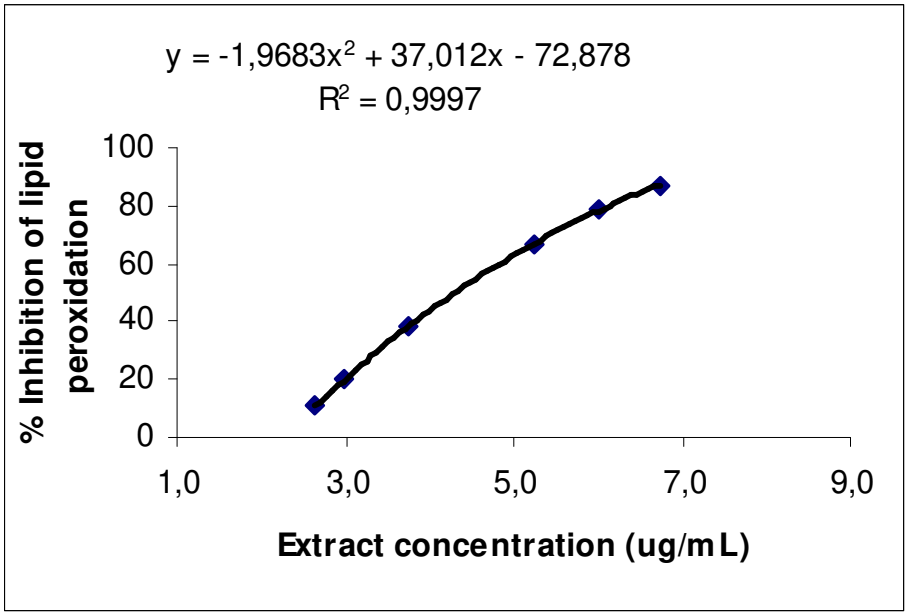


Figure 5. Inhibition of lipid peroxidation by slightly ripe araticum seed extract

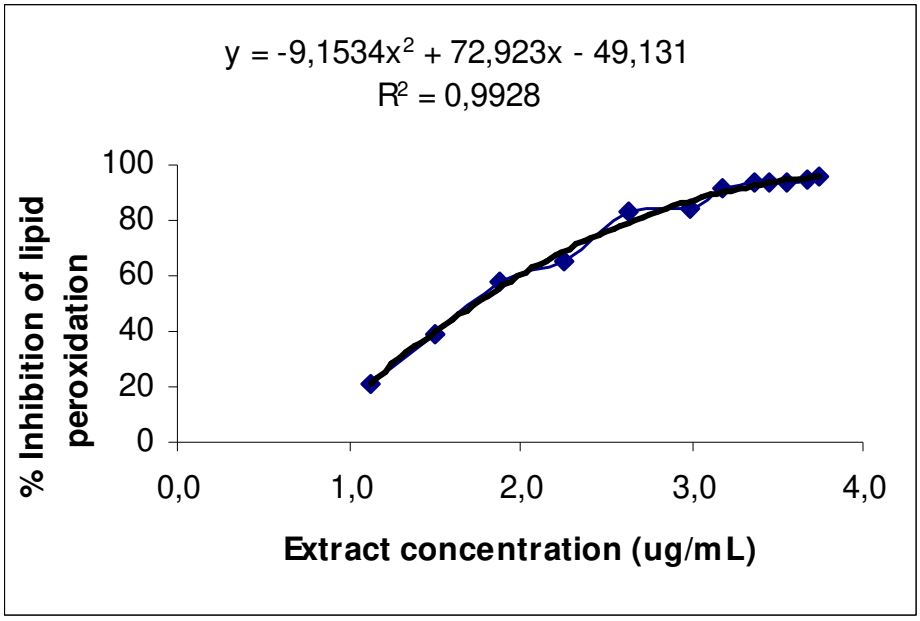
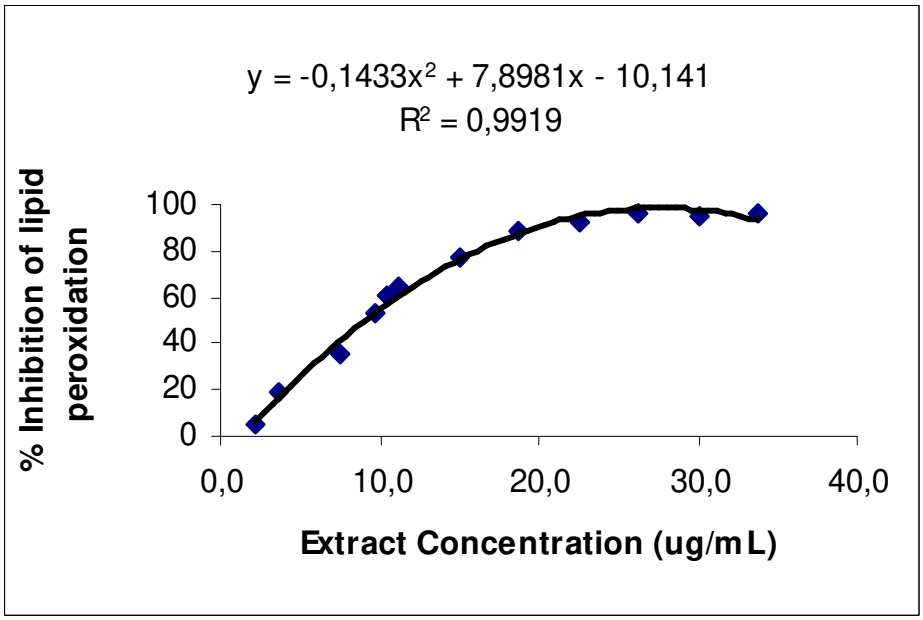


Figure 6. Inhibition of lipid peroxidation by slightly ripe araticum pulp extract



CONCLUSION

In conclusion, the results of the present work indicate the presence of compounds possessing excellent antioxidant activity from peel and seed of slightly ripe araticum fruits with the seeds as the best source of the antioxidants exhibiting higher activity as compared to peel. In addition, the extraction with ethanol gives higher antioxidant activity that the extraction with water. Thus, the results of the present work indicate that the selective extraction of antioxidant form natural sources by appropriate solvent is very important.

Regarding the ripeness, the results obtained from the two different methods chosen to evaluate the antioxidant activity suggest that the compounds responsible for the antioxidant activity of the araticum extracts probably are lost during the ripeness. The extracts from overripe fruits of araticum showed lower antioxidant activity. This is the first report on the descriptions of antioxidants from the Brazilian exotic fruit araticum, further studies are needed on the isolation and characterization of individual phenolic compounds to elucidate their different antioxidant mechanisms and the existence of possible synergism, if any, among the compounds.

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CAPÍTULO 3 - Antioxidant Activity of Annona crassiflora: Characterization of major components by Electrospray ionization mass spectrometry.

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ABSTRACT

Annona crassiflora known commonly as araticum is a tropical fruit consumed mainly by native people of the Brazilian Cerrado (second biggest biome of Brazil). Our previous study indicated that *Annona crassiflora* is a very good source of natural antioxidants. In this study, polar components of araticum pulp, peel and seeds ethanolic extracts were investigated by direct infusion electrospray ionization mass spectrometry (ESI-MS) both in the negative ion mode. Characteristic ESI mass spectra with many diagnostic ions were obtained for the extracts, serving for fast and reliable information. The technique provided information of component structures revealing the presence of important bioactive components widely reported as potent antioxidants such as ascorbic acid, caffeic acid, quinic acid, ferulic acid, xanthoxylin, rutin, caffeoyltartaric acid, caffeoyl glucose and [quercetin + hexose + pentose – H]-1 This is the first report on the composition by ESI-MS of araticum peel and seed ethanolic extracts demonstrating excellent antioxidant activity .

KEYWORDS: araticum, electrospray ionization mass spectrometry, total phenols, antioxidant activity, DPPH.

INTRODUCTION

The Cerrado is the second largest biome in South America, after the Amazon rainforest. It occupies almost 25% of Brazil (Proença, Oliveira, & Silva, 2000). Its very rich flora, which is just beginning to be studied, is estimated to be comprised of about 1000 species of trees, 3000 species of herbs and shrubs, and about 500 of climbers (Medonça et al., 1988). In the last 30 years, progressive mechanization with improved techniques for clearing and fertilizing the land have contributed to the accelerated destruction of the natural vegetation, and it is estimated that 40% of the cerrado biome has already been deforested (Ratter, Ribeiro & Bridgewater, 1997). The *Anonnaceas* family has a great variety of exotics fruits which are apparently rustic and have typical form such as conde fruit (*Annona squamosa*), graviola fruit (*Annona muricata*) and araticum of cerrado or marolo (*Annona crassiflora*) (Silva & Tassara, 2001). Many members of annonaceae are used in folk medicine for antiparasitic or antitumoral treatment of intestinal diseases. In recent years, many interesting compounds termed tetrahydrofuranic acetogenins (or annonaceous acetogenins) have been reported and have gained organic chemist's and biochemist's attention because of their novel structure and wide–range of bioactivities (Araya, 2004). *Annona crassiflora* is a tree that bears a typical fruit known as araticum of cerrado or cerradão. Its fruits are highly consumed "in natura" by native people or used to prepare juice, ice-cream or jelly. The fruits are sold in regional markets and have no commercial value in Brazil. The oil from the seeds are used against scalp infections and in the folk medicine, the leaves and seeds infusion are used against diarrhea and as antitumoral (Lorenzi, 1988; Almeida, Proença, Sano & Ribeiro, 1994). There are very few studies examining the relationship between the biological activities and the ethnobotanical uses by traditional herbal medicine

in order to validate them. Recently, the antioxidant activity of araticum (*Annona crassiflora)* was evaluated by using "in vitro" models and the results indicated that ethanolic extracts of araticum seeds and peel have compounds possessing excellent antioxidant activity so high as others know antioxidant plant products. There was a noticeable correlation between total polyphenols and free radical DPPH scavenging activity (R^2 = 0,821) (Roesler, Malta, Carrasco & Pastore, 2006). The role of antioxidants in preventing oxygen radical and hydrogen peroxide induced cytotoxicity and tissue damage in various human diseases is becoming increasingly recognized. The importance of the antioxidants constituents of plant materials in the maintenance of health and protection against heart diseases and cancer is also raising interest among scientists, food manufacturers and consumers as the trend for the future is toward functional food with specific health effects (Loliger, 1991). Typical compounds that possess antioxidant activity include phenols, phenolic acids and their derivatives, flavonoids, tocols, phospholipids, amino acids and peptides, phytic acid, ascorbic acid, pigments, and sterols. Phenolic antioxidant are primary antioxidants which act as free-radical terminators (Xing & White, 1996). Electrospray (ESI) is a soft and wide-ranging ionization technique that has revolutionized the way the molecules are ionized and transferred to mass spectrometers for mass and property measurements as well as structural characterization (Fenn, Mann, Meng, Wong, Whitehouse., 1989). ESI has therefore greatly expanded the applicability of mass spectrometry to a variety of new classes of molecules with thermal instability, high polarity and mass (Catharino, Haddad, Cabrini, Cunha, Sawaya, & Eberlin, 2005). ESI-MS proves very fast and versatile employing little sample preparation to yield immediate compositional information of the most polar ESI-ionisable compounds. These unique

features of direct infusion ESI-MS have recently been applied for fingerprinting of complex mixtures such as bee propolis (Sawaya et al., 2004), beer (Araujo et al., 2005), wine (Cooper & Marshall, 2001; Catharino et al, 2006), whisky (Moller, Catharino & Eberlin, 2005) and vegetable oil (Catharino et al, 2005; Wu, Rodgers, Marshall, 2004). Therefore the objective of the present work is to explore the ability of the fast and versatile ESI-MS technique with direct infusion to characterize the ethanolic extracts of araticum which demonstrated high antioxidant activity.

MATERIAL AND METHODS

Reagents and standards.

Solvents, reagents and standards were purchased from Sigma Aldrich Chemical Co (St. Louis, MO, USA).

Plant Material.

Araticum fruits *(Annona crassiflora)* were obtained from Erlow Farm, Km 07 Br 070 Goiânia Brazil, in the center west of Brazil. Fruit harvests were conduct twice and special care was taken to avoid damaged fruits. Fruits were transported to the Univ. of Campinas (UNICAMP) and stored at 5°C until used within 2 months.

Ethanolic extraction.

Undamaged fruits were selected and peel, pulp and seeds were manually separated. About 100g of each part of the fruit (pulp, peel and seeds) were cut in small parts, mixed and extracted twice with 300 mL of aqueous ethanol (5:95, v/v, water: ethanol) using a household mixer for 20 min. The extracts were filtered through cotton membrane and the residue was re-extracted under the same conditions. The residues were kept for further evaluations. The resultant material was subjected to vacuum rotary evaporation at 40°C to

remove ethanol. The concentrated ethanolic extracts were lyophilized and stored at – 18°C in amber glass bottles until used.

Determination of total phenols assay.

Estimation of the global phenol content in the extracts was performed by the Folin-Ciocalteu method that involves reduction of the reagent by phenolic compounds, with concomitant formation for a blue complex, its intensity at 760 nm increases linearly with the concentration of phenols in the reaction medium as described by Swain and Hillis, (1959). Ethanolic extracts were dissolved in methanol to obtain a concentration of 0.5 mg/ml. Samples (0.5 mL) were passed through a 0.45 um membrane filter and mixed with 2.5 mL of 10 fold diluted Folin-Ciocalteu reagent and 2.0 mL of 7.5% sodium carbonate solution. After the mixture had been allowed to stand for 5 min at 50°C, the absorbance was measured at 760 nm. In this study, gallic acid was used as spectrophotometric standards and the total phenolic content of the fruit extracts were expressed as gallic acid equivalents (GAE/100g). Estimation of the phenolic compounds was carried out in triplicate and mean values reported.

Electrospray ionization mass spectrometry.

For fingerprinting ESI-MS analysis a hybrid high-resolution and high-accuracy (5 ppm) Micromass Q-TOF mass spectrometer (Micromass, Manchester, UK) was used. The general conditions were: source temperature of 100°C, capillary voltage of 3.0 kV and cone voltage of 40 V. For measurements in the negative ion mode, ESI(-)-MS, 10.0 μL of concentrated NH_4OH were added to the sample mixture having a total volume of 1mL yielding 0.1% as final concentration. For measurements in the positive ion mode ESI(+)-MS, 10.0 μL of concentrated formic acid were added giving a final concentration of 0.1%.

ESI-MS was preformed by direct infusion with a flow rate of 10 μL min^{-1} using a syringe pump (Harvard Apparatus). Mass spectra were acquired and accumulated over 60 sec and spectra were scanned in the range between 50 and 1000 *m/z*. Structural analysis of single ions in the mass spectra from pulp, peel and seeds extracts was performed by ESI-MS/MS. The ion with the *m/z* of interest was selected and submitted to 15-45 eV collisions with argon in the collision quadrupole. The collision gas pressure was optimized to produce extensive fragmentation of the ion under investigation.

Determination of radical scavenging activity.

2,2-diphenyl-1-picrylhydrazyl (DPPH) has been widely used to evaluate the free radical scavenging of natural antioxidants (28,29,30). The scavenger activity of main compounds of araticum extracts (quercetin, caffeic acid , ascorbic acid, ferrulic acid and rutin) was tested against the DPPH radical as well as other compounds for comparative porpoise (gallic acid and alfa-tocopherol). Ethanolic solutions in different concentrations were prepared by adding 1000 uL of DPPH (0,004% w/v) and the final volume was brought to 1200 uL with ethanol. Final concentrations of test materials in the cuvettes were in the range of 1.0 to 100 uM. Each tube was incubating for 30 minutes at room temperature in the dark. The control was prepared as above without any extract, and ethanol was used for the baseline correction. The DPPH' solution was freshly prepared daily, stored in a flask covered with aluminum foil, and kept in the dark at 4°C between measurements. The percent decrease in absorbance was recorded for each concentration and percent quenching of DPPH' was calculated on the basis of the observed decrease in absorbance of the radical. Changes in the absorbance of the samples were measured at 517 nm. Radical scavenging

was expressed as the inhibition percentage and was calculated using the following formula (31):

% Inhibition = $((A_{DPPH} - A_{Extr})/A_{DPPH})*100$

where A_{DPPH} is the absorbance value of the DPPH' blank sample and A_{Extr} is the absorbance value of the test solution. A_{Extr} was evaluated as the difference between the absorbance value of the test solution and the absorbance value of its blank. The IC_{50} values are reported as final concentration of extract in the cuvettes defined as ug/mL of dried extracts required to decrease the initial DPPH concentration by 50%.

RESULTS AND DISCUSSION

Recently, the total phenol and the antioxidant activity of ethanolic extracts of araticum pulp, peel and seeds had been reported in various "in vitro" models (Roesler, Malta, Carrasco & Pastore, 2006). The results showed that araticum extracts contain extremely high contents of total phenols. The highest amount was 111.42g kg^{-1} dry matter for peel ethanolic extract and 136.98g kg^{-1} dry matter for the seed ethanolic extract. Overall, the lowest concentration was found in the ethanolic pulp extract at 31.08g kg^{-1} dry matter. Considering the high total phenol content of araticum extracts compared to recent results of fruits extracts published in the literatures such as pomegranate (Singh, Chidambar Murthy, & Jayaprakasha, 2002), apple, plum and pear (Imeh & Khokhar, 2002), guava (Jimenez, Rincon, Pulido, & Fulgencio, 2001) and berries (Benvenuti, Pellati, Melegari & Bertelli, 2004) - (Table 1), the antioxidant activity of araticum extracts was determined by using "in vitro" methods. Free radical scavenging potentials of araticum peel and seed extracts at different concentrations were tested by the DPPH method and seed, peel and pulp presented

IC_{50} of 31.14 μg mL^{-1}, 48.82 μg mL^{-1} and 148.82 μg mL^{-1} respectively. In addition, the inhibition of lipid peroxidation by using rat liver microsomes as an oxidative system was also evaluated. Fifty percent inhibition of lipid peroxidation of microsomes to thiobarbituric acid reactive species (TBARS) by hydroxyl radicals, generated by $FeCl_3$ system, requires 1.72 μg mL^{-1} of seed ethanolic extract, 4.44 μg mL^{-1} of peel ethanolic extract and 8.62 μg mL^{-1} of pulp etanolic extract. The results are summarized in Table 2. Reports on antioxidant activity of other plant extracts such as *Emblica officinalis* (Jose & Kuttan, 1995), *Phoenix dactylifera* (Vayalil, 2002) and *Punica granatum* (Singh, Chidambar Murthy, & Jayaprakasha, 2002) require a IC_{50} of 3.4 mg ml^{-1}, 2.2 mg ml^{-1} and around 100 ppm respectively of extracts solution. Expressing the antioxidant activity of the extract solution instead of the final cuvettes concentration as mentioning before, the IC_{50} for seed, peel and pulp was 0.043 mg mL^{-1} (43 ppm), 0.12 mg mL^{-1} or 115 ppm and 0.243 mg mL^{-1} or 243 ppm (Roesler, Malta, Carrasco & Pastore, 2006). The ESI-MS Fingerprints technique with direct infusion was used to characterize the presence of compounds with potent free-radical-scavenging activity in araticum fruit. The extracts were analyzed by direct insertion both in the negative and positive ion modes. However, ESI(+)-MS fingerprints produce by far the most characteristic mass spectra; hence only the ESI(-)-MS data will be presented and discussed. This method in the negative ion mode provides a sensitive and selective method for the identification of polar organic compounds with acidic sites, such as the phenolic, organic acids and sugars compounds found in araticum. De-protonated forms of the compounds of interest were then selected and dissociated and their ESI-MS/MS were compared to those of standards. The ESI-MS fingerprints of the samples of araticum extracts (Figure 1) show similarities and some important differences. Figure 1C

shows an ESI(-)-MS typical for pulp extracts. Only sugars and organic acids anions are observed as [malic acid - H_2O - H]$^-$ of *m/z* 115, [malic acid - H]$^-$ of *m/z* 133, [hexose - H_2O - H]$^-$ of *m/z* 161, [hexose - H]$^-$ of *m/z* 179, [2 hexose - H_2O - H]$^-$ of *m/z* 341, [3 hexose - $2H_2O$ - H]$^-$ of *m/z* 503, [3 hexose - H_2O - H]$^-$ of *m/z* 521 and [4 hexose - $2H_2O$ - H]$^-$ of *m/z* 683. Therefore, the small content of total phenols (31.08g kg^{-1}), lack of detection of bioactives and the predominance of sugars and acids in the pulp extract possibly explains its non antioxidant activity results by the "in vitro" models DPPH and inhibition of lipid peroxidation. In the fingerprint of the peel extracts (Figure 1B), the following components were identified in their deprotonated forms: anydric malic acid (*m/z* 115), malic acid –(*m/z* 133), ascorbic acid (*m/z* 175), caffeic acid –(*m/z* 179), quinic acid –(*m/z* 191), ferulic acid (*m/z* 193), xanthoxylin –(*m/z* 195) and rutin –(*m/z* 609). The ESI-MS fingerprints of the seed extracts (Figure 1A) identify a much greater variety of major components wich include: anydric malic acid (*m/z* 115), malic acid (*m/z* 133), ascorbic acid –(*m/z* 175), caffeic acid –(*m/z* 179), quinic acid –(*m/z* 191), ferulic acid –(*m/z* 193), xanthoxylin –(*m/z* 195), caffeoyltartaric acid –(*m/z* 312), caffeoyl glucose (*m/z* 341), [quercetin + hexose + pentose – H]$^-$ of *m/z* 595 and rutin (*m/z* 609). Besides these common components, other ions reveal the presence of unique components for the seed extracts, that is caffeoyltartaric acid (*m/z* 312), caffeoyl glucose (*m/z* 341) and [quercetin + hexose + pentose] of *m/z* 595. The richer composition of the seed extract particularly the presence of phenolic components possibly explain its higher antioxidant activity as compared to the peel and pulp extracts of araticum. The summary of the main compounds found in each part of the araticum fruit is shown in the Table 2. In addition, an association between the antioxidant activity of araticum and its compounds is presented in Table 3. The investigation by direct infusion

electrospray ionization mass spectrometry (ESI-MS) provided important information of biactive components present in the araticum extracts that are widely reported as potent antioxidants probably explaining the antioxidant activity of the extracts (Gülçin, 2006; Kim, Kim, Seo, Shin, Jin & Lee, 2006; Kweon, Hwang & Sung, 2001; Roche, Dufour, Mora & Dangles, 2005). The major compounds found were malic acid, ascorbic acid, caffeic acid, quinic acid, ferulic acid , xanthoxylin, caffeoyltartaric acid, caffeoyl glucose, [quercetin + hexose + pentose – H]- and rutin. The scavenger activity against DPPH radical (IC_{50}) of the main araticum compounds is reported in Table 4. In addition, Table 4 also displays recently published data about some other compounds and its derivatives presented in the araticum extracts. On the whole, araticum major compounds were very effective scavenger and the best results were obtained by caffeic acid IC_{50} = 1.9 mg ml^{-1}, quercetina IC_{50} = 3.1 mg ml^{-1} and rutin IC_{50} = 7.5 mg ml^{-1} respectively. It is important to point out that results for scavenger abilities against DPPH are in good agreement with previous data reported for these compounds (Kim et al., 2006; Saleem et al., 2004; Baratto et al., 2003 and Kweon et al., 2001). Saleem et al., 2004 reported excellent results for quercetin IC_{50} = 4.75 mg ml^{-1} and dimethyl ester of caffeoyltartaric acid IC_{50} = 9.67 mg ml^{-1}. According to Kweon et al., 2001, great results were given by epigallocatechin IC_{50} = 3.7 μM, 3-O-caffeoyl-1-methylquinic acid IC_{50} = 6.9 μM, 5-O-caffeoyl-4-methylquinic acid IC_{50} = 8.8 μM, chlorogenic acid IC_{50} = 12.3 μM and caffeic acid 13.7 μM. Concerning the results found by Kim et al., 2006, the lowest IC_{50} was found for caffeic acid IC_{50} = 3.2 mg ml^{-1} , quinic acid derivatives (methyl 3,5-di-O-caffeoyl-4-O-(3-hydroxy-3-methyl) glutaroylquinate IC_{50} = 4.4 mg ml^{-1} , 3,5-dicaffeoylquinic acid IC_{50} = 5.6 mg ml^{-1} and 4,5-dicaffeoylquinic acid IC_{50} = 5.9 mg ml^{-1}) and quercetin IC_{50} = 5.9 mg

ml^{-1} . In conclusion, the results of the present work indicate the presence of compounds possessing excellent antioxidant activity from peel and seed of araticum fruits with the seeds as the best source of the antioxidants exhibiting higher activity as compared to peel and pulp. Besides, it is highly important to emphasize that the araticum fruit may be considered a very cost-effective natural antioxidant if considered the following relevant aspects: very low IC_{50}; seed and peel are remains of human consumption and often dismissed; the extraction process is extremely simple and fast and the possibility of even more effective extracts by optimisation of extraction, purification and isolation of the identified phenolic compounds. As a consequence, the present results may encourage additional and more in-depth studies on the pharmacological and functional properties of araticum extracts in order to evaluate its possibility as a natural source for the development of a dietary supplement or functional food due to its high antioxidant activity.

Table 1. Total phenol contents of fruit extracts recently published.

	Total Phenols	Reference
Rubus fruticosus L. (Blackberry)	289.3 ± 55,8 (a)	Benvenuti and others 2004
Rubus idaeus L. (Raspberry)	177.5 ± 52,2 (a)	Benvenuti and others 2004
Ribes nigrum L . (Black currant)	639.8 ± 112,9 (a)	Benvenuti and others 2004
Ribus rubrum L. (Red currant)	417.9 ± 72,6 (a)	Benvenuti and others 2004
Aronia melanocarpa Elliott (Black chokeberry)	690.2 ± 8,8 (a)	Benvenuti and others 2004
Psidium guajava **peel**	58.7 ± 4,0 (b)	Jimenez and others 2001
Psidium guajava **pulp**	26.3 ± 0,8 (b)	Jimenez and others 2001
Malus pumila (Apple Red Delicious)	2866 ± 102 (c)	Imeh and Khokhar 2002
Pyrus communis (Pear Forelle)	1194 ± 83 (c)	Imeh and Khokhar 2002
Prunus domestica (Plum Royal Garnet)	2643 ± 112 (c)	Imeh and Khokhar 2002
Punica granatum **Peel** (Pomegranate - ethanolic extration)	18.0 (d)	Singh and others 2002
Punica granatum **Seed** (Pomegranate - ethanolic extration)	2.1 (d)	Singh and others 2002
Annona crassiflora Peel (Araticum - ethanolic extraction)	111.42 ± 8.57 (b)	Roesler and others 2006
Annona crassiflora Peel (Araticum - ethanolic extraction)	136.98 ± 7.56 (b)	Roesler and others 2006
Annona crassiflora Peel (Araticum - ethanolic extraction)	31.08 ± 1.23 (b)	Roesler and others 2006

(a) Total polyphenols expressed as mg of gallic acid per 100g fresh weight (FW)
(b) Total polyphenols expressed as g of gallic acid per Kg dry matter (dm)
(c) Total polyphenols expressed as mg of catechin per 100g dry matter (dm)
(d) Total polyphenols expressed as % w/w of tannic acid - dry matter (dm)

Table 2. Compounds identified in ethanolic extracts of araticum using ESI(-) – MS/MS

ESI- MS ions (*m/z*)					
Compound	**A. crassiflora**			**Deprotonated Ions**	**MS/MS íons**
	Seed	**Peel**	**Pulp**	**$[M-H]^-$ *m/z***	***m/z***
[malic acid - H_2O - H]$^-$	√	√	√	115	-
[malic acid - H]$^-$	√	√	√	133	115
[hexose - H2O - H]$^-$	n.d	n.d	√	161	89
ascorbic acid	√	√	n.d	175	143
caffeic acid	√	√	n.d	179	179; 135
[hexose - H]$^-$			√	179	162; 89
quinic acid	√	√	n.d	191	173; 127; 111; 93; 85
ferulic acid	√	√	n.d	193	193; 178; 149; 134
Xanthoxylin	√	√	n.d	195	-
caffeoyltartaric acid	√	n.d	n.d	311	179
caffeoyl glucose	√	n.d	n.d	341	179
[2 hexose - H2O - H]$^-$	n.d	n.d	√	341	162; 89
[3 hexose - 2H2O - H]$^-$	n.d	n.d	√	503	162; 89
[3 hexose - H2O - H]$^-$	n.d	n.d	√	521	162; 89
[quercetin + hexose + pentose]$^-$	√	n.d	n.d	595	301
rutin hydrate	√	√	n.d	609	301
[4 hexose - 2H2O - H]$^-$	n.d		√	683	162; 89

√, detected; n.d, not detected.

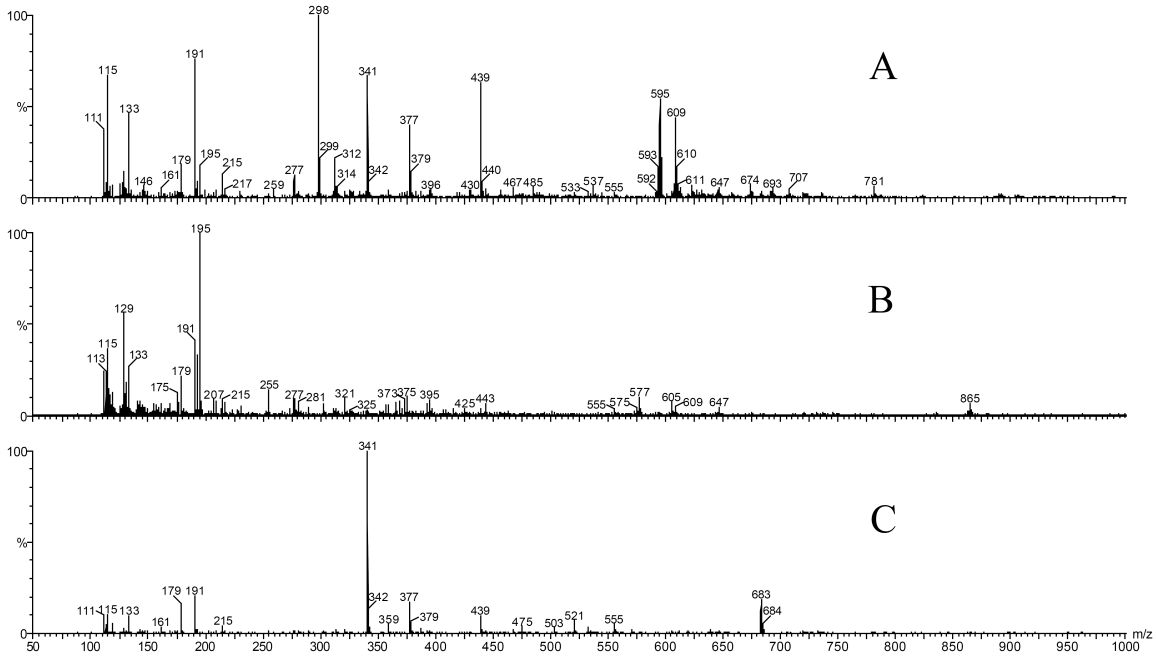
Table 3. Antioxidant activity of Araticum Ethanolic Extracts and respective compounds identified by ESI(-) – MS/MS

	TP (g Kg -1)	**IC 50 (ug mL -1)**		**Mainly componds (ESI-MS)**
		DPPH	**Lipid Peroxidation**	
A. crassiflora pulp	31.08	148.82	8.62	sugar and organic acids ions
A. crassiflora peel	111.42	48.82	4.44	ascorbic acid, caffeic acid, quinic acid, ferulic acid, xanthoxylin and rutin
A. crassiflora seed	136.98	31.14	1.72	ascorbic acid, caffeic acid, quinic acid, ferulic acid, xanthoxylin, caffeoyltartaric acid, caffeoyl glucose, quercetin and rutin

Table 4. Free radical scavenging activity of araticum componds and other natural antioxidants by DPPH.

		DPPH free radical scanenging activity					
		Kim et al., 2006	Kweon et al., 2001	Saleem et al., 2004	Baratto et al., 2003	Experiments Data	
		IC_{50} (ug/mL)	IC_{50} (uM)	IC_{50} (ug/mL)	IC_{50} (uM)	IC_{50} (ug/mL)	IC_{50} (uM)
methyl 5-O-caffeoyl-3-O-sinapoylquinate	**quinic acid derivates**	8.3 ± 0.2					
ethyl 5-O-caffeoyl-3-O-sinapoylquinate		6.1 ± 0.1					
methyl 5-O-caffeoyl-4-O-sinapoylquinate		8.5 ± 0.4					
ethyl 5-O-caffeoyl-4-O-sinapoylquinate		9.5 ± 0.3					
methyl 3,5-di-O-caffeoyl-4-O-(3-hydroxy-3-methyl) glutaroylquinate		4.4 ± 0.1					
ethyl 5-O-caffeoylquinate		7.1 ± 0.4					
3,5-dicaffeoylquinic acid		5.6 ± 0.1					
4,5-dicaffeoylquinic acid		5.9 ± 0.2					
3-O-(3'-methylcaffeoyl)quinic acid	**quinic acid derivates**		16.0 ± 0.34				
5-O-caffeoyl-4-methylquinic acid			8.8 ± 0.06				
3-O-caffeoyl-1-methylquinic acid			6.9 ± 0.07				
dimethyl ester of caffeoyltartaric acid	**caffeic acid derivates**			9.67 ± 1,97			
dimethyl ester of caffeoyltartronic acid				10.71 ± 1.63			
monomethyl ester of caffeoyltartronic acid				14.17 ± 4.15			
methyl ester of caffeic acid				13.13 ± 0.96			
5-O-galloyl quinic	**galloyl quinic derivates**				18.7 ± 2.1		
3,5-O-digalloyl quinic					7.1 ± 0.8		
3,4,5-O-trigalloyl quinic					3.9 ± 0.6		
quercetin		5.9 ± 0.7		4.75 ± 0.57		3.1 ± 0.1	9,2 ± 0,1
quercetin 3-O-glucopyranoside		16.6 ±1.0					
caffeic acid		3.2 ± 0.1	13.7 ± 0.1			1.9 ± 0.1	10,4 ± 0,7
ascorbic acid		5.5 ± 0.1	49.5 ± 0.35	6.49 ± 1.07		7.7 ± 0.3	43.5 ± 1,6
ferulic acid			36.5 ± 0.23			9.9 ± 0.2	51.3 ± 0,9
rutin						7.5 ± 0.1	12.4 ± 0,1
chlorogenic acid			12.3 ± 0.12				
gallic acid					11.2 ± 0.9	1.4 ± 0.1	9.6 ± 0,3
alfa-tocopherol			40.6 ± 0.29	12.64 ± 0.42		7.0 ± 0.1	14.8 ± 0.1
epigallocatechin			3.7 ± 0.03				

Figure 1: ESI-MS fingerprints of araticum: A) Seed, B) Peel and C) Pulp.



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CAPÍTULO 4 - Antioxidant Activity of *Caryocar brasiliense* (pequi) and Characterization of major components by Electrospray Ionization Mass Spectrometry.

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ABSTRACT

The *Caryocar brasiliense* known commonly as pequi is a tropical fruit of Brazilian Cerrado and is considered an important option of income and food for the populations living in this biome. Nowadays, the Brazilian cerrado biome is challenged by two different realities: the great possibility of food production and the extremely rich Biodiversity. Thus it is estimated that 40% of the cerrado biome has already been deforested. Our previous study indicated that *Caryocar brasiliense* had high total phenol content (209g as gallic acid equivalent Kg^{-1}) and excellent scavenging activity against 2,2-diphenyl-1-picrylhydrazyl radical (IC_{50} of 9.44 μg ml^{-1}). In this study we validate the highly efficient antioxidant activity of *C. brasiliense* using the biological relevant method of chemically induced lipid peroxidation. The half inhibition concentration did not exceed 0.8 μg ml^{-1}. In addition, polar components of pequi ethanolic extract were investigated by direct infusion electrospray ionization mass spectrometry (ESI-MS). The technique revealed the presence of important bioactive components widely reported as potent antioxidants such as gallic acid, quinic acid, quercetin and quercetin 3-O-arabinose possibly explaining its higher antioxidant activity. This is the first report on the composition by ESI-MS of pequi extract demonstrating excellent antioxidant activity. Owing to this composition and properties, the studies can be further extending to exploit its possible application as natural antioxidant for cosmetic, pharmaceutical and food products.

KEYWORDS: pequi, electrospray ionization mass spectrometry, total phenols, antioxidant activity, TBA.

INTRODUCTION

Searching for an economically viable and environmentally friend application of Brazilian Cerrado natural resources is highly important and equally urgent in order to avoid the total destruction of natural vegetation and Cerrado Biodiversity. The Cerrado, the second largest biome in South America after the Amazon rainforest, had not suffered human interference until 40 yeas ago. Nowadays, the Brazilian cerrado biome is challenged by two different realities: the great possibility of food production, being considered one of the most important agriculture celery of the world and the extremely rich Biodiversity. It occupies almost 25% of Brazil (Proença, Oliveira, & Silva, 2000) and its very rich flora, which is just beginning to be studied, is estimated to be comprised of about 1000 species of trees, 3000 species of herbs and shrubs, and about 500 of climbers (Medonça et al., 1988). In the last 30 years, progressive mechanization with improved techniques for clearing and fertilizing the soil have contributed to the accelerated destruction of the natural vegetation, and it is estimated that 40% of the cerrado biome has already been deforested (Ratter, Ribeiro & Bridgewater, 1997). Within the framework of our research on natural resources of Cerrado, investigations were conducted on to *Caryocar brasiliense* (pequi). The fruits of pequi (*C. brasiliense*) are an important option of income and food for the populations living in the Cerrado regions of Brazil. Its fruits are widely consumed "in natura" as well as to prepare juice, ice cream, liquor, jelly and specially traditional dishes famous thought out Brazil. The pulp of the pequi has good quantity of edible oil, vitamin A and proteins. On the other side, the nut has also oil, which is applied in cosmetic products such as soaps and skin emulsions (Silva & Tassara, 2001). From the peel and leaf it is extracted high quality yellow color that are used for dying fibers. Regarding the folk medicine, the pulp oil is

traditionally used as tonic agent against asthma, influenza, cold and bronchopulmonary diseases (Almeida, Proença, Sano & Ribeiro, 1994). There are very few scientific studies examining the relationship between the biological activities and the ethnobotanical uses by traditional herbal medicine in order to validate them. Recently, Roesler et al., 2006 demonstrated the scavenging activity of pequi extracts against free radical DPPH. The antifungal activity against *Cryptococcus neoformans* of different parts of *C. brasiliense* had been investigated by Passos, et al., 2002. Oliveira et al., 1968 mentioned that ethanolic extract from leaves could have antitumor proprieties. In recent years, growing efforts have been made in the fields pharmaceuticals and foodstuffs to study so called antioxidants, especially those of natural origin. Oxidation is a metabolic process that leads to energy production necessary for essential cell activities. However, metabolism of oxygen in living cells also leads to the unavoidable production of oxygen-derived free radicals, commonly known as reactive oxygen species (ROS) (Mccord 1994, Adegoke and others 1998). In the absence of adequate endogenous antioxidant defenses, the propagation of free radical-producing events can lead to the co-oxidation of nucleophilic cellular constituents and the reaction of secondary lipid autoxidation products with nucleophilic macromolecules such as membrane constituents, enzymes and DNA (Yan & Kitts, 1996). Therefore the excess free radicals circulating in the body oxidize the low density lipoproteins (LDL), making them potentially lethal, the excess free radicals can also accelerate ageing processes and have been linked to other very serious pathologies, such as brain stroke, diabetes mellitus, rheumatoid arthritis, Parknson`s disease, Alzheimer`s disease and cancer (Ames and others 1993, Ames and others 1995, Diaz and others 1997, Lang and Lozano 1998, Christen 2000, Esterbauer and others 1991, Esterbauer and others 1992). The role of antioxidants in

preventing oxygen radical and hydrogen peroxide induced cytotoxicity and tissue damage in various human diseases is becoming increasingly recognized. Antioxidants is any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate. The terms oxidizable substrate includes every type of molecule found "in vivo". Antioxidant defenses comprise agents that catalytically remove free radicals and other reactive species, proteins that minimize the availability of pro oxidants such as iron ions, copper ions and haem, proteins that protect biomolecules against damage and low molecular agents that scavenger ROS such as glutathione and alfa tocopherol (Halliwell & Guitteridge, 1999). The importance of the antioxidants constituents of plant materials in the maintenance of health and protection against heart diseases and cancer is also raising interest among scientists, food manufacturers and consumers as the trend for the future is toward functional food with specific health effects (Loliger, 1991). Typical compounds that possess antioxidant activity include phenols, phenolic acids and their derivatives, flavonoids, tocols, phospholipids, amino acids and peptides, phytic acid, ascorbic acid, pigments, and sterols. (Xing & White, 1996). A phenol contains an –OH a group attached to a benzene ring. Many phenols exert powerful antioxidant effects "in vitro", inhibiting lipid peroxidation by acting as chain breaking peroxyl radicals scavengers. Phenols with two adjacent –OH groups, or other chelantin structures, can also bind transition metal ions in forms poorly active in promoting free-radical reacting. Phenols can also directly scanvenge ROS, such as OH, ONOOH and HOCl (Halliwell & Guitteridge, 1999). The aim of the present work was to prepare different extracts from pequi fruits and validated the antioxidant activity of this fruit by using the "in vitro" model lipid peroxidation by using rat liver microsomes as an oxidative

system. In addition, the present work explores the ability of the fast and versatile Electrospray Ionization Mass Spectrometry (ESI-MS) technique with direct infusion to characterize the extracts of pequi, which demonstrated high antioxidant activity. Electrospray (ESI) is a soft and wide-ranging ionization technique that has revolutionized the way the molecules are ionized and transferred to mass spectrometers (MS) for mass and property measurements as well as structural characterization (Fenn, Mann, Meng, Wong, Whitehouse, 1989). ESI has therefore greatly expanded the applicability of mass spectrometry to a variety of new classes of molecules with thermal instability, high polarity and mass (Catharino, Haddad, Cabrini, Cunha, Sawaya, & Eberlin, 2005).

MATERIAL AND METHODS

Reagents and standards.

Solvents and reagents were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA).

Plant Material.

Pequi fruits *(Caryocar brasiliense)* were obtained from Erlow Farm, Km 07 Br 070 Goiânia Brazil, in the center west of Brazil. Fruit harvests were conducted twice and special care was taken to avoid damaged fruits. Fruits were transported to the Univ. of Campinas (UNICAMP) and stored at 5°C until used which was usually within 2 months.

Aqueous extraction.

Undamaged fruits were selected and peel, pulp and seeds were manually separated. About 100 g of each part of the fruit (pulp, peel and seeds) was cut in small parts, mixed and extracted twice with 300 mL of fresh water using a household mixer for 20 minutes. The

extracts were filtered through cotton membrane and the residue was re-extracted under the same conditions. The residues were kept for further evaluations. The pooled filtrates were lyophilized and stored at – 18°C in amber glass bottles until used.

Ethanolic extraction.

Undamaged fruits were selected and peel, pulp and seeds were manually separated. About 100g of each part of the fruit (pulp, peel and seeds) were cut in small parts, mixed and extracted twice with 300 mL of aqueous ethanol (5:95, v/v, water: ethanol) using a household mixer for 20 min. The extracts were filtered through cotton membrane and the residue was re-extracted under the same conditions. The residues were kept for further evaluations. The resultant material was subjected to vacuum rotary evaporation at 40°C to remove ethanol. The concentrated ethanolic extracts were lyophilized and stored at – 18°C in amber glass bottles until used.

Determination of total phenols assay.

Estimation of the global phenol content in the extracts was performed by the Folin-Ciocalteu method that involves reduction of the reagent by phenolic compounds, with concomitant formation for a blue complex, its intensity at 760 nm increases linearly with the concentration of phenols in the reaction medium as described by Swain and Hillis, (1959). Ethanolic extracts were dissolved in methanol to obtain a concentration of 0.5 mg/ml. Samples (0.5 mL) were passed through a 0.45 um membrane filter and mixed with 2.5 mL of 10 fold diluted Folin-Ciocalteu reagent and 2.0 mL of 7.5% sodium carbonate solution. After mixture had been allowed to stand for 5 min at 50°C, the absorbance was measured at 760 nm. In this study, gallic acid was used as spectrophotometric standards and the total phenolic content of the fruit extracts were expressed as gallic acid equivalents

(GAE/100g). Estimation of the phenolic compounds was carried out in triplicate and mean values reported.

Determination of radical scavenging activity.

2,2-diphenyl-1-picrylhydrazyl (DPPH) has been widely used to evaluate the free radical scavenging of natural antioxidants (Brand-Williams and others 1995, Bondet and others 1997, Sanchez-Moreno and others 1998). From aqueous and ethanolic extracts, ethanolic solutions in different concentrations were prepared by adding 1000 µL of DPPH (0,004% w/v) and the final volume was brought to 1200 µL with ethanol. Final concentration of test materials in the cuvettes were between 1,0 to 2000,0 µg mL^{-1}. Each tube was incubating for 30 minutes at room temperature in the dark. The same procedure was taken for galic acid and commercial rosmarinus extract for comparative purpose. The control was prepared as above without any extract, and ethanol was used for the baseline correction. The DPPH solution was freshly prepared daily, stored in a flask covered with aluminum foil, and kept in the dark at 4°C between measurements. The percent decrease in absorbance was recorded for each concentration and percent quenching of DPPH was calculated on the basis of the observed decrease in absorbance of the radical. Changes in the absorbance of the samples were measured at 517 nm. Radical scavenging was expressed as the inhibition percentage and was calculated using the following formula (Yen and Duh 1994):

$\%\ \text{Inhibition} = ((A_{DPPH} - A_{Extr})/A_{DPPH})*100$

where A_{DPPH} is the absorbance value of the DPPH' blank sample and A_{Extr} is the absorbance value of the test solution. A_{Extr} was evaluated as the difference between the absorbance value of the test solution and the absorbance value of its blank. The IC_{50} values are reported

as final concentration of extract in the cuvettes defined as μg/mL of dried extracts required to decrease the initial DPPH concentration by 50%.

Preparation of rat liver microsomes.

Male Wistar Rats receiving normal diets and weighing 200 – 300 g were killed by decapitation after overnight starvation. Livers were removed and homogenized (1:2 w/v) in ice-cold phosphate buffer (100 mM, ph 7,4) containing 1 mM EDTA and 1,15% KCl. The homogenate was centrifuged (10000g, 20 min, 4°C), and the supernatant was collected and centrifuged (105000g, 60 min, 4°C). To be stored, the pellet was suspended (0,5mg liver/mL) in ice-cold phosphate buffer (100 mM, pH 7,4) containing 0,1 mM EDTA and 20% glycerol (Omura and Sato 1964). Microsomal protein concentrations were determined by the Lowry method (Lowry and others 1951) with bovine serum albumin used as standard.

Antioxidant Activity.

Thiobarbituric acid reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm (Van der Sluis and others 2000). It was optimized to be able to use microtiter plates and ELISA reader, which makes it possible to analyze large numbers of samples in run. Microsomes were thawed on ice and were diluted with Tris-HCl buffer (50mM, pH 7,4) containing 150 mM KCl to 1mg/mL protein and centrifuged (100000g, 60 min, 4°C). The pellet was ressuspended with 1ml of the Tris buffer and diluted to the concentration needed (final concentration 0.5 mg/mL protein unless otherwise stated) (Omura and Sato 1964, Van der Sluis and others 2000, Singh and others 2002).

The microsomes (aliquots of 240 μL) were pre incubated in a 48-well plate for 5 min at 37°C. Samples of 30 mL of different concentration of extracts dissolved in ethanol or blank

(corresponding with the solvent ethanol) were added. Lipid peroxidation (LPO) was induced by adding 15 µL of ascorbic acid (4mM) and 15 µL of $FeCl_3$ (0.2 mM). After incubation for 60 min at 37°C the reaction was stopped by addition of 0.5 mL of 0.83% thiobarbituric acid dissolved in TCA-HCl (16.8% w/v trichloroacetic acid in 0.125N HCl). LPO was assessed by measuring thiobarbituric acid reactive species (TBARS) after the plates were heated for 15 min at 80°C and subsequent centrifugation (2500 rpm, 15 min). A 250 µL sample of each incubation was transferred to 96-well plates, and absorption was read at 540 nm (color) vs 620 nm (turbidity correction) by ELISA reader (Van der Sluis and others 2000, Singh and others 2002). Final concentration of test materials in the cuvettes were between 1.0 to 50.0 µg/mL .

Calculations. The percentage of inhibition produced by a sample at a given concentration can be calculated from the absorbance readings. The percentage of inhibition is expressed as the inhibition of lipid peroxidation of that sample compared to the lipid peroxidation in a blank.

% Inhibition = $((A_{blank} - A_{sample})/ A_{blank})*100$

where A_{blank} = absorbance of the blank ($A_{540} - A_{620}$), and A_{sample} = absorbance of the sample ($A_{540} - A_{620}$).

Electrospray ionization mass spectrometry.

For fingerprinting ESI-MS analysis a hybrid high-resolution and high-accuracy (5 ppm) Micromass Q-TOF mass spectrometer (Micromass, Manchester, UK) was used. The general conditions were: source temperature of 100°C, capillary voltage of 3.0 kV and cone voltage of 40 V. For measurements in the negative ion mode, ESI(-)-MS, 10.0 µL of concentrated NH_4OH were added to the sample mixture having a total volume of 1000 µl

yielding 0.1% as final concentration. For measurements in the positive ion mode ESI(+)-MS, 10.0 μL of concentrated formic acid were added giving a final concentration of 0.1%. ESI-MS was preformed by direct infusion with a flow rate of 10 μL min^{-1} using a syringe pump (Harvard Apparatus). Mass spectra were acquired and accumulated over 60 sec and spectra were scanned in the range between 50 and 1000 *m/z*. Structural analysis of single ions in the mass spectra from pulp, peel and seeds extracts was performed by ESI-MS/MS. The ion with the *m/z* of interest was selected and submitted to 15-45 eV collisions with argon in the collision quadrupole. The collision gas pressure was optimized to produce extensive fragmentation of the ion under investigation. The compounds were identified by comparison of their ESI-MS/MS fragmentation spectra with literature whenever possible. The Equipment was calibrated with a solution of Phosphoric acid, permitting a resolution of less than 20 ppm. The compounds whose fragmentation spectra were not found in literature were tentatively identified based on their high resolution mass.

RESULTS AND DISCUSSION

It has been widely accepted that diet may significantly influence human health and life quality. Recently, more consumers are interested in food products that either reduce the risk of, or manage, a specific health condition. In this study, two different parts isolated from pequi fruits were compared for their total phenolic contents, antioxidant properties and polar composition by ESI-MS. The pequi fruit is a very important income of regional population and it has been used as food complement, for vegetable oil production (food and cosmetics applications) and also for tradition medicine. The pequi most consumed part is the pulp. The pulp +seed represents just 25% of the fruit (Roesler et al., 2006). The

mesocarp (pulp) has approximately 76% of oil (dry matter), 3% of proteins, 14% of fiber and 11% of other carbohydrates (Marx, Andrade & Maia, 1997). On the other hand, the endocarp (seed) has 6.8% of carbohydrates, 1.0% of proteins and 10% de lipids (Silva; Silva, Junqueira & Andrade, 1994). Although the epicarp (peel) represents 75% of the fruit, there are few studies on this part of fruit. Probably the lack of information is because the peel is considered a residue and is always dismissed by regional population.

Total Phenolic Content.

Folin-Ciocalteau's method allows the estimation of all flavonoids, anthocyanins and nonflavonoid phenolic compounds, that is, of all the phenolics present in the sample. The different ethanolic and aqueous pequi extracts were examined and compared for their total phenolic contents (TPC) expressed as gallic acid equivalent (GAE). As shown in Table 1, the greatest TPC of 209.37 g of GAE Kg^{-1} dry matter (dm) was detected in pequi peel ethanolic extract, whereas aqueous pequi pulp + seed extract had the lowest TPC value of 20.88 g of GAE Kg^{-1} dm. The results shown that peel has great quantity of TPC compared to pulp + seed independently of the solvent used for extraction. The aqueous extraction of total phenolic compounds was so efficient as ethanolic extraction for pequi peel. It is important to point out that the ethanolic extraction of peel was not fully completed considering that its residue still gave high TPC of 161.77g kg^{-1} dm. Thus the total phenol content could probably be improved by further studies such as increasing the solvent quantity, the time of extraction and the number of re-extractions from the residue. A commercial *Rosmarinus officinalis* extract often used as a antioxidant in food and cosmetic industry was also measured in this study in order to be compared with the pequi extracts. Considering the high total phenol content of pequi extracts compared to recent results of fruits extracts published in the literatures such as araticum (Roesler et al., 2006), olives

(Boskou et al., 2006), pistachio (Goli, Barzegar & Sahari, 2005), pomegranate (Singh and others 2002), apple, plum and pear (Imeh and Khokhar 2002), guava (Jimenez and others 2001) and berries (Benvenuti and others 2004), the antioxidant activity of pequi extracts was determined by two different methods.

Table 1. Total Phenol Content (TPC) expressed as Galic Acid Equivalents (GAE)[a].

	TP (g GAE/Kg dm)			
	Extracts		Residues	
	Ethanolic	Aqueous	Ethanolic	Aqueous
Caryocar brasilense **Peel**	209.37±3.57	208.42±1,35	161.77±1.14	n.d.
Caryocar brasilense **Pulp + Seed**	27.19±1.25	20.88±3.45	15.03±2.84	20.59±0.64
Rosmarinus officinalis	35.68*		n.d.	

a Each value is the mean ± standard deviation of at least three replicate experiments. n.d. = not determined. dm = dry matter. * Total phenols per kg of commercial extract.

Determination of radical scavenging activity.

A radical scavenging activity toward the stable radical 2,2-diphenyl-1-picryl hydrazyl DPPH was firstly used since it is a very simple, sensitive and rapid method which was very convenient for the screening of large numbers of samples of different polarity (Koleva and others 2002). The pequi extracts were analyzed and compared with gallic acid and quercetin for free radical scavenging activity. The concentration required to scavenge 50% of the free radicals in the reaction mixture, the IC_{50} value, was also determined. A smaller IC_{50} value corresponds to a greater DPPH scavenging activity. These data indicated that the lowest IC_{50} was given by the gallic acid (1.4 μg mL^{-1}), quercetin (3.1 μg mL^{-1}), ethanolic pequi peel extract (9.44μg mL^{-1}) and aqueous pequi peel extract (17.98μg mL^{-1}). Thus

pequi peel extracts, particularly ethanolic extract, has excellent radical scavenging activity comparable with remarkable antioxidants as quercetin and gallic acid. The IC_{50} value for commercial rosemary extract was 80.82μg mL^{-1}. The water extracts gave higher IC_{50} in all the extracts tested. Even the residue left from the preparation of ethanolic extract pequi peel showed a good scavenging activity which could be used to enhance the extracts activity by improvements in the extraction methods. Regression analyses of the scavenging activity of cerrado fruits showed a high linear dependence of extracts concentration as illustrated by Figures 1-3. The slopes were 0.155; 2.6289 and 2.4844 for ethanolic pulp + seed pequi extract, peel aqueous pequi extract and peel ethanolic pequi extract, respectively. In addition, there was a noticeable correlation between free radical DPPH scavenging activity and total polyphenols (R^2 = 0.9792) for pequi extracts and residues (Figure 4). Reports on the relationship between total phenols and antioxidant activities are contradictory; whereas some authors have observed a high correlation (USDA, ARS, Roesler et al., 2006, Jimenez and others 2001, Benvenuti and others 2004, Kahkonen and others 1999), others have found no direct correlation (Imeh and Khokhar 2002, Eberhardt and others 2001, Wu and others 2004).

Figure 1: Radical scavenging activity of ethanolic pequi peel extract.

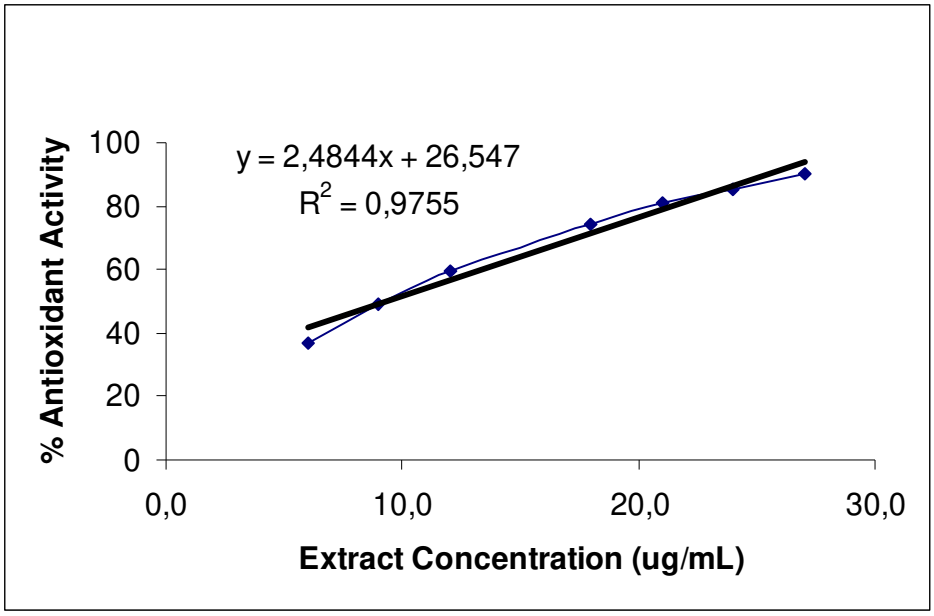


Figure 2: Radical scavenging activity of aqueous pequi peel extract.

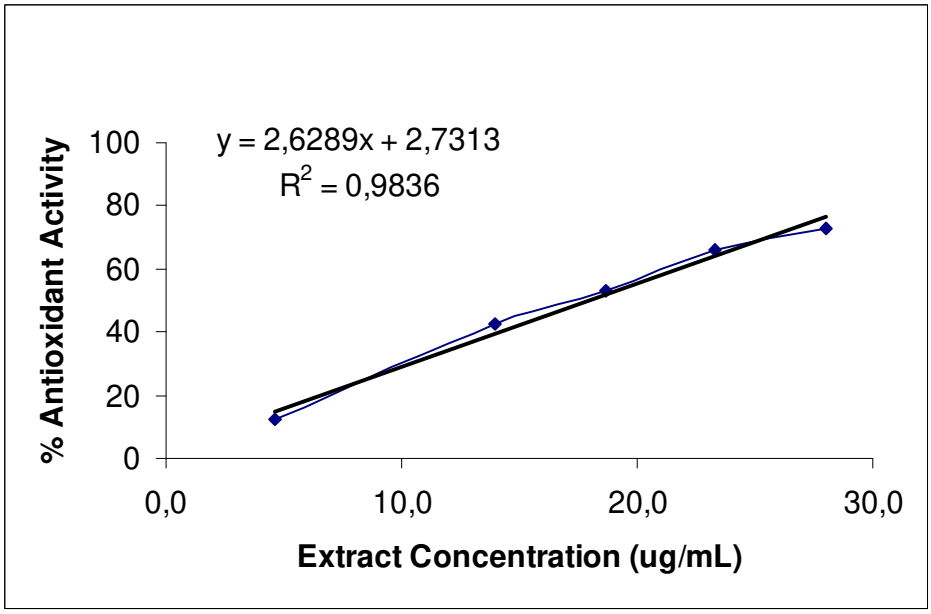


Figure 3: Radical scavenging activity of ethanolic pequi pulp + seed extract.

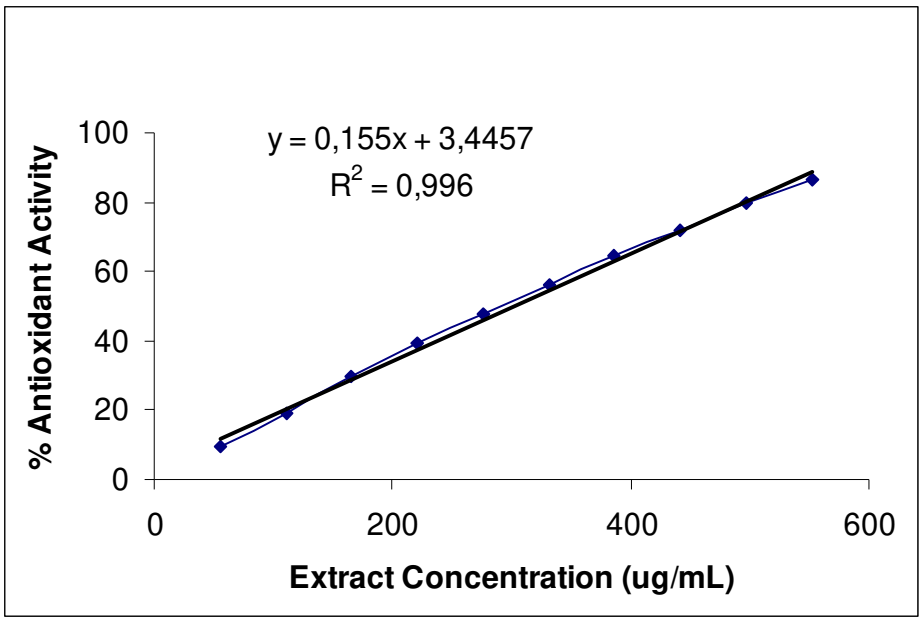
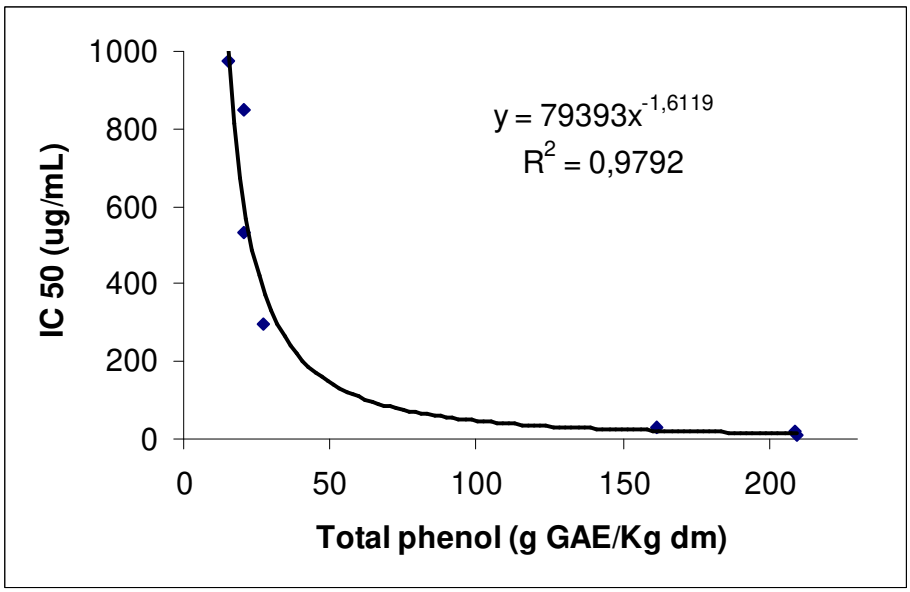


Figure 4: Correlation between free radical DPPH scavenging activity and total phenols content of pequi differente extracts.



Inhibition of Lipid Peroxidation.

The rat liver microsome was chosen as an oxidative system because it is close to the "in vivo" situation where both an aqueous phase and a lipid phase are present. The extent of lipid peroxidation after chemical induction by radical formation is monitored by the thiobarbituric acid (TBA) test. Fifty percent inhibition of lipid peroxidation of microsomes to TBARS by hydroxyl radicals, generated by $FeCl_3$ system, requires 0.78 μg mL^{-1} of peel ethanolic extract and 33.84 μg mL^{-1} of pulp + seed etanolic extract. For comparative purposes, the IC_{50} for gallic acid and quercetin were also measured. The IC_{50} achieved for gallic acid was 1.01 μg mL^{-1} or 5.94 uM and for quercetin 1.18 μg mL^{-1} or 3.5 μM. Van der Sluis and others 2000 reported about the same order of antioxidant potency for catechin and quercetin using the same method. Thus pequi peel ethanolic extract was highly efficient antioxidant using the biological relevant model of iron chemically induced microssome peroxidation by scavenging peroxy radical. The results of the iron induced lipid peroxidation are shown in Table 2 and Figures 5 and 6. Regression analysis of lipid peroxidation inhibition results showed a high dependence of pequi extracts concentration as demonstrated in the Figures 5 and 6. The best adjustment correlation was obtained by using quadratic model (R^2 = 0.99 and 0.97 for pequi peel and seed+pulp respectively). The excellent performance of pequi extracts, particularly pequi peel extract, as natural antioxidant was also achieved with the ability to scavenge stable radical DPPH. As expected, the DPPH and TBA assays did not presented the same correlation tendency or absolute IC_{50} results mainly due to the different reaction medium conditions (hydrophilic and hydrophobic), different substrates (chemical radical and biological membranes) and different product monitored of each assay. Antioxidant activity and mechanisms are system-dependent and can vary with radical substrates targets, solvent, antioxidant phase

localisation, etc. Owing to the complexity of the oxidation-antioxidation processes, no single method is capable of providing a comprehensive picture of the antioxidant profile of a studied sample (Koleva et al., 2002). Integrating results from multiple assays with different mechanisms is important to elucidate differences in reactivity between compounds, as well as changes in reaction rates and mechanisms with solvent, environment and antioxidant concentration. According to Schaich et al., 1993, for example, curcumins scavenge radicals rapidly in lipids but when water is present metal complexation dominates. Pequi peel ethanolic extract had high antioxidant activity (smallest IC_{50} and highest slope value for the correlation between antioxidant activity and extract concentration) whatever the oxidative substrates or assay chosen thus indicating the presence of compounds with potent antioxidant activity in pequi peel with so high activity as others well known antioxidant plant products. On the other hand, pequi seed+peel ethanolic extract demonstrated better potential to inhibit lipid peroxidation than to scavenge stable radical DPPH as shown on Table 2. Considering that the lipid peroxidation substrate (microsomes) contains both an aqueous phase and a lipid phase and DPPH radical substrate is just hydrophilic, it may be possible that pequi seed+peel ethanolic extract is more efficient when lipid phase is present.

Table 2. Half-inhibition concentration (IC50) of Pequi Extracts against DPPH and Lipid Peroxidation

	DPPH		DPPH		TBA
	IC 50 (ug/mL)		IC 50 (ug/mL)		IC 50 (ug/mL)
	Extract		Residue		Extract
	Ethanolic	Aqueous	Ethanolic	Aqueous	Ethanolic
Caryocar brasilense **Peel**	9.44±0.3	17.98±0.4	28.49±0.45	nd	0.78 ± 0.1
Caryocar brasilense **Pulp + Seed**	298.75±3.8	534.43±7.3	974.55±1.29	847.23±34.83	33.84 ± 4.2
Rosmarinus officinalis	80.84 ± 4.5	n.d.	n.d.		n.d.
Galic Acid	1.4 ± 0.1	n.d.	n.d.		1.01 ± 0.1
Quercetin	3.1 ± 0.1	n.d.	n.d.		3.5 ± 0.2

Figure 5: Inhibition of lipid peroxidation by ethanolic pequi peel extract

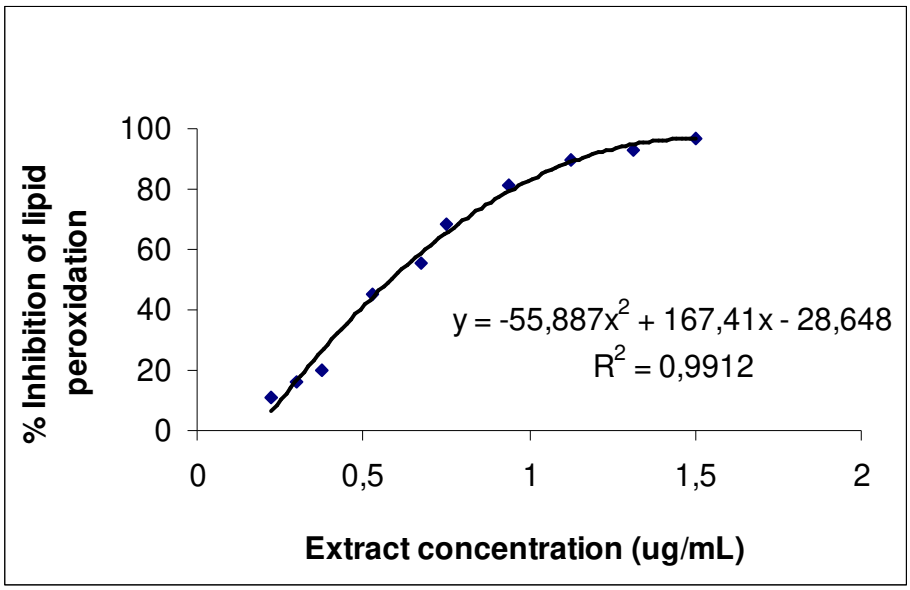
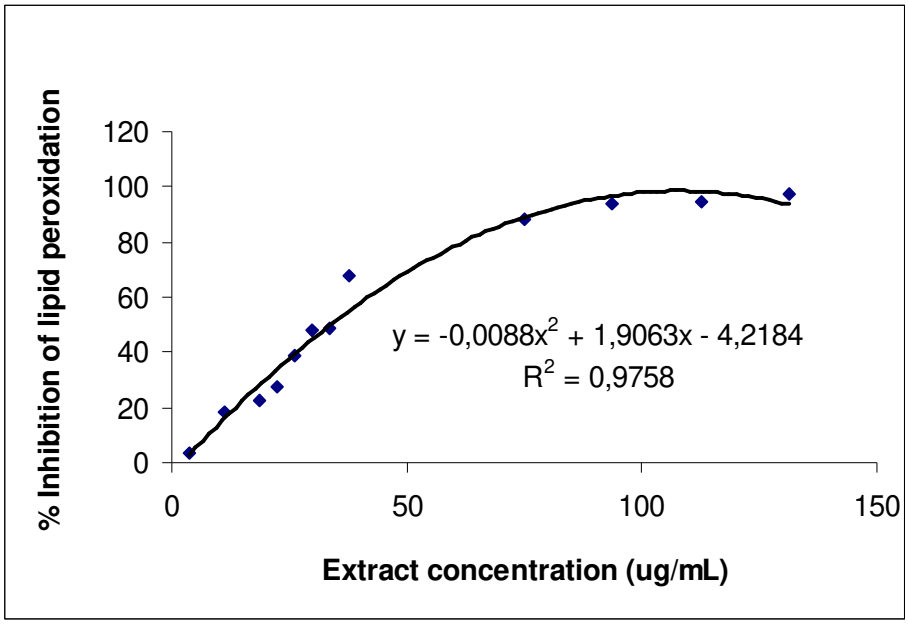


Figure 6: Inhibition of lipid peroxidation by ethanolic pequi pulp+seed extract



ESI-MS Fingerprints.

Electrospray Ionization (ESI) is a soft and wide-ranging ionization technique that has revolutionized the way the molecules are ionized and transferred to mass spectrometers for mass and property measurements as well as structural characterization (Fenn, Mann, Meng, Wong, Whitehouse, 1989). ESI has therefore greatly expanded the applicability of mass spectrometry to a variety of new classes of molecules with thermal instability, high polarity and mass (Catharino, Haddad, Cabrini, Cunha, Sawaya, & Eberlin, 2005). ESI-MS proves very fast and versatile employing little sample preparation to yield immediate compositional information of the most polar ESI-ionisable compounds. These unique features of direct infusion ESI-MS have recently been applied for fingerprinting of complex mixtures such as cerrado fruit araticum (Roesler et al., 2007), bee propolis (Sawaya et al., 2004), beer (Araujo et al., 2005), wine (Cooper & Marshall, 2001; Catharino et al, 2006),

whisky (Moller, Catharino & Eberlin, 2005) and vegetable oil (Catharino et al, 2005; Wu, Rodgers, Marshall, 2004). The extracts were analyzed by direct insertion ESI-MS both in the negative and positive ion modes. However, ESI(-)-MS fingerprints produce by far the most characteristic data; hence only ESI(-)-MS data will be presented and discussed.

ESI(-)-MS provides a very sensitive and selective method for the identification of polar organic compounds with acidic sites, including therefore the major bioactive phenolic, organic acids and sugars components expected to be found in tropical fruits. De-protonated forms of the compounds of interest were then selected and dissociated and their ESI-MS/MS were compared to literature.

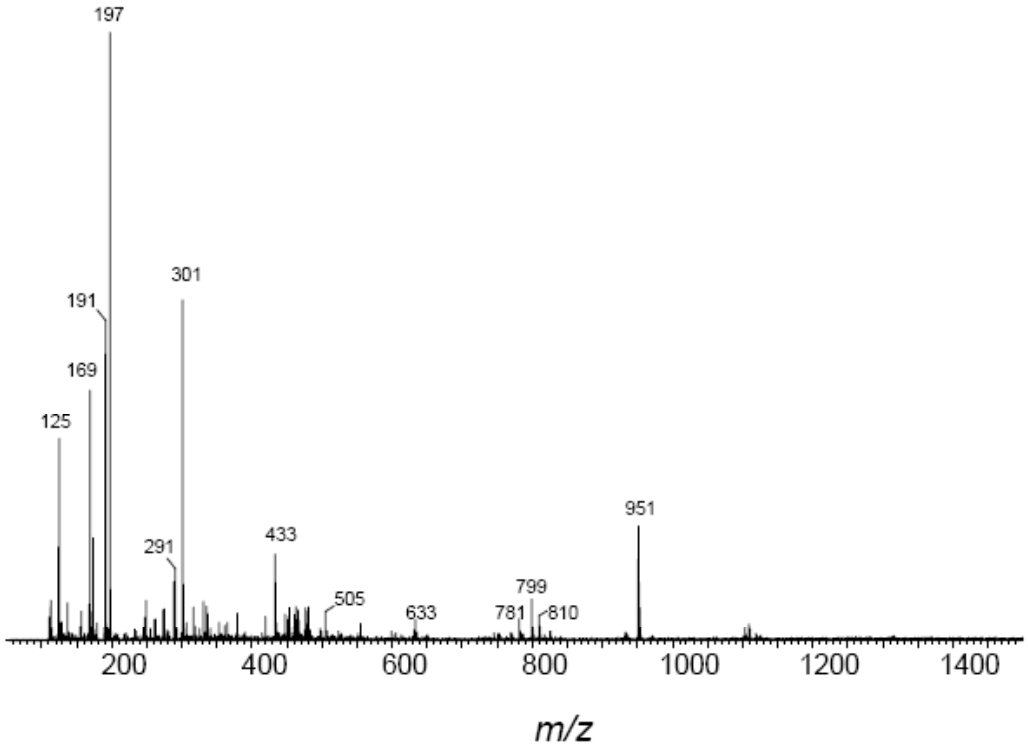
The investigation by direct infusion electrospray ionization mass spectrometry (ESI-MS) provided important information of biactive components of pequi peel ethanolic extracts which are widely reported as potent antioxidants probably explaining the antioxidant activity of the extracts (Kim et al., 2006; Kweon et al., 2001; Roche, Dufour, Mora & Dangles, 2005). The major phenolic components identified for pequi ethanolic peel extract by ESI-MS fingerprints were: p-hydro benzoic acid of *m/z* 137, gallic acid of *m/z* 169, quinic acid of *m/z* 191, quercetin of *m/z* 301 and quercetin 3-O-arabinose of *m/z* 433 (Figure 7 and Table 3) by comparison from data found in literature (Bastos et al., 2007, Møller et al., 2007).

Table 3. Compounds identified in pequi peel ethanolic extract using ESI(-) – MS/MS

ESI- MS ions (*m/z*)		
Compound		MS/MS íons
	$[M-H]^-$ *m/z*	*m/z*
gallic acid	169	125, 79
quinic acid	191	111, 173, 85, 93, 127
quercetin	301	179, 151, 121,107
quercetin 3-O-arabinose	443	301

As reported on Table 2, the phenolic compounds found in pequi peel ethanolic extract (quercetin and gallic acid) were also tested and demonstrated high antioxidant activity in the same bioassays system chosen for pequi extracts. The IC_{50} found for quercetin and gallic acid are in the same concentration range as previously reported by Kim et al., 2006; Saleem et al., 2004; Barrato et al., 2003 and Van der Sluis et al., 2000. According to Saleem et al., 2004 and Kim et al., 2006, the results found for quercetin against DPPH radical was IC_{50} = 4.75 μg ml^{-1} and IC_{50} = 5.9 μg ml^{-1} while the result found in this study was 3.1 μg ml^{-1} for the same assay. Regarding the results obtained by Barrato et al., 2003, the IC_{50} for gallic acid was 11.2 μM and the result showed in this study was 9.6 μM for DPPH assay. Concerning quercetin 3-O glucopyranoside, the IC_{50} reported by Kim et al., was 16.6 μg ml^{-1}. For quinic acid derivatives, the IC_{50} results are in the range of 4.4 to 9.5 μg ml^{-1} according to Kim et al., 2006 and in the range of 6.9 to 16 μM for Kweon et al., 2001, depending on the derivative of the quinic acid. Barrato et al., 2003 studied also some galloyl derivatives, which results varied between 4.0 to 19 μM.

Figure 7: ESI-MS fingerprints of Pequi Peel Ethanolic Extract



The phenolic compounds act by scavenging free radicals and quenching the lipid peroxides. The hydroxyl and phenoxy groups of phenolic compounds donate their electron to the free radials and neutralize them, forming phenolic radical and quinine methide intermediate, which is excreted via bile (Pan et al., 1999). An epidemiological study in the Netherlands (the Zutphen study) suggested an inverse relation of the incidence of coronary heart disease and stroke in elderly men with the dietary intake of flavonoids (especially quercetin) (Keli et al., 1996). In addition, lipid peroxidation may be prevented at the initiation stage by free radical scavengers, while the chain propagation reaction can be intercepted by peroxy-radical scavengers such as phenolic antioxidants (Takahama, 1983). Hence the phenolic compounds achieved in pequi peel ethanolic extract probably are the responsible for its antioxidant activity. The correlation analysis between total phenol content of pequi

different extracts and scavenging activity against DPPH radical showed a remarkable correlation (R^2= 0.9792) which clearly demonstrates the a strong relation between these phenolic compounds and antioxidant activity (Figure 4).

In conclusion, pequi peel ethanolic extract showed strong free radical scavenging activity and inhibition of lipid peroxidation. It is likely that the rich composition of the pequi peel extract particularly the presence of phenolic components such as gallic acid, quinic acid, quercetin and quercetin 3-O-arabinose possibly explain its higher antioxidant activity. Thus pequi peel may serve important pharmaceutical, cosmetic or food complement roles, linked to its antioxidant behaviour, as those related to prevention of diseases induced by oxidation stress. In addition, pequi peel can be considered a cost effective natural antioxidant source since pequi peel is actually considered a residue from pequi fruit applications. Further studies should be done to provide even more effective extracts by more optimised extraction, purification and isolation of the identified phenolic compounds. "In vivo" models are needed to evaluate the possibility of pequi extract as a natural source for the development of a dietary supplement due to its high antioxidant activity. This research is part of our continuous effort to promote the value-added utilization of cerrado natural resources and in improving human nutrition for disease prevention and health promotion.

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CAPÍTULO 5 - Effect of Extracts from Araticum (*Annona crassiflora*) on CCl4-Induced Liver Damage in Rats

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ABSTRACT

The influence of ethanolic extracts of *Annona crassiflora* fruit on the activities of hepatic antioxidant enzymes was examined. Extracts of *A. crassiflora* seeds and peel were administered orally (50 mg of galic acid equivalents/kg) to Wistar rats for 14 consecutive days followed by a single oral dose of carbon tetrachloride (CCl_4, 2 g kg^{-1}). Lipid peroxidation and the activities of hepatic catalase (CAT), cytochromes P450 (CP450) and b5, glutathione peroxidase (GPx), glutathione reductase (GRed), superoxide dismutase (SOD), and the content of glutathione equivalents (GSH), were evaluated. Treatment with CCl_4 increased lipid peroxidation by 44%, increased the level of GSH equivalents by 140% and the content of cytochrome b5 by 32%; and concomitant reduction of the activities of CAT, SOD and CP450 by 23, 34 and 39% respectively. Treatment with *A. crassiflora* seeds and peel extracts alone inhibited lipid peroxidation by 27 and 22% and preserved CP450 content. Pretreatment with the extract of *A. crassiflora* prevented lipid peroxidation, reduced CAT activity and the induction of GSH equivalents content caused by CCl_4, but had no effect on CCl_4-mediated changes in the activities of CP450 and b5 and SOD. These results show that *A. crassiflora* seeds and peel contain antioxidant activity in vivo and could be of potential therapeutic use.

KEYWORDS: *Annona crassiflora*, antioxidant activity, lipid peroxidation, CCl4, liver enzymes.

INTRODUCTION

Reactive oxygen species (ROS) are products of oxidative metabolism and their production can be stimulated by radiation and xenobiotic agents derived from air pollution or chemicals such as carbon tetrachloride, paraquat and cigarette smoke. In the absence of adequate endogenous antioxidant defenses, the propagation of free radical-producing events can lead to the co-oxidation of nucleophilic cellular constituents and the reaction of secondary lipid autoxidation products with nucleophilic macromolecules such as membrane constituents, enzymes and DNA (Yan & Kitts, 1996).

Oxidative stress has been associated with the development of chronic and degenerative diseases, including cancer, heart disease, and neuronal degeneration such as in Alzheimer's disease, as well as being involved in aging *(*Ames and others 1993, Ames and others 1995, Diaz and others 1997, Lang and Lozano 1998, Christen 2000*).* The potential of plant antioxidants in contributing to human health and in protecting against heart diseases and cancer has attracted considerable interest among scientists, food manufacturers and consumers, and has led to the development of functional foods with specific health effects *(*Lolinger, 1991*).* Typical compounds with antioxidant activity include phenols, phenolic acids and their derivatives, flavonoids, tocols, phospholipids, amino acids and peptides, phytic acid, ascorbic acid, pigments and sterols. Phenolic compounds are primary antioxidants that act as free-radical scavengers *(*Xing and White 1996*).*

Annona crassiflora, commonly known as araticum, is a tropical fruit consumed mainly by native people of the Brazilian cerrado (the second biggest biome of Brazil). In previous studies, the seeds and peel of slightly ripe fruit were found to have significant antioxidant

activity in various models "in vitro" *(*Roesler et al., 2006*)*. Electrospray ionization mass spectrometry (ESI-MS) also revealed the presence of important bioactive components widely recognized as antioxidants, including malic acid, ascorbic acid, caffeic acid, quinic acid, ferulic acid, xanthoxylin, rutin, caffeoyltartaric acid, caffeoyl glucose and [quercetin + hexose + pentose - H]-1 *(*Roesler et al., 2007*)*. The aim of the present study was to assess the protective effect of an ethanolic extract of *A. crassiflora* seeds and peel in Wistar rats treated with carbon tetrachloride (CCl4). The protective effect of the extract was assessed by quantifying the level of lipid peroxidation and the activities of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), cytochromes P450 and b5, glutathione peroxidase (GPx), glutathione reductase (GRed) and glutathione (GSH) equivalents in liver homogenates.

MATERIAL AND METHODS

Reagents and standards.

All solvents and reagents were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA).

Plant material.

Araticum fruits (*A. crassiflora*) were obtained from Fazenda Erlow, Km 7 Br 070 in the state of Goiânia, in western central Brazil. Fruits were harvested on two occasions, with special care to avoid damaging the flesh, and were transported to UNICAMP where they were stored at 5°C until used (usually no longer than two months).

Preparations of the ethanolic extraction.

Undamaged fruits were selected and the peel, pulp and seeds were manually separated. About 100 g of each part of the fruit (peel and seeds) was cut into small parts, mixed and

extracted twice (for 20 min each) with 300 mL of aqueous ethanol (5:95, v/v, water:ethanol) using a household mixer. The extracts were filtered through cotton membranes and the residue was re-extracted under the same conditions. The residues were kept for additional evaluations. The ethanol was removed from the resulting material by vacuum rotary evaporation at 40°C. The concentrated ethanolic extracts were lyophilized and stored at -18°C in amber glass bottles until used.

Determination of the total phenol content.

The total polyphenol content of the extracts was determined by the Folin-Ciocalteu method, which involves reduction of the reagent by phenolic compounds and the concomitant formation for a blue complex; the resulting absorbance at 760 nm increases linearly with the concentration of phenols in the reaction medium (Swain and Hillis 1959). Briefly, ethanolic extracts were dissolved in methanol to a concentration of 0.5 mg/mL. Samples (0.5 mL) were passed through a 0.45 μm membrane filter and mixed with 2.5 mL of 10 fold diluted Folin-Ciocalteu reagent and 2.0 mL of 7.5% sodium carbonate solution. After incubation for 5 min at 50°C, the resulting absorbance was measured at 760 nm. Galic acid was used as the spectrophotometric standard and the total phenolic content of the fruit extracts was expressed as galic acid equivalents (GAE/100 g). The content of phenolic compounds was assayed in triplicate.

Experimental procedure.

Male Wistar rats (230-265 g) were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB) at UNICAMP and were housed on a 12 h light/dark cycle at 23^{o}C, with free access to food and water. The rats were allocated to one of six groups containing seven rats each. The first group (G1) served as the control, the second

group (G2) received CCl4 alone, the third group (G3) and fifth group (G5) received an aqueous solution of *A. crassiflora* peel extract (50 mg of galic acid equivalents/kg), the fourth (G4) and sixth group (G6) received an aqueous solution of *A. crassiflora* seed extract (50 mg of galic acid equivalents/kg) for 14 consecutive days. The dose of extract used was based on the LD_{50} of polyphenols *(*Bombardelli and Morazzoni, 1995*).* Groups G1 and G2 were given saline until the 14th day, whereas groups G2, G5 and G6 received a single oral dose of CCl4 (2 g/kg) in olive oil (1:1, v/v) 6 h after the last administration of extract or saline on the 14th day. All of the rats were killed with an overdose of anesthetic halotano 24 h later. The liver was excised and microsomal and cytosolic fractions were prepared by differential centrifugation *(*Omura and Sato 1964*)* and stored in aliquots at -80°C until analyzed. The protein concentrations of the microsomal and cytosolic fractions were measured by the Lowry method *(*Lowry et al., 1951*)* with bovine serum albumin as the standard.

Lipid peroxidation.

Thiobarbituric acid reacts with malondialdehyde (MDA) to form a diadduct (pink chromogen) that can be detected spectrophotometrically at 532 nm (Van der Sluis et al., 2000). The assay used here was modified for ELISA microtiter plates, which allowed the analysis of a large number of samples per run. Microsomes were thawed on ice, diluted to a concentration of 1 mg of protein/mL with 50 mM Tris-HCl (pH 7.4) containing 150 mM KCl and then centrifuged (100,000 *g*, 60 min, 4°C). The pellet was resuspended in 1 mL of Tris buffer and diluted to a final concentration of 0.5 mg/mL, unless stated otherwise *(*Omura and Sato 1964, Van der Sluis et al., 2000, Singh et al., 2002*).*

Microsomes (aliquots of 240 µL) were preincubated in a 48-well plate for 5 min at 37°C, after which lipid peroxidation (LPO) was induced by adding 15 µL of 4 mM ascorbic acid

and 15 µL of 0.2 mM $FeCl_3$. After a 60 min incubation at 37°C, the reaction was stopped by adding 0.5 mL of 0.83% thiobarbituric acid dissolved in a solution of trichloroacetic acid (TCA)-HCl (16.8%, w/v, TCA in 0.125 N HCl). LPO was quantified by measuring the thiobarbituric acid reactive species (TBARS) after heating the plates for 15 min at 80°C followed by centrifugation (2,500 rpm, 15 min). A 250 µL sample of each incubation was transferred to 96-well plates and the absorption was read at 540 nm (color) vs 620 nm (turbidity correction) using a SpectroMax 340 ELISA reader *(*Van der Sluis et al., 2000, Singh et al., 2002*).* The results were calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and were expressed as MDA equivalents. One unit of lipid peroxidation activity was defined as the amount of TBA that was converted to TBARS, with the specific activity being expressed as units/mg of protein.

Enzyme assays.

The total microsomal cytochrome P450 and b5 content was quantified as described by Omura and Sato (1964). Catalase was assayed as described by Aebi (1984); glutathione peroxidase (GPx) and glutathione reductase (GRed) were estimated by the method of Albrecht Wendel (1981). The content of glutathione equivalents was determined by the method of Theodorus et al. (1981) and superoxide dismutase (SOD) was assayed as described by Misra & Fridovich (1972).

Statistical analysis

The results were expressed as the mean ± S.E.M. and statistical comparisons were done using Student´s t-test. A value of $P \leq 0.05$ indicated significance.

RESULTS

Body and organ weight and general observations.

The oral administration of the extracts did not produce any clinical signs or gross alterations attributable to heptic toxicity or other organ damage. However, rats that received the seed extract showed some anxiety and were restless, perhaps because of the phenolic acids and their derivatives (caffeic acid, caffeoyl glucose and caffeoyl tartaric) present in this extract *(*Roesler et al., 2007*).* There were significant differences among the body weight gains of the groups that received seed extract (G4 and G6) if compared with control group (G1). Groups 4 and 6 showed reduction in body weight gain profile of approximately 28 and 23% compared with control. Table 1 shows the body weight, hepatic index and microsomal and cytosolic protein concentrations of the different groups.

Table 1 – Modulatory influence on the two investigate extracts of *Annona crassiflora* on weight gain profiles, protein levels and toxicity related parameters.

Groups & Treatment	Body Weight Gain Profile	Liver wt x 100/ Final body wt	Protein (mg/ml)	
			Microsome	Cytosol
Group 1 Control	50.43 ± 7.91	4.78 ± 0.17	22.90 ± 0.43	47.10 ± 0.93
		100.00*	100.00*	100.00*
Group 2 CCl4 (negative control)	63.53 ± 10.06 (1)	4.72 ± 0.09	20.55 ± 0.98	48.20 ± 0.63
		98.49*	89.74*	102.33*
Group 3 Peel extract	46.00 ± 7.92	4.43 ± 0.15 (1)	24.40 ± 0.36 (1)	46.70 ± 0.75
		92.58*	106.55*	99.15*
Group 4 Seed extract	36.29 ± 12.00 (1)	4.95 ± 0.19	21.00 ± 1.42	42.70 ± 1.92
		103.53*	91.70*	90.66*
Group 5 Peel extract + CCl4	62.43 ± 15.06	4.38 ± 0.07 (1)	17.92 ± 0.87 (1.3)	46.50 ± 0.42
		91.35*	78.25*	98.73*
Group 6 Seed extract + CCl4	38.70 ± 5.88 (1.2)	4.46 ± 0.08 (1.4)	17.30 ± 1.16 (1.4)	46.20 ± 0.79
		93.05*	75.55*	98.09*

Values are expressed as mean ± SD of 6-7 animals

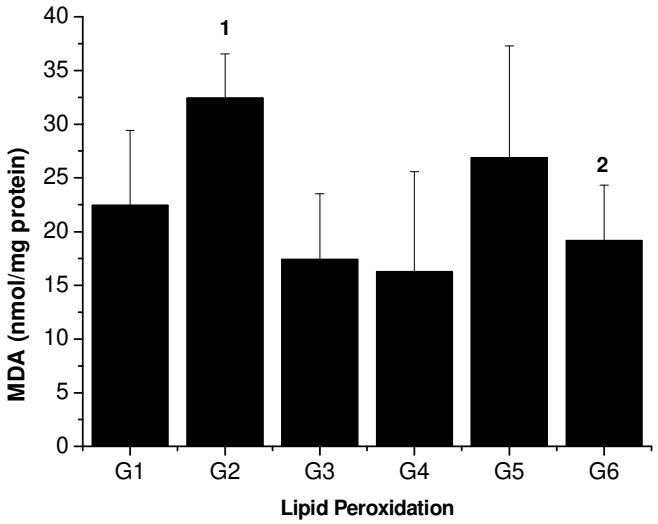
(*) Represents relative changes in parameters assessed (i.e., levels of parameter assessed in livers of rats receiving test substance to that of control rats)

(1) Represents significant difference from group 1 at $P<0.05$; (2) represents significant difference from group 2 at $P<0.05$; (3) represents significant difference from group 3 at $P<0.05$, (4) represents significant difference from group 4 at $P<0.05$.

Influence of *A. crassiflora* seed and peel extracts on hepatic lipid peroxidation.

Enhanced lipid peroxidation associated with a depletion of tissue antioxidants is characteristic of CCl4-treated rats (Weber et al., 2003). In agreement with this, CCl_4 significantly increased the level of hepatic TBARS (by 44%) of G2 in this study compared to the control group (G1). The *A. crassiflora* seed extract (G6) significantly decreased the levels of TBARS compared to CCl4-treated rats (-41%) as shown in the Figure 1. The *A. crassiflora* peel (G3) and seed extracts (G4) alone inhibited TBARS formation by 22% and 27%, respectively, compared to the saline controls.

Figure 1. Hepatic lipid peroxidation in rats treated with CCl_4 in the absence or presence of *A. crassiflora* seed and peel extracts. The columns represent the mean + S.E.M. of at least 5 rats.



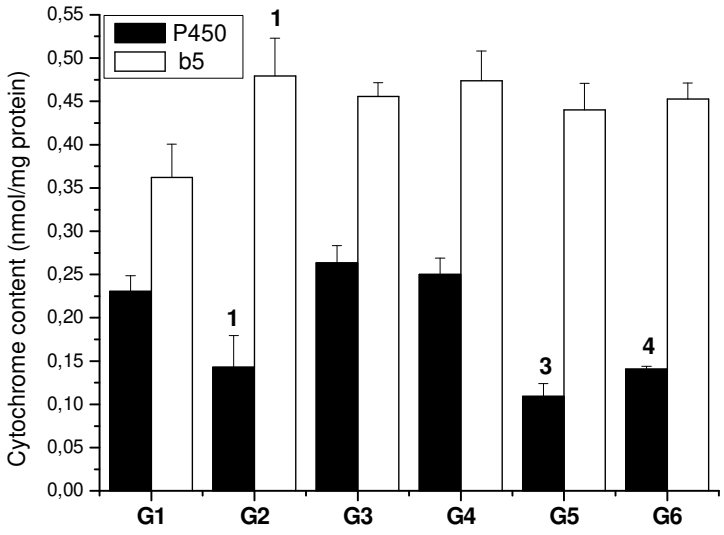
(1) Represents significant difference from group 1 at P<0.05; (2) represents significant difference from group 2 at P<0.05;

Influence of *A. crassiflora* seed and peel extracts on the hepatic content of cytochrome P450 and b5.

The recognition of clinically significant interactions between plant-based drugs and conventional medicines has raised concerns regarding the potential of herbal products to interfere with the metabolism of medications such as kava-kava and echinacea *(*Gorski et al, 2003; Zou et al, 2004*).* However, as shown here (Figure 2), the extracts alone did not alter the basal levels of cytochrome P450. CCl_4 decreased the content of cytochrome P450 and the *A. crassiflora* seed and peel extracts were unable to prevent this reduction.

However, there was no additive effect in rats treated with the extracts and CCl_4 (Figure 2). On the other hand, both extracts induced cytochrome b5, even under CCl_4 effect.

Figure 2. Hepatic content of cytochrome P450 and b5 in rats treated with CCl_4 in the absence and presence of *A. crassiflora* seed and peel extracts. The columns represent the mean + S.E.M. of at lest 5 rats.



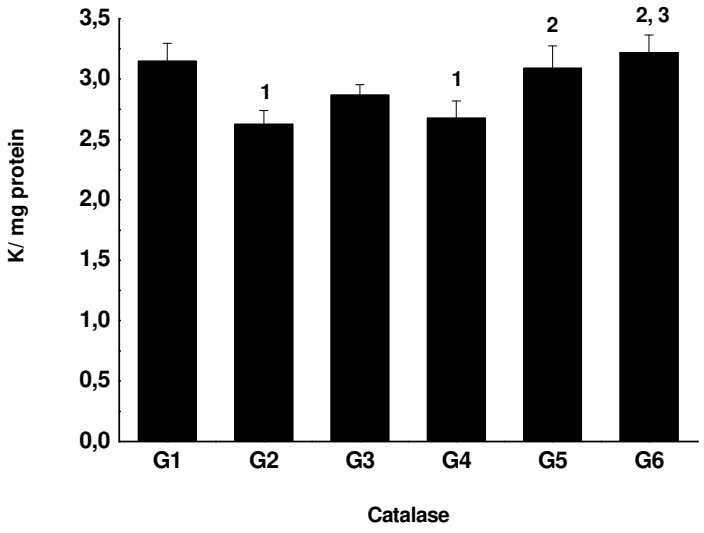
(1) Represents significant difference from group 1 at P<0.05; (3) represents significant difference from group 3 at P<0.05 and (4) represents significant difference from group 4 at P<0.05

Influence of *A. crassiflora* seed and peel extracts on hepatic catalase activity.

Catalase converts H_2O_2 to water and oxygen and also converts hydroperoxides to the corresponding alcohols in the presence of a hydrogen donor (Aebi, 1984). As shown in Figure 3, the single dose of CCl_4 produced a reduction in catalase activity by 23% (G2).

Both of the *A. crassiflora* extracts prevented the decrease in catalase activity caused by CCl_4 (G5 and G6).

Figure 3. Hepatic catalase activity in rats treated with CCl_4 in the absence and presence of *A. crassiflora* seed and peel extracts. The columns represent the mean+S.E.M. of at least 5 rats.



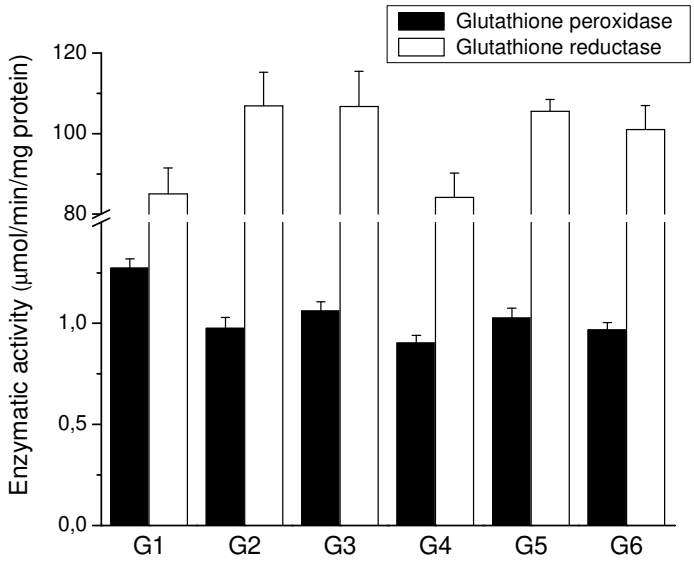
(1) Represents significant difference from group 1 at P<0.05; (2) represents significant difference from group 2 at P<0.05 and (3) represents significant difference from group 3 at P<0.05.

Influence of *Annona crassiflora* seed and peel extracts on hepatic glutathione peroxidase (G-Px) and glutathione reductase (GRed) activities.

Enzymes involved in the detoxification of ROS and lipid hydroperoxides include the glutathione redox cycling enzymes glutathione peroxidase (GPx) and glutathione reductase (GRed). GPx catalyzes the inactivation of H_2O_2 and activity is greatest when the enzyme is

in its reduced form. Other enzymes, notably catalase and glutathione-S-transferase, also influence the activity of GPx. Whereas catalase competes with GPx for H_2O_2, glutathione-S-transferase competes with GPx only for hydroperoxides *(*Yuan and Kitts, 1996*).* In our study, there were no significant changes in the GPx and GRed activities among the different groups, including in rats treated with CCl_4 (Figure 4), possibly because of competition with enzymes such as catalase.

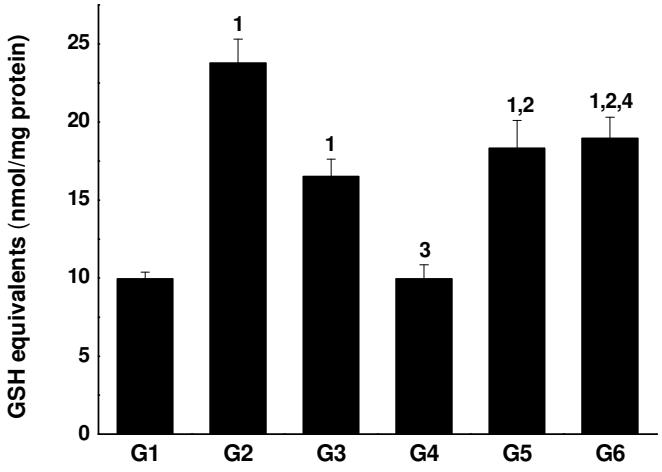
Figure 4. Hepatic glutathione peroxidase and glutathione reductase activities in rats treated with CCl_4 in the absence and presence of *A. crassiflora* seed and peel extracts. The columns represent the mean+S.E.M. of at least 5 rats.



Influence of *A. crassiflora* seed and peel extracts on the level of glutathione equivalents (GSH equivalents).

Under physiological conditions, GSH occurs in its reduced form, with oxidation leading to the formation of glutathione disulfide (GSSG). Considerable interest has focused on the redox state of GSH and GSSG *(*Theodorus et al., 1981*).* In rats, treatment with the peel extract increased the GSH levels whereas the seed extract had no effect. CCl_4 significantly increased the level of GSH equivalents and this increase was partially prevented by both *A. crassiflora* extracts (Figure 5). Since the oxidation of GSH "in vivo" can lead to the formation of GSSH and hydrogen peroxide, the increased level of GSH equivalents caused by CCl_4 probably corresponded to GSSH.

Figure 5. Hepatic glutathione (GSH) equivalents in rats treated with CCl_4 in the absence and presence of *A. crassiflora* seed and peel extracts. The columns represent the mean+S.E.M. of at least 5 rats.

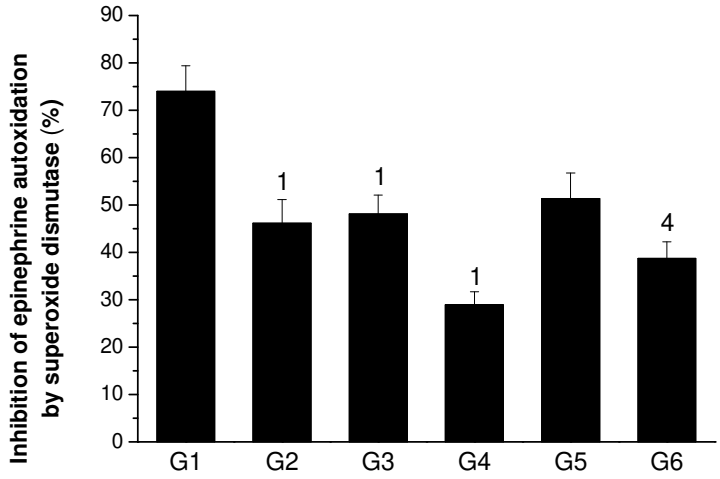


(1) represents significant difference from group 1 at P<0.05; (2) represents significant difference from group 2 at P<0.05; (3) represents significant difference from group 3 at P<0.05 and (4) represents significant difference from group 4 at P<0.05

Influence of *A. crassiflora* seed and peel extracts on hepatic SOD activity.

The ability of superoxide dismutase to inhibit the autoxidation of epinephrine at pH 10.2 has been used as the basis of a convenient and sensitive assay for this enzyme. SOD was determined by measuring the inhibition of the adrenochrome formation rate (Misra and Fridovich, 1972) in reaction medium containing 1mM-adrenaline (epinephrine) and 50 mM-glycine/NAOH, pH 9.6 (Boveris et al., 1983). Treatment with CCl_4 induced the autoxidation of epinephrine so the inhibition of autoxidation had a reduction of 34% if compared with the control group. As shown in Figure 6, the two *A. crassiflora* extracts alone significantly decreased the SOD activity and consequently increased the autoxidation of the epinephrine. The *A. crassiflora* peel (G3) and seed (G4) inhibited the autoxidation of epinephrine by only 48.1 and 28.9% respectively while the control (G1) shown an inhibition of approximately 74%. The profile of SOD activity seen with the extracts was unaltered by concomitant treatment with CCl_4.

Figure 6. Hepatic SOD inhibition of autoxidation of epinephrine in rats treated with CCl4 in the absence and presence of A. crassiflora seed and peel extracts. The columns represent the mean+S.E.M. of at least 5 rats.



(1) Represents significant difference from group 1 at P<0.05 and (4) represents significant difference from group 4 at P<0.05

DISCUSSION

Araticum (*A. crassiflora)* is a tree that bears a typical fruit known as araticum of cerrado or cerradão. Its fruits are consumed extensively "in natura" but have no commercial value in Brazil. The seeds in oil are used against scalp infections and in the folk medicine, the leaves and seeds infusion are used against diarrhea and as antitumoral (Lorenzi 1988, Almeida and others 1994). Many members of annonaceae are used in folk medicine for antiparasitic or

antitumoral treatment of intestinal diseases. Previous studies have shown the *in vitro* free radical scavenging potential of *Annona crassiflora* peel and seed extracts at different concentrations by the DPPH method and seed and peel presented IC_{50} of 31.14 and 48.82 $\mu g\ mL^{-1}$, respectively. The inhibition of lipid peroxidation by using rat liver microsomes as an oxidative system was evaluated and fifty percent inhibition of lipid peroxidation of microsomes to TBARS requires 1.72 $\mu g\ mL^{-1}$ of seed ethanolic extract, 4.44 $\mu g\ mL^{-1}$ of peel ethanolic extract and 8.62 $\mu g\ mL^{-1}$ of pulp etanolic extract *(*Roesler et al., 2006*).*

In the present study, an experimental model of acute hepatotoxicity in Wistar Rats was induced by oral administration of CCl_4. CCl_4 has been extensively used in experimental models to elucidate the cellular mechanisms behind oxidative damage *(*Basu, 2003*).* CCl_4 is activated by cytochrome P450 (CYP) 2E1, 2B1/B2 and possibly 3A to form trichloromethyl radicals (CCl_3*) and trichloromethyl peroxy radicals (CCl_3OO*) that cause lipid peroxidation and subsequent tissue damage *(*Weber and Boo, 2003*).* Enhanced lipid peroxidation associated with a depletion of tissue antioxidants is characteristic of CCl_4-treated rats *(*Sipes et al., 1977*).*

Direct evidence of this hepatotoxicity was noted in the occurrence of alterations in hepatic parameters in the second group (G2) such as increased on hepatic lipid peroxidation (MDA concentration), decrease of phase I cytochrome P450 content, decreased phase II CAT and SOD activity, increased GSH-equivalents level. The present results agree with previously reported data (Jayakumar, et al., 2006, Srinivasan et al., 2005; Singab et al., 2005 and Chidambara Murthy et al., 2002; Zhu et al., 1999). Since free radicals play an important role in CCl_4- induced hepatotoxicity, it seems logical that compounds that neutralize such radicals may have an hepatoprotective effect.

The oral administration of *A. crassiflora* peel (G3) and seed extract (G4) inhibited the TBARS formation by 22 and 27%, respectively. *A. crassiflora* seed extract (G6) significantly decreased the levels of TBARS when compared with the CCl_4 treated group (G2) by 41%. Thus, the maintenance of normal levels of hepatic MDA in group 6 (administrated *A. crassiflora* seed extract + CCl_4) is of great interest since it provides evidence to suggest a hepatoprotective effect. The lipid peroxidation control in vivo is important for several reasons, in particular because it contributes to the development of atherosclerosis. The products of lipid peroxidation, particularly the citotoxic aldehydes such as malondialdehyde (MDA) are important because they can also cause damage to DNA *(*Halliwell and Aruoma, 1960*).* Thus the maintenance of normal levels of hepatic MDA is of great interest since MDA is a major aldehyde resulting from the peroxidation of biological tissue and it is an indicator of tissue damage. Lipid peroxidation may be prevented at the initiation stage by free radical scavengers, while the chain propagation reaction can be intercepted by peroxy-radical scavengers such as phenolic antioxidants (Takahama, 1983). The investigation by direct infusion electrospray ionization mass spectrometry (ESI-MS) provided important information of biactive components of araticum extracts mainly phenolic antioxidants such as caffeic acid, quinic acid, ferulic acid, xanthoxylin, caffeoyltartaric acid, caffeoyl glucose, [quercetin + hexose + pentose – H]- and rutin *(*Roesler et al., 2007) .The phenolic compounds act by scavenging free radicals and quenching the lipid peroxides. The hydroxyl and phenoxy groups of phenolic compounds donate their electron to the free racials and neutralize them, forming phenolic radical and quinine methide intermediate, which is excreted via bile (Pan et al., 1999).

Consumption of vegetables and fruits is known to reduce the risk of cancer (Wattenberg, 1992). In general, the induction of phase 2 drug-metabolizing enzymes, the suppression of phase 1 enzymes or the combination of these actions have been reported to be the mechanisms responsible for the protection against toxic and neoplastic effects of carcinogens (Talalay, 1989; 2000).

The phase 1 enzyme cytochrome P450 dependent monooxygenase system is responsible for the oxidative and reductive metabolism of a variety of drugs, carcinogens, pesticides and steroid hormones. The monooxygenase system is subject to the inductive and inhibitory effects of environmental and genetic factors, which may play an important role in determining the biological fate of foreign chemicals that require P450–mediated detoxication or bioactivation. *A. crassiflora* extracts preserves cytochrome P450 content which are comparable with the control values of the enzyme. The pre-treatment with the *A. crassiflora* extracts was unable to alleviate the inhibition of the cytochrome P450 activity of the CCl_4 treated group. On the other hand, both extracts induced cytochrome b5, even under CCl_4 effect. The role of this enzyme includes electron transfer, coupled catalysis and allosteric regulation of cytochrome P450 during metabolism of endogenous and xenobiotic compounds *(*Porter, 2002*).* Outer microssomes, cytochrome b5 is required in several reactions, such as fatty acids and hormones synthesis *(*Schenkman and Jansson, 2003*).* Sheweita *et al.*, 2001 described cytochrome b5 induction after concomitant administration of CCl_4 and antioxidants, but no changes were observed with single compound treatment. The mechanism of induction and the role of cytochrome b5 under CCl_4 and antioxidants exposure still require further investigation.

Phase 2 enzymes play important roles in the detoxification of xenobiotics, and thus their up-regulation gives protection against potentially harmful insults from the environment (Kong et al., 2001). Living tissues are endowed with innate antioxidant defence mechanisms, such as the presence of the enzymes CAT, SOD and GPx. A reduction in the activities of enzymes associated with the accumulation of highly reactive free radicals, leading to deleterious effects such as loss of integrity and function of cell membranes (Reedy and Lokesh, 1992, Krishnakantha and Lokesh, 1993, Sheela and Angusti, 1995). Administration of CCl_4 leads to generation of peroxy radical with is associated with inactivation of CAT and SOD enzymes. This probably explains the significantly reduced activities of CAT and SOD in the rats challenge with CCl_4 (G2). Extracts of *A. crassiflora* are capable of enhancing/maintaining phase 2 enzymes activity. The only exception was observed for SOD phase 2 enzyme which activity was unexpectedly depleted by *A. crassiflora* extracts as much as for CCl_4.

Nutritional factors and dietary habits have a significant effect on the regulation of antioxidant enzyme activity. According to L´Abbe et al., (1991), the SOD activity can be depleted in liver and heart by fish oil diet. In addition, the decrease in the SOD activity in heart has been already associated with weight loss *(*Wohaieb and Godin, 1978). As reported in Table 1, there was significant difference between the body weight gain profile between the control group and the seed extract group (-23%). For the peel extract group, there was a tendency of decrease in the body gain profile as well (-9%). Besides, araticum seeds have approximately 16% of lipids (Roesler et al., 2006). The lipids from the seeds were not removed before preparing the extracts and the solvent used for extraction process also removed lipids, so it is possible to affirm that the seed extract has approximately 16%

of lipids as well. The composition of araticum oil had already been reported by Zuppa (2001). It contains approximately 15% of saturated fatty acids (palmitic and estearic acids), 51% of monounsaturated fatty acid (oleic acid) and 32% of polyunsaturated fatty acid (linoleic and linolenic acids). Fish oil has approximately 32% of saturated fatty acids (miristic and palmitic acids), 25% of monounsaturated fatty acids (oleic and palmitoleic acids) and 40% of polyunsaturated fatty acids (20:5 n-3 and 22:6 n-3) (Gamboa and Gioielli, 2006). The araticum oil has quantities of polyunsaturated fatty acid as high as fish oil, but fish oil has more fatty acids with long chain. Therefore, further studies are needed for better understanding of *A. crassiflora* seed and peel extracts, SOD activity and nutrition and metabolic factors that can regulate antioxidant enzyme activity.

Glutatione is a major, non-protein thiol in living organisms which performs as a key role in coordinating antioxidant defense mechanisms. The main role of GSH (L-y-glutamyl-L-cysteinyl-glycine) in vivo is to be a primary agent involved in deactivating electrophilic free radicals. Oxidation of GSH as an antioxidant can occur in vivo with oxidized glutathione (GSSH) *(*Yuan and Kitts, 1996). Treatment with CCl_4 induced the GSH-equivalents by 140%. Since the oxidation of GSH in vivo can lead to the formation of GSSH and hydrogen peroxide, the increased level of GSH equivalents caused by CCl_4 probably corresponded to GSSH. This increasing of GSH-equivalents, probably GSSH, was partially prevented by both *A. crassiflora* extracts.

Treatment of rats with single dose of CCl_4 at 2.0 g kg^{-1} of body weight and the pre-treatment with the *Annona crassiflora* seed and peel extracts at 50 g kg^{-1} (in terms of galic acid equivalents) were not able to alter significantly the level of glutathione peroxidase (G-Px) and glutathione redutase (G-red).

In conclusion, our results show that ethanolic extracts of *A. crassiflora* can enhance or maintain the activity of hepatic antioxidant enzymes, except for SOD, which was unexpectedly depleted by the extracts to a similar extent as that seen with CCl_4. Pretreatment with *A. crassiflora* extracts protected against CCl_4 toxicity, as shown mainly by the lipid peroxidation assay, catalase activity and level of GSH equivalents. In contrast, treatment with CCl_4 and pretreatment with *A. crassiflora* extracts did not significantly alter the level of glutathione peroxidase and glutathione reductase. It is also important to emphasize that the *A. crassiflora* extracts preserved cytochrome P450 content which are comparable with the control values of the enzyme. Thus there is probably no need to be concerned about interactions between plant-based drugs and conventional medicines. The antioxidant activity of the *A. crassiflora* extracts *in vitro* (Roesler et al., 2006) and demonstrated here *in vivo* may be mediated by compounds such as ascorbic acid, caffeic acid, quinic acid, ferulic acid, xanthoxylin, caffeoyltartaric acid, caffeoyl glucose, [quercetin + hexose + pentose-H] and rutin. These compounds are widely reported as potent antioxidants and probably can explain the antioxidant activity of the *A. crassiflora* extracts (Gülçin, 2006; Kim et al., 2006; Roche et al., 2005; Kweon et al.,2001). The present study represents the first attempt in determining whether the *Annona crassiflora* extracts can protect Phase 1 and Phase 2 hepatic enzymes against CCL4 induced liver damage. Further studies are needed to elucidate the mechanisms involved in this hepatic protection and to explore possible synergism among components of the extracts.

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CAPÍTULO 6 - Brazilian Cerrado Antioxidant Sources: Cytotoxicity and Phototoxicity in Vitro

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ABSTRACT

There is an increasing need for search of new natural antioxidants as the the role of antioxidants in preventing oxygen radical and hydrogen peroxide induced cytotoxicity and tissue damage in various human diseases is becoming increasingly recognized. *Annona crassiflora* (araticum), *Eugenia dysenterica (*cagaita), *Caryocar brasiliense* (pequi) and *Swartzia langsdorfii* (banha de galinha) are tropical fruits of the second biggest biome of Brazil called Cerrado. Previous studies showed that extracts of cerrado fruits demonstrated high content of total phenols and excellent antioxidant activity by using the "in vitro" models. The fingerprinting analysis of cerrado fruits revealed the presence of important bioactive molecules probably responsible for their antioxidant activity. In this study, cytotoxicity and phototocixity of the cerrado fruit extracts were evaluated using the "in vitro" 3T3 neutral red uptake (NRU) method in order to explore their toxicity. None of the extracts showed phototoxicity in the dosage levels tested. Regarding cytotoxicity, araticum peel and cagaita seed extracts did not shown any inhibition of cell grown up to 300 $\mu g\ ml^{-1}$, maximum solubility. Araticum seed and pequi peel extracts presented an estimated IC_{50} of 100 and 4800 $\mu g\ mL^{-1}$ respectively. Thus the estimated LD_{50} for araticum seed and for pequi peel extracts were 809.1 and 2849.3 mg kg^{-1} respectively. The "in vitro" NRU tests estimated starting dose for in vivo acute lethality studies and can reduce the number of animals used in "in vivo" studies (i.e., reduction), minimize the number of animals that receive lethal doses (i.e., refinement), and avoid underestimating hazard. This is the first attempt to screening the toxicity of cerrado fruits extracts with high antioxidant activity.

KEYWORDS: tropical fruits, cerrado, antioxidant activity, 3T3 neutral red uptake, cytotoxicity, phototoxicity.

INTRODUCTION

The importance of the search for and exploitation of natural antioxidants, especially of plant origin, has greatly increased in recent years (Jayaprakasha & Jaganmohan, 2000). Both epidemiological and clinical studies have provided evidence that phenolic

antioxidants present in cereals, fruits, and vegetables are principal contributing factors in accounting for the significant reduced incidences of chronic and degenerative diseases encountered by populations whose diet is high in the intake of these foods (Shahid, 1996). Typical compounds that possess antioxidant activity include phenols, phenolic acids and their derivatives, flavonoids, tocols, phospholipids, amino acids and peptides, phytic acid, ascorbic acid, pigments, and sterols (Xing & White, 1996). A phenol contains an –OH group attached to a benzene ring. Many phenols exert powerful antioxidant effects "in vitro", inhibiting lipid peroxidation by acting as chain breaking peroxyl radicals scavengers. Phenols with two adjacent –OH groups, or other chelantin structures, can also bind transition metal ions in forms poorly active in promoting free-radical reacting. Phenols can also directly scavenge reactive oxygen species (ROS), such as OH, ONOOH and HOCl (Halliwell & Guitteridge, 2000).

Annona crassiflora (araticum), *Eugenia dysenterica* (cagaita) and *Caryocar brasiliense* (pequi) are tropical fruits of the second biggest biome of Brazil called Cerrado. However these fruits are highly used by traditional herbal medicine as healing, there are few scientific studies on the relationship between the biological activities and the ethnobotanical uses in order to validate them. Our previous studies shown that ethanolic extracts of these fruits demonstrated high content of total phenols and excellent antioxidant activity using the "in vitro" model 2,2-diphenyl-1-picrylhydrazyl (DPPH) and chemically induced lipid peroxidation using rat liver microsome as oxidative substrate. The best results were found for ethanolic extracts of pequi peel, cagaita seeds, araticum seeds and araticum peel. The IC_{50} results were in the range of 14 – 50 μg mL^{-1} for DPPH assay and 0.7 to 4.5 μg mL^{-1} lipid peroxidation model. The investigating by direct infusion electrospray

ionization mass (ESI-MS) and tandem mass spectrometries (MS) revealed the presence of important bioactive molecules in the cerrado fruits extracts probably responsible for their antioxidant activity such as ascorbic acid, gallic acid, quinic acid, dicaffeoylquinic acid, quercetin, quercetin 3-O-arabinose, ferulic acid, rutin, xanthoxylin, caffeic acid and its derivatives such as caffeoyltartaric acid and caffeoyl glucose. (Roesler et al., 2006 , Roesler et al., 2007). In order to ensure that all potentially hazardous products, especially new substances, have proper warning labels and uses, regulatory agencies require determination of acute toxicity hazard potential of substances and products. This determination for acute toxicity hazard is currently made using a test that requires laboratory rats. Historically, lethality estimated by LD_{50} (i. e. the dose of a substance that produces 50% of animal death) has been primary toxicological endpoint in acute toxicity tests. The conventional LD_{50} acute oral toxicity "in vivo" test method has been modified in various ways to reduce and refine animal use in toxicity testing (OECD 2001a, c, d; EPA 2002a). "in vitro" cytotoxicity methods have been evaluated as another means to reduce and refine the use of animals and these methods may be helpful in predicting "in vivo" acute toxicity. Since moving the staring dose closer to the LD_{50} reduces the number of animals necessary for the acute oral systemic toxicity test, the use of "in vitro" cytotoxicity assays to predict a starting dose close to the LD_{50} may reduce animal use. ICCVAM recommended (ICCVAM 2001 a) further evaluation of the use of "in vitro" cytotoxicity data as one of the factors used to estimated starting dose for "in vivo" acute lethality studies based on preliminary information that this approach could reduce the number of animals used in "in vivo" studies (i.e., reduction), minimize the number of animals that receive lethal doses (i.e., refinement), and avoid underestimating hazard.

Cytotoxicity has been defined as the adverse effects resulting from interference with structures and/or processes essential for cell survival, proliferation, and/or function (Ekwall, 1983). The endpoint measured in acute systemic toxicity assays is usually animal death. Cell death and animal death may be similar since animals are comprised of organ systems consisting of tissues, which are comprised of cells. (Ekwall, 1983) and others (Grisham and Smith 1984) concluded that, since the actions of substances that produce injury and death are ultimately exerted at cellular level, "in vitro" cytotoxicity assays may be useful for the prediction of acute lethal potency. The endpoint measured in the "in vitro" neutral red uptake (NRU) cytotoxicity test methods is cell death (neutral red –NR is taken up only by live cells) and the major endpoint of interest is the concentration at 50% inhibition of NRU (i.e., the IC_{50}). Neutral red is a weakly cationic water-soluble dye that stains living cells (Borenfreund and Puerner 1985). It readily diffuses through the plasma membrane and concentrates in lysosomes where it electrostatically binds to the anionic lysosomal matrix. Toxins can alter the cell surface or the lysosomal membrane seeming to cause lysosomal fragility and other adverse changes that gradually become irreversible. Thus, cell death and/or inhibition of cell growth decreases the amount of neutral red taken up by the culture.

The dermal phototoxicity of a chemical is defined as a toxic response that is elicited after exposure of skin to the chemical or systemic administration of the chemical, and subsequent exposure to light. As demonstrated by several validation studies (NIH, 2001; ZEBET/ECVAM/COLIPA, 1998; Borenfreund & Puerner, 1985), the phototoxic potential of chemicals can be effectively assessed by "in vitro" methods. In 1996, an OECD workshop recommended an "in vitro" tier-testing approach for phototoxicity assessment

(Spielmann et al., 1994). In 2000, the Commission of European Communities put into force Directive 200033EC, which introduces the "in vitro" 3T3 NRU, phototoxicity test as a validated replacement for testing methods involving the use of laboratory animals. The essence of the "in vitro" test 3T3 NRU phototoxicity test is to compare the cytotoxicity of a chemical when tested in the presence and absence of exposure to a non-cytotoxic dose of UVA light. Cytotoxicity is expressed as the concentration-dependent reduction of the uptake of the vital dye neutral red, 24 hours after treatment with the chemical (Peters & Holzhutter, 2002).

The aim of this study is to evaluate the toxicity of the Brazilian Cerrado fruits extracts *Annona crassiflora* (araticum), *Eugenia dysenterica* (cagaita) and *Caryocar brasiliense* (pequi) by using the "in vitro" 3T3 NRU cytotoxicity and phototoxicity methods in order to estimate the applicability of these fruits as a sustainable source of natural antioxidant for cosmetic, foods and pharmaceutical applications

MATERIAL AND METHODS

Cell Lines.

BALB/C 3T3 mouse fibroblasts, clone 31 were obtained from ATCC.

Chemicals and Media.

Dulbecco's Modified Eagle Medium (DMEM), Phosphate buffered saline (PBS), Neutral Red (NR) dye –tissue culture grade (SIGMA-ALDRICH), New born calf serum (NBCS or NCS), Etanol (ETOH) analytical grade, Glacial acetic acid analytical grade, Sodium Lauryl Sulfate (positive control).

Plant Material.

Cerrado fruits were obtained from Erlow Farm, Km 07 Br 070 Goiânia Brazil, in the center west of Brazil. Fruit harvests were conducted twice and special care was taken to avoid damaged fruits. Fruits were transported to the Univ. of Campinas (UNICAMP) and stored at 5°C until use, which was no longer than 2 months.

Ethanolic extraction.

Undamaged fruits were selected and peel, pulp and seeds were manually separated. About 100 g of each part of the fruit (pulp, peel and seeds) was cut in small parts, mixed and extracted twice with 300 mL of aqueous ethanol (5:95, v/v, water: ethanol) using a household mixer for 20 minutes. The extracts were filtered through cotton membrane and the residue was re-extracted under the same conditions. The residues were kept for further evaluations. The resultant material was subjected to vacuum rotary evaporation at 40°C to remove ethanol. The concentrated ethanolic extracts were lyophilized and stored at – 18°C in amber glass bottles until used.

"In vitro" Cytotoxicity.

The assays were carried according to the official protocol from OECD. Briefly, 3T3 cells were seeded into 96-well plates to form a sub confluent monolayer (density of 1x104 cells/well). Culture medium was removed and different concentration of test chemicals in medium were added to cells for 24 hours/ 37°C/ 5% CO2. The untreated vehicle control was equal to the treatment medium. The cells were washed once with (PBS) and Neutral Red (NR) dye medium was added for 3 hours/ 37°C/ 5% CO2. The cells were washed with PBS and NR desorbing fixative (ETOH: acetic acid:water (50:1:49)) was added to the plates. Plates were shaken for 20 minutes and NR absorption was measured at optical

density (OD) 540 ±10 nm. Cell viability was calculated as the % of control values to define IC_{50} substance concentrations (μg/mL) (NICEATM, 2003).

"In vitro" Phototoxicity.

The assays were performed according to the official protocol from NIH (2001). The cells were prepared as in the cytotoxicity protocol, considering two plates to each test sample. The 3T3 NRU test for phototoxicity requires a 60 minutes exposure to sample dilution in PBS followed by an ultraviolet (UVA) (5 J/cm^2) exposure of one of the plates (the other plate is used as non irradiated control). After washing both plates with PBS, the cells were incubated for 24 hours/ 37°C/ 5% CO_2. The plates were washed, uncubated with NR dye medium and re-washed. The NR desorbing fixative was added and NR absorption was measured at optical density (OD) 540 ±10 nm. Cell viability was calculated to each treatment. Photoxicity is assessed by comparing the differences in cytotoxicity between negative control test plates that have not been exposed to UVA and test plates exposed to UVA. The photo-irritancy factor (PIF) relates the half-effective concentration value EC_{50}(-UV) of the curve for darkness, to the half-effective concentration value EC_{50}(+UV) of the curve in the presence of light, by means of the following formula: PIF = EC_{50}(-UV) / EC_{50}(+UV) – Equation 1.

Depending on whether the PIF value is larger or smaller than the cut-off value of 5 (PIFc), the chemical is classified as phototoxic or non-phototoxic. A shortcoming of the measure in Equation 1 is that additional ad hoc definitions are required to cope with situations where no effective concentration values can be derived from the corresponding concentration-response curve: a) if no EC_{50} value can be derived form one of the two curves, the corresponding EC_{50} value in Equation 1 is replaced by the highest concentration tested, and

the chemical is classified as phototoxic if this modified PIF value is larger than one unity; b) if no EC_{50} value exists for both curves, the chemical is considered non-phototoxic (NIH, 2001; ZEBET/ECVAM/COLIPA, 1998; Borenfreund & Puerner, 1985).

Solubility protocol.

For test chemicals prepared in chemical dilution medium, the highest test article concentration that may be applied to cell in the main experiments will be either 100 mg mL^{-1}, or the maximum soluble dose. For test chemicals prepared in either DMSO or ethanol, the highest test article concentration that may be applied to the cells in the main experiments will be either 2.5 mg mL^{-1}, or less, depending upon the maximum solubility in solvent (NICEATM, 2006; NICEATM, 2003).

RESULTS AND DISCUSSION

Brazil has a strong tradition of herbal medicine and, like most developing countries; its rural and traditional population still depends mainly on the indigenous system of medicine for their healthy related matters. Herbal medicines are frequently used to treat a large variety of ailments and symptoms, e.g., fever, inflammation, and pain; however, there is little information about their efficacy and lack of acute toxicity.

The *Anonnaceas* family has a great variety of exotics fruits, which are apparently rustic and have typical form such as conde fruit (*Annona squamosa*), graviola fruit (*Annona muricata*) and araticum of cerrado or marolo (*Annona crassiflora*) (Silva and Tassara 2001). Many members of annonaceae are used in folk medicine for antiparasitic or antitumoral treatment of intestinal diseases. In Brazil, the fruits are sold in regional markets and have no commercial value in Brazil. The seeds in oil are used against scalp infections and in the

folk medicine, the leaves and seeds infusion are used against diarrhea and as antitumoral (Lorenzi 1988, Almeida and others 1994). In addition, the seeds of *Annona crassiflora* are believed to be efficacious against snake venom. Weinberg and colleagues (1993) found that hydro-alcoholic extract of *Annona crassiflora* seed showed a no-especific inhibitory effect on drug-induced contractions of guinea-pig ileum that could be responsible for the effect against snake venom. Londershausen and collaborators (1991) showed that some annonins isolated from seeds of *Annona squamosa* revealed interesting insecticidal properties. The fruits of pequi (*C. brasiliense*) represent an important option of income and food for the populations living in the Cerrado regions of Brazil. The pulp of the pequi has good quantity of edible oil, vitamin A and proteins. On the other side, the nut has also oil, which is applied in cosmetic products such as soaps and skin emulsions (Silva & Tassara, 2001). From the peel and leaf is extracted high quality yellow color that is used for dying fibers. Regarding the folk medicine, the pulp oil is traditionally used as tonic agent against asthma, influenza, cold and bronchopulmonary diseases (Almeida, Proença, Sano & Ribeiro, 1994; Rodrigues & Carvalho, 2001).

The *Eugenia dysenterica* is a shrubby tree with edible cherry-like fruits called cagateira (Correa, 1984). The plants are well known in Brazilian cerrado medicine and the leaves are part of preparations used for medical diarrheic care and dysentery (Septimio, 1994). The fruits are consumed in nature with moderation because of its laxative activity or by homemade preparations as juice, ice creams and jelly (Silva, 1994). The volatile constituents of *Eugenia dysenterica* leaf oil had been investigated for its antifungal activity (Costa et al., 2000).

Table 1. Ethnobotanical data of selected cerrado fruits

Plant Name	Family	Local name	Plant used	Local uses
Annona crassiflora; Annona rodriguesii	Annonaceae	araticum, marolo, cabeça de negro	seed	snake bite (1)
			seed, leaves	antidiarrhea (2,3)
			seed	hair antiparasitic (2)
Eugenia dysenterica; Stenocalyx	Myrtaceae	cagaita	fruits	laxative (2)
			leaves	antidiarrhea (2)
Caryocar brasilense	Caryocaraceae	pequi,piqui, piquia	nut oil	asthma, influenza, cold and bronchopulmonary diseases (2,3)
			seed	aphrodisiac tonic (3)
			leaves	regulate women period (2)

(1) Weinberg et. al., 1993; (2) Almeida et. al., 1998; (3) Rodrigues et. al., 2001

Cytotoxicity.

Neutral red is a weakly cationic, water-soluble dye that stains living cells by readily diffusing through the plasma membrane and concentrating in lysosomes. The intensity of the dye in culture is directly proportional to the number of living cells. In addition, since altering the cell surface or the lysosomal membrane by a toxicological agent causes lysosomal fragility and other adverse changes that graduallyu become irreversible, cell death and/or inhibition of cell growth decreases the amount of neutral red taken up by culture. A calculation of cell viability is expressed as NRU for each concentration of the chemical by using the mean NRU of four replicate values (blanks is subtracted). These values are compared with the control mean values. Relative cell viability is then expressed as percent of untreated vehicle control. Table 1 presents the results of Cerrado fruits extracts cytotoxicity. For comparative purpouses, Table 2 shows some chemicals data concerning LD_{50} and IC_{50}.

Cagaita seed and Araticum peel ethanolic extracts did not decrease cell viability up to 300 $\mu g\ mL^{-1}$ as shown in Figure 1 and 5. Thus no significant general toxicity was observed and the IC_{50} values were higher than 300 $\mu g\ mL^{-1}$. Due to the low solubility of these fruits extracts it was not possible to increase the concentration in order to obtain an IC_{50} value.

Pequi peel extract was tested up to 3000 μg mL^{-1}. At this concentration, pequi peel extract start producing precipitated in the cell culture medium and also caused a decreasing in cell viability. If considered the linear correlation of cell viability decreasing and pequi peel ethanolic extract concentration, the IC_{50} would be 4.8 mg mL^{-1}. Figure 3 showed the cell viability behavior against pequi peel ethanolic extract concentration.

Regarding araticum seed ethanolic extract, the results suggested some evidence of moderate toxicity as shown in Figure 7. The IC_{50} was determined using a Hill function which is a four parameter logistic mathematical model relating the concentration of the reference substance to the response (typically following a sigmoidal shape). The IC_{50} value for araticum seed ethanolic extract was 100 μg mL^{-1}.

To obtain a model for the prediction of lethal dose LD_{50} values from IC_{50} values, Halle (2003) calculated a linear regression from pairs of the log-transformed IC_{50x} (in mM) and log transformed rodent oral LD_{50} values (in mmol kg^{-1}). The regression obtained between "in vitro" Cytotoxicity and Rat and Mouse Oral LD_{50} values from 347 chemicals was Log LD_{50} (mmol kg^{-1}) = 0.435 x log IC_{50x} (mM) + 0.625. This regression was denominated as RC regression. Presumably, the substance units were expressed in moles because moles are the units that produce biological activity and, hence, are expected to produce the best fitting regression. IC_{50} values for 347 substances were obtained from 157 original publications (Halle 2003). The 1,912 IC_{50} values, two to 32 per substance, were averaged using geometric means to produce one IC_{50x} value for each substance.

To improve the RC regression with respect to the prediction of LD_{50} values by "in vitro" NRU IC50 values, NICEATM/ECVAN, 2006 developed regressions using RC data in weight units to exclude (1) mouse data (i.e., the RC rat-only regression) and (2) exclude

substances with mechanisms of toxicity that were not expected to be active in the 3T3 cell cultures (i.e., the RC rat-only regression excluding substances with specific mechanisms of toxicity regression).

The regression formula for the RC rat-only weight regression was Log LD_{50} (mg kg^{-1}) = 0.357 x log IC_{50} (μg mL^{-1}) + 2.194 and the regression formula for the RC rat-only weight regression excluding substances with specific mechanisms of toxicity was Log LD_{50} (mg kg^{-1}) = 0.372 x log IC_{50} (μg mL^{-1}) + 2.024.

Considering the formula for the RC rat-only weight regression [Log LD_{50} (mg kg^{-1}) = 0.357 x log IC_{50} (μg mL^{-1}) + 2.194], the LD_{50} result obtained for araticum seed extract was 809.1 mg kg^{-1} and for pequi peel extract was 2849.3 mg kg^{-1}.

Using the regression formula for the RC rat-only weight regression excluding substances with specific mechanisms of toxicity [Log LD_{50} (mg kg^{-1}) = 0.372 x log IC_{50} (μg mL^{-1}) + 2.024] the LD_{50} results were 586.1 mg kg^{-1} and 2176.3 mg kg^{-1} for araticum seed and pequi peel extracts respectively. .

Agreement of the “in vitro” predicted GHS (Globally Harmonized System of Classification and Labelling of Chemicals with LD_{50}) toxicity categories with those based on the reference rat oral LD_{50} values was shown in the Table 2 for araticum seed and pequi peel extract.

Previous studies “in vivo” with two groups of seven rats each showed that orally administrated aqueous solution of *A. crassiflora* seed extract (50 mg of gallic acid equivalents/kg, i.e., 767 mg of crude extract/kg of rats) for 14 consecutive days did not produce any death, clinical signs or gross alterations attributable to hepatic toxicity or other organ damage if compared to a control group with received saline until the 14^{th} day.

However, rats that received the *A. crassiflora* seed extract showed some anxiety and were restless, perhaps because of the phenolic acids and their derivatives (caffeic acid, caffeoyl glucose and caffeoyl tartaric) present in this extract. In addition, it was observed that there was significant reduction in the body weight gains (approximately 28 and 23%) of the two groups that received seed extract if compared with control group (Roesler et al., 2007). The same study was done for the *A. crassiflora* peel extract where two groups of seven rats each received oraly aquous solution of 50 mg of gallic acid equivalents/kg, i.e., 358 mg of crude extract/kg of rats for 14 days. No different behaviour was observed for these two groups of rats. Thus, the "in vitro" result is in agreement with the previous "in vivo" study once there was no sign of ceel viability decreasing up to the maximum concentration tested. In addition, the "in vitro" method was able to estimate that the *A. crassiflora* seed extract could have some type of injury.

Considering the results obtained "in vivo" it is possible that the "in vitro" NRU cytotoxicity test method over predicted the toxicity of the *A. crassiflora* seed extract by estimating a lower LD_{50} value than that necessary to produce lethal death. The endpoint measured in the "in vitro" NRU cytotoxicity test methods is cell death and the endpoint measured in acute systemic toxicity assays is usually animal death. Cell death and animal death may be similar since animals are comprised of organ systems consisting of tissues, which are comprised of cells. All cells, regardless of whether they are in animals or "in vitro" cells cultures, have similar cellular mechanisms of energy production and utilization and maintenance of cell membrane integrity. Animal death and death of cells in culture due to toxicity are similar in that both involve some type of injury. For the animal, the cellular injury produces tissue and organ injury to the most sensitive target organ, which may then

cause the death of the whole organism. Organ system failure can be due either to the death of cells in the affected organ or to the loss of function of the surviving cells in the organ, which results in cell death or loss of function in other organs (Gennari et al., 2004). Death of an animal is produced by major organ system failure. Ultimately the cardiovascular and respiratory system fail. Respiratory depression may be due to depression of the central nervous system rather than a direct assault on the respiratory system. Other major organ system failures, such as liver and kidney failure, gastrointestinal corrosion, and bone marrow depression, also produce death. Cell death is a culture system involves the death of a single cell type. Cell death and animal death may be produced by the same mechanisms, such as disruption of membrane structure or function, inhibition of mitochondrial function, disturbance of protein turnover, disruption of energy production, etc. (Gennari et al., 2004). Animal and cell culture systems are different with respect to how a substance or toxin is delivered to cell and how it is distributed, metabolised, and excreted. The 3T3 cell culture system includes serum and have little or no capacity to metabolise xenobiotic compounds. Excretion from the cell culture milieu cannot occur since cell culture systems have no excretory system. The culture cells are exposed to substances for the entire duration of exposure in the test system.

Phototoxicity.

Differently from the “in vitro”cytotoxicity results that are used to screening toxicity and estimate the LD_{50} of chemicals, the NRU assay using 3T3 cells was validated by ECVAM and accepted for regulatory use to detect the phototoxic potencial of substances. Phototoxic potential is assessed by comparing the differences in totoxicity between negative control plates that were not exposed to ultraviolet (UVA, 315-400 nm) and test plates exposed to UVA. The phototoxicity results for the cerrado fruits extract are shown in the Table 1 and

Figures 2 and 4. None of the extracts showed photoxicity in the dosage levels tested. Again, due to the low solubility of cagaita seed and araticum peel extracts it was not possible to increase the concentration in order to obtain an IC_{50} values. Pequi peel and araticum seed ethanolic extract presented cell viability reduction during the exposure to ultraviolet (UVA) light. The PIF obtained for pequi and araticum seed were 1.1 and 1.2 respectively. Although there was cell viability reduction, it occurred even for the no exposed plates so the PIF value was relatively low and the extracts were not considered phototoxic (PIF <5). Table 2 presented some chemicals PIF values for comparative purposes. The phototoxicity results obtained by "in vitro" NRU method are the great importance especially for cosmetic and pharmaceutical application once topycal formulations are commonly used during the day with exposure to the sun and artificial ligh.

In conclusion, the "in vitro" NRU methods showed that the excellent antioxidant extracts of araticum seed and peel cagaita seed and pequi peel did not presented phototoxic. Regarding cytotoxicity, araticum peel and cagaita seed extracts did not shown any inhibition of cell grown up to 300 $\mu g\ ml^{-1}$, maximum solubility of these extracts. Araticum seed extract presented an IC_{50} of 100 $\mu g\ mL^{-1}$ and pequi peel extract had an estimated IC_{50} of 4800 $\mu g\ mL^{-1}$. The LD_{50} obtained by using the RC rat-only weight regression for araticum seed extract was 809.1 mg $kg^{-1}$and for pequi peel extract was 2849.3 mg kg^{-1}. While the "in vitro" NRU phototocixity method is validated and accepted for regulatory uses, the cytotoxicity data should be used as a screening procedure to assess cytotoxicity as well as one of the factors used to estimated starting dose for "in vivo" acute lethality studies based on preliminary information that this approach could reduce the number of animals used in "in vivo" studies (i.e., reduction), minimize the number of animals that receive lethal doses

(i.e., refinement), and avoid underestimating hazard. A detailed evaluation of tropical plants used in local health traditions and ethnopharmacological evaluation to verify their efficacy and safety can lead to the development of invaluable herbal drugs or isolation of compounds of therapeutic value. It is expected that the searching for an economically viable and environmentally correct application of Cerrado natural resources such natural antioxidant could increase the value of these fruits and help to avoid the destruction of natural vegetation and Cerrado Biodiversity.

Table 2. Cytotocixity and Phototoxicity by in vitro NRU and Oral LD50

	Cytotoxicity IC 50 (mg/mL)	PIF	Oral DL50 (mg/kg) (4)
Annona crassiflora **Peel**	> 0.3	n.d.	
Annona crassiflora **Seed**	0.10 ± 0.01	1.23	809.1
Eugenia dysenterica **Seed**	> 0.3	n.d.	
Caryocar brasilense **Peel**	4.8	1.08	2849.3
Sodium lauril sulfate (SLS)	0.04 ± 0.00 (1)	1.3 (2)	
Phenol	0.05 ± 0.01 (3)		414 (4)
Nicotine	0.27 ± 0.07 (3)		50 (4)
Glycerol	24.65 (3)		12691 (4)
Bergamot oil		15.2 (2)	
Caffeine			192 (4)

(1) Value from Edgewood Chemical Biological Center (NICEATM/ECVAN, 2006); (2) Peters & Holzhutter, 2002; (3) Globally harmonized system of classification and labeling of chemicals for acute oral toxicity (UN 2005); (4) Halle, 2003

Table 3. Prediction of Globally Harmonized System of Classification and Labeling of Chemicals with LD50 Category by Regression 1 and 2

		Araticum seed extract		Pequi peel extract	
		LD50 Category (300 - 2000 mg/kg)		LD50 Category (2000 - 5000 mg/kg)	
		Reg 1	Reg 2	Reg1	Reg 2
LD50 (mg/kg)		809.1	586.1	2849.3	2176.3
Accuracy (%)		78	78	67	44
Toxicity	Overpredicted (%)	0	22		56
	Underpredicted (%)	22	0	22	
LD50 Category (300 - 2000 mg/kg)	Predicitivity (%)	35	32	60	36
	Overpredicitivity (%)	25	23		
	Underedicitivity (%)	40	46	40	64

Regression 1: RC rat-only weight regression; Regression 2: RC rat-only weight regression excluding substances with specific mechanisms of toxicity.

Figure 1: Cytotoxicity for pequi peel extract.

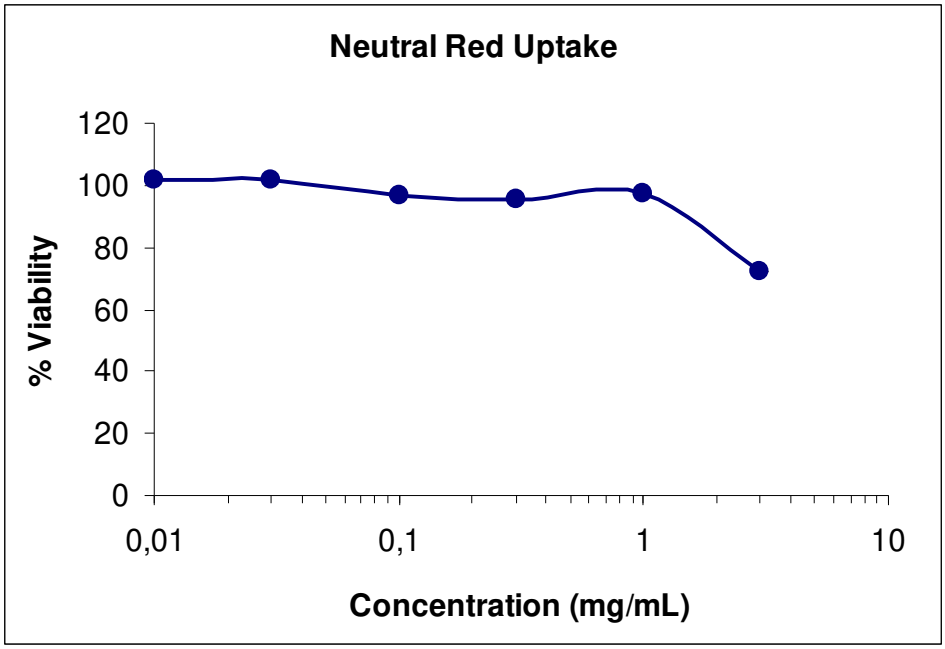


Figure 2: Photocytotoxicity for pequi peel extract.

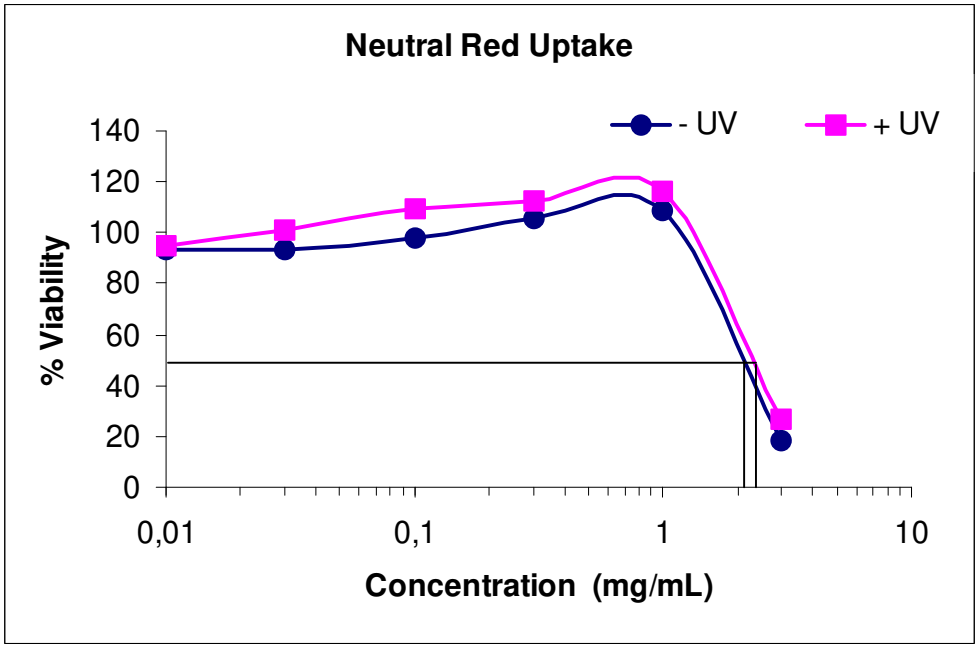


Figure 3: Cytotoxicity for araticum seed extract.

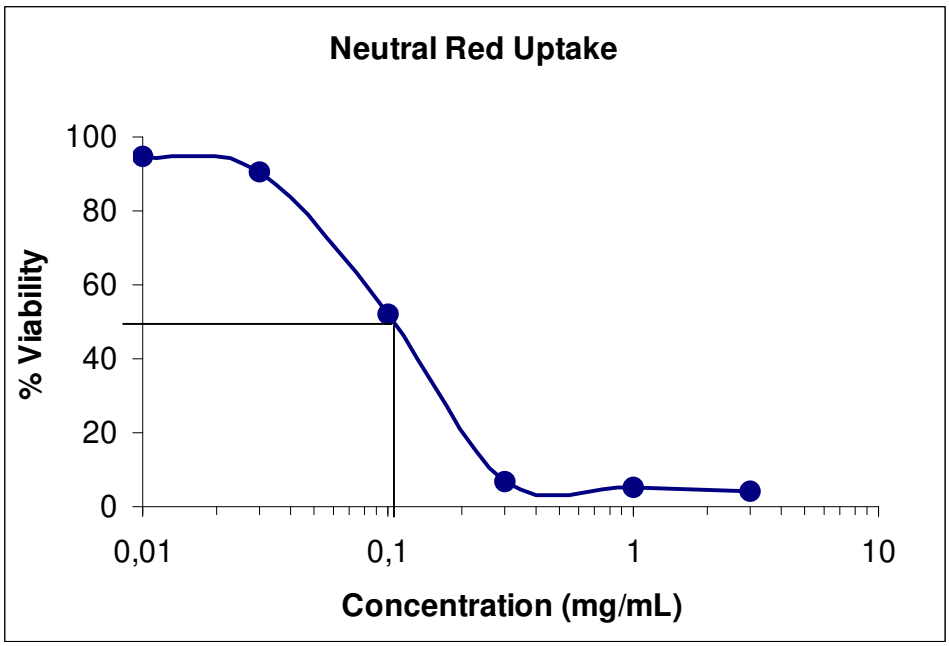
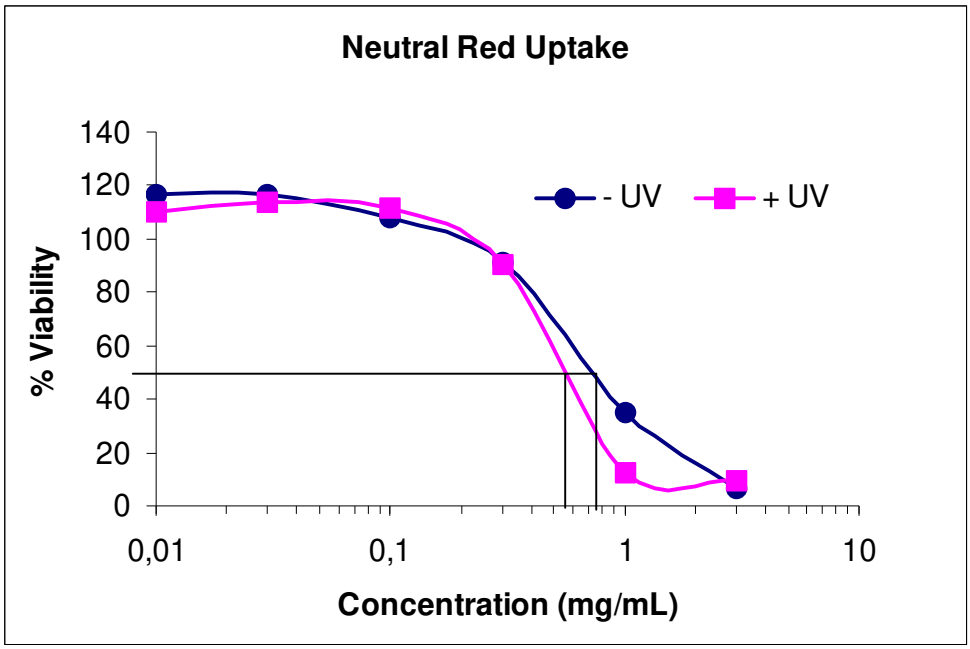


Figure 4: Photocytotoxicity for araticum seed extract.



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CONCLUSÕES GERAIS

O processo de extração das cinco frutas nativas do cerrado produziu 24 diferentes extratos e respectivos resíduos. A avaliação da AAO dos diferentes extratos revelou que os extratos etanólicos de semente e casca de *Annona crassiflora* (araticum), semente de *Eugenia dysenterica* (cagaita) e casca de *Caryocar brasiliense* (pequi) apresentam excelente capacidade antioxidante "in vitro" e portanto possuem alto potencial para aplicação como antioxidante natural nos setores farmacêutico, cosmético e alimentício. As frutas *Solanum lycocarpum* (lobeira) e *Swartzia langsdorfii* (banha de galinha) não demonstraram potencial antioxidante "in vitro".

A capacidade de seqüestrar radicais livres em relação ao radical estável DPPH foi inicialmente avaliada por se tratar de uma metodologia simples, rápida e sensitiva, conveniente para realização de "screening" de um grande número de amostras com diferentes polaridades. O IC_{50} (quantidade necessária de extrato para inibir a oxidação do radical DPPH em 50%) obtido para os extratos etanólicos de semente e casca de araticum, casca de pequi e semente de cagaita está na faixa de 14 – 50 $\mu g\ mL^{-1}$. A fim de validar a metodologia, bem como para efeitos comparativos, substâncias mundialmente reconhecidas como antioxidantes (ácido gálico, ácido cafeico, ácido ascórbico, ácido ferrúlico, ácido clorogênico e rutina) foram avaliados e os resultados obtidos estão em concordância com a literatura (IC_{50} 1,4 – 10 $\mu g\ mL^{-1}$). Por meio desse ensaio, as frutas de pequi, araticum e cagaita revelaram-se excelentes fontes de compostos antioxidantes.

O potencial dos extratos em inibir a peroxidação lipídica foi avaliado nos extratos de maior potencial antioxidante previamente escolhidos. Neste modelo "in vitro" há uma indução química da peroxidação lipídica dos microssomas de fígado de rato e quantificação dos malonaldeídos (MDA). Os resultados obtidos para os extratos encontram-se na faixa de 0,7 to 4,5 $\mu g\ mL^{-1}$. Esse ensaio confirmou os resultados previamente obtidos pelo radical DPPH, ou seja, a alta AAO proporcionada pelos extratos das frutas pequi, araticum e cagaita .

As propriedades hidrofílicas e lipofílicas dos modelos bem como a polaridade dos compostos antioxidantes presentes nos extratos possivelmente causam as diferenças nos IC_{50} obtidos para os mesmos extratos. Demonstra-se desta forma a importância de pelo

menos dois modelos com substratos diferentes de forma a obter resultados confiáveis. Entretanto, embora os ensaios não tenham apresentado quantitativamente os mesmos resultados de IC_{50} para os diferentes extratos, independentemente do modelo empregado, os extratos etanólicos de semente e casca de araticum, casca de pequi e semente de cagaita são os melhores extratos quanto à AAO.

A alta quantidade de compostos fenólicos determinada por Folin-Ciocalteau e principalmente a identificação desses compostos por espectrofotometria de massa com fonte de ionização por electrospray (ESI-MS) comprovaram que substâncias bioativas amplamente reconhecidos como antioxidantes naturais estão presentes nos extratos etanólicos de sementes e cascas dos frutos do cerrado, indicando portanto que a AAO desses extratos está diretamente relacionada a compostos fenólicos como ácido cafêico e seus derivados como cafeoil e dicafeoil tartárico e cafeoil glucose; ácido quínico, ácido ferrulico, rutina, xantoxilina, ácido gálico e seus derivados como mono-galoil glicopiranosídio; quercetina e quercetina 3-O-arabinose.

Sementes e cascas de frutos do cerrado apresentam maiores quantidades de compostos fenólicos e maior AAO independente do modelo utilizado ou do solvente escolhido para extração dos mesmos. As polpas apresentaram baixa quantidade de substâncias fenólicas e baixíssima atividade antioxidante. O "fingerprinting" obtido para as polpas por meio de ESI-MS revelou apenas a presença de ácidos orgânicos e açúcares, ressaltando mais uma vez que a AAO dos extratos está correlacionada com o conteúdo fenólico dos mesmos.

A correlação obtida entre a AAO e conteúdo de compostos fenólicos para o modelo do radical DPPH foi muito boa, ou seja, o valor de R^2 ficou entre 0,82 e 0,97. Em relação ao modelo de peroxidação lipídica, menos experimentos foram realizados em função da complexidade e morosidade do mesmo. Desta forma, apenas para o araticum a correlação foi notável ($R^2 = 0,99$).

Em função da excelente AAO demonstrada "in vitro", os extratos etanólicos de casca e semente de araticum foram escolhidos para validação "in vivo". A atividade das enzimas hepáticas responsáveis pela detoxificação de ROS e peróxidos de lipídios foi monitorada em grupos de ratos com e sem intoxicação por CCl_4 a fim de avaliar o efeito protetor dos extratos em condições normais e contra compostos tóxicos. Em concordância

com os dados de literatura, o tratamento com CCl_4 induziu significativamente a peroxidação lipídica, aumentou o nível de equivalentes de glutationa e o conteúdo de Cb5, bem como reduziu a atividade das enzimas CAT, SOD e CP450. O tratamento com os extratos de araticum inibiu a peroxidação lipídica nos ratos saudáveis quando comparados ao controle em 27 e 22% respectivamente, bem como preservou o conteúdo de CP450. Em relação aos animais que receberam CCl_4, os extratos de araticum também preveniram a peroxidação lipídica, a redução na atividade da CAT e a indução dos equivalentes de glutationa em comparação aos animais do grupo controle que receberam CCl_4. Os extratos de araticum não conseguiram impedir os danos causados por CCl_4 no conteúdo de CP450, b5 e na atividade de SOD. Portanto, os extratos etanólicos de araticum possuem AAO "in vivo". O controle da peroxidação lipídica "in vivo" é muito importante principalmente em função da aterosclerose. Os malonaldeídos, compostos resultantes da peroxidação lipídica de tecidos biológicos, são citotoxicos e causam danos ao DNA. A redução dos malonaldeídos e consequentemente da peroxidação lipídica é um benefício de grande importância demonstrado "in vivo" pelos extratos de araticum. Foi evidenciado também que os extratos não interferem no conteúdo de CP450, reduzindo a probabilidade de interações medicamentosas entre esses extratos e medicamentos convencionais, atual preocupação quanto à utilização de produtos naturais com poucos estudos científicos.

Foi possível estimar o potencial tóxico dos melhores extratos por meio da avaliação citotoxicológica e fototoxicológica "in vitro" utilizando células de fibroblastos da pele de camundongo. Nenhum dos extratos apresentou fototoxicidade nas concentrações testadas. Em relação à citotoxicidade, extratos etanólicos de casca de araticum e semente de cagaita não apresentaram inibição do crescimento celular até a concentração de 300 $\mu g\ ml^{-1}$, máxima solubilidade dos mesmos conforme protocolo. Extratos etanólicos de semente de araticum e casca de pequi apresentaram baixo potencial citotóxico e por meio de regressões lineares, a dose letal 50 estimada é de 809,1 e 2849,3 $mg\ kg^{-1}$ para os extratos de semente de araticum e casca de pequi respectivamente. Enquanto os estudos de fototoxicidade são validados e aceitos mundialmente para utilização legal, estudos de citotoxicidade "in vitro" são utilizados para estimar o potencial tóxico de substâncias e contribuem para a redução do numero de animais e minimização de animais que recebem dose letal nos estudos "in vivo".

Embora o Brasil possua forte tradicição popular na utilização de plantas para o tratamente de uma série de sintomas e doenças como febre, inflamação e dores diversas, há pouquíssimos estudos científicos que visam comprovar a eficácia dessas plantas. Este é o primeiro estudo científico que avaliou as frutas do bioma cerrado a fim de verificar a presença de bioativos naturais e comporvar sua atividade antioxidante por meio de testes "in vitro" e "in vivo" de forma a validar sua utilização como ingrediente funcional para os setores alimentício, cosmético e farmacêutico. A busca por produtos ou ingredientes naturais, denominados mundialmente como "natural health products", que promovam benefícios como prevenção de doenças crônicas e degenerativas tem gerado muito interesse não apenas por parte dos pesquisadores científicos, mas também pelos consumidores. Consequentemente, indústrias de diferentes segmentos procuram atender a demanda desses consumidores cada vez mais exigentes em relação à origem natural dos ingredientes e principalmente a comprovação dos benefícios de bioativos presentes em produtos como nutraceuticos/cosmeceuticos. A qualidade, quantidade e variedade de dados obtidos nesse trabalho deverão conduzir o desenvolvimento de produtos naturais com comprovação científica que atendam a demanda por produtos naturais e saudáveis nos diversos setores industriais. Consequentemente, a importância da valorização dos recursos naturais do bioma cerrado deverá proporcionar o desenvolvimento sustentável dessa região, gerando renda para a população local e protegendo os recursos naturais.

Por último, é importante ressaltar que as sementes e cascas dos frutos de cagaita, araticum e pequi não são utilizados para a alimentação, sendo descartados como resíduos sem qualquer valor comercial. Desta forma, esses materiais tornam-se fontes importantes e renováveis de compostos antioxidantes para os diversos setores industriais, com custo-benefício altamente vantajoso.

ANEXO 1:

CEEA/Unicamp

Comissão de Ética na Experimentação Animal
CEEA/Unicamp

CERTIFICADO

Certificamos que o Protocolo nº **1286-1**, sobre "**Avaliação do efeito de extratos de *Araticum* (*A. crassiflora*) nos danos causados por CCl4 em fígados de ratos**", sob a responsabilidade de **Profa. Dra. Glaucia Maria Pastore / Roberta Roesler / Thomaz A. A. Rocha e Silva**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal – CEEA/Unicamp em reunião de **25 de junho de 2007**.

CERTIFICATE

We certify that the protocol nº **1286-1**, entitled "**Effect of extracts from *Araticum* (*A. crassiflora*) on CCl4-induced liver damage in rats**", is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **June 25, 2007**.

Campinas, 25 de junho de 2007.

Profa. Dra. Ana Maria A. Guaraldo
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