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AVALIAÇÃO DA ATIVIDADE ANTIOXIDANTE, ANTIINFLAMATÓRIA E ANALGÉSICA DE SEMENTES DE RESÍDUOS DE VINIFICAÇÃO

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Dissertação apresentada ao Programa de Pós-Graduação em Biotecnologia da Universidade de Caxias do Sul, visando à obtenção do grau de Mestre em Biotecnologia.

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ÍNDICE

LISTA DE TABELAS

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

AAPH = 2,2´-azobis (metilpropionamidina) diidrocloreto

BSA = Albumina sérica bovina

CLAE (HPLC) = Cromatografia líquida de alta eficiência

CT = (+)-catequina

$DPPH^{\bullet}$ = 2,2 -difenil-1-picrilhidrazil

ECT = (-)-epicatequina

EGC = (-)-epigalocatequina

ERO = Espécies reativas do oxigênio

GA = Ácido gálico

GSE = Extratos de semente de uva

LDH = Lactato desidrogenase

MDA = Malondialdeído

QL = Quimioluminescência

t-BOOH = *tert*-butil hidroperóxido

TAR = Reatividade antioxidante total

TBARS = Espécies reativas ao ácido tiobarbitúrico

TCA = Ácido tricloroacético

TNF-α = Fator de necrose tumoral α

TPC = Conteúdo polifenólico total

TRAP = Potencial antioxidante reativo total

TROLOX = ácido 6-hidróxi-2,5,7,8 tetrametilchroman-2-carboxílico

RESUMO

A produção mundial de vinho, estimada em torno de 149 milhões de hectolitros por ano, gera uma grande quantidade de resíduos (aproximadamente 13% do peso da uva processada), o qual é utilizado como fertilizante ou simplesmente descartado. Estes resíduos são ricos em polifenóis, capazes de prevenir diversas patologias, incluindo câncer, aterosclerose e doenças neurodegenerativas. Neste trabalho, avaliou-se o conteúdo de polifenóis totais, atividade antioxidante, antiinflamatória e analgésica de extratos aquosos obtidos a partir de sementes de resíduos de vinificação de *Vitis labrusca* (cv. Bordo e Isabel) e *Vitis vinifera* (cv. Cabernet Sauvignon e Merlot). Os compostos fenólicos majoritários encontrados nos extratos foram a (+)-catequina, (-)-epicatequina e (-)-epigalocatequina, dímeros de procianidinas B1, B2, B3, B4 e o ácido gálico. Todos os extratos mostraram importante atividade antioxidante, tanto *in vitro,* como em células eucarióticas da levedura *Saccharomyces cerevisiae.* Observou-se uma correlação positiva entre a atividade antioxidante e o conteúdo de polifenóis totais, sugerindo que esses compostos são responsáveis, ao menos, em parte, pelo efeito biológico observado. Nenhum dos extratos apresentou atividade analgésica, mas todos mostraram-se capazes de diminuir a migração linfocitária em ratos tratados com carragenina. Esses dados sugerem que é possível obter-se extratos aquosos a partir de resíduos de vinificação, tanto de *V. vinifera* como de *V. labrusca*, com importante atividade biológica.

ABSTRACT

The worldwide wine production is around 149 millions of hectoliters per year which generate around 13% by weight of the grapes processed as wastes, which are generally used as fertilizer or simply discarded. These residues are sources of phenolic compounds that contribute to the prevention of several diseases, including cancer, atherosclerosis and neurodegenerative disorders. This work evaluated the total phenolic content, antioxidant, anti-inflammatory and analgesic activities of aqueous grape seed extracts from winery wastes of *Vitis labrusca* (cv. Bordo and Isabella) and *Vitis vinifera* (cv. Cabernet Sauvignon and Merlot). The major phenolic compounds found in extracts were (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, procyanidins dimers B1, B2, B3, B4 and gallic acid. All extracts showed an important antioxidant activity both *in vitro* and *in vivo* using eukaryotic cells of *Saccharomyces cerevisiae*. This effect showed a positive correlation with total phenolic content, suggesting that these compounds are responsible, at least in part, for the biological benefits observed. None of the extracts showed analgesic activity, however, all extracts were able to diminish the migration of lymphocytes in rats treated with carrageenan. These data suggest that it is possible to obtain aqueous extracts from seeds of winery by-products (both from *V. vinifera* and *V. labrusca)* with important biological activity.

1. INTRODUÇÃO

O Estado do Rio Grande do Sul é o maior produtor de vinho do País, com cerca de 465 milhões de litros produzidos no ano de 2007 (Ribeiro de Mello, 2008). Para a produção de vinhos finos, utiliza-se a espécie *Vitis vinifera,* principalmente as variedades Cabernet Sauvignon e Merlot. A produção de vinhos de mesa e sucos é feita, principalmente, a partir de *Vitis labrusca*, variedades Bordo e Isabel. Cerca de 13% do peso da uva processada durante a elaboração do vinho resulta em resíduo (casca, galhos e sementes), no qual, normalmente, é usado como adubo ou simplesmente descartado (Torres, *et al.,* 2002). Sabe-se que, mesmo após o processamento industrial, ainda restam, nestes resíduos, quantidades significativas de compostos com atividade biológica, principalmente, antioxidante (Alonso *et al*., 2002; Yilmaz & Toledo, 2004, Makris *et al*., 2007).

Extratos de resíduos de vinificação de *V. vinifera,* preparados com solventes orgânicos (acetato de etila, água/hexano, metanol/etanol/água, água/etanol), já tiveram sua atividade antioxidante avaliada (Torres *et al.*, 2002; Yilmaz & Toledo, 2004, Pinelo *et al*., 2005; Makris *et al*., 2007). No entanto, esses produtos apresentam utilização limitada, tanto para a indústria farmacêutica como cosmética, devido à presença de resíduos de solventes. Até o momento, não existem dados sobre a possibilidade de obtenção de extratos com atividade biológica a partir de *V. labrusca,* ou utilizando água

como solvente.

Em vista disso, este trabalho teve como objetivo avaliar as atividades antioxidante, antiinflamatória e analgésica de extratos aquosos de sementes de resíduos de vinificação de *V. labrusca L.* (variedades Bordo e Isabel) e de *V. vinifera L.* (variedades Cabernet Sauvignon e Merlot), bem como determinar os compostos fenólicos majoritários presentes nesses extratos.

Os resultados estão apresentados em dois capítulos. No primeiro, estudou-se a composição fenólica, a atividade antioxidante *in vitro* (capacidade de varredura do radical livre 2,2-difenil 1-picrilhidrazil) e a atividade antioxidante *in vivo* (células da levedura *Saccharomyces cerevisiae*) dos diferentes extratos. No segundo capítulo, avaliou-se a atividade antioxidante *ex vivo* (empregando-se fatias de fígado de ratos Wistar), e a atividade antiinflamatória e analgésica *in vivo* dos quatro extratos de sementes provenientes de resíduos de vinificação.

2. REVISÃO DA LITERATURA

2.1 Uvas: composição e atividade biológica

A videira (*Vitis* sp) foi introduzida no Brasil em 1532, com a espécie V*itis vinifera*, originária de Portugal (Camargo, 1994). Entre os anos de 1830 à 1850, foram trazidas cultivares de origem americana, denominadas *Vitis labrusca.* Essas videiras difundiram-se rapidamente em todo país (Camargo, 1994; Giovannini, 1999) e, a partir da segunda metade do século XIX, a vitivinicultura passou a ter importância comercial principalmente para os estados do Rio Grande do Sul, Santa Catarina, São Paulo, Minas Gerais, Bahia e Pernambuco (Camargo, 1994).

A espécie *V. vinifera* é a mais utilizada para a elaboração de vinhos finos (Winkler *et al*., 1997), principalmente a partir das cultivares Cabernet Sauvignon e Merlot (Giovannini, 1999). Já a *V. labrusca* é usualmente empregada na produção de vinhos comuns e sucos (Soares de Moura *et al*., 2002). Entre as principais cultivares americanas no Brasil, estão a Bordo, também conhecida como Yves e a Isabel, também chamada de Isabella (Giovannini, 1999).

As uvas são ricas em compostos polifenólicos (Gabetta *et al.*, 2000; Saucier *et al*., 2001; Monagas *et al.*, 2003; Ashraf-Khorassani & Taylor, 2004; Kammerer *et al.*, 2004; Nicoletti *et al*., 2008), conhecidos por sua importante atividade antioxidante (para revisão, ver Ferguson, 2001 e Ferguson e Philpott, 2008). De forma geral, os polifenóis

podem ser classificados em flavonóides e não flavonóides. Os flavonóides apresentam uma estrutura hidrocarbonada (Figura 1) do tipo C_6-C_3-C_6 (difenilpropano), derivada do ácido chiquímico e de três restos de acetato (Trueba & Sanchéz, 2001; Aron & Kennedy, 2008) e são divididos em diversas classes (flavan-3-ol, flavona, flavonol, flavanona, flavononol, antocianidina, chalcona, aurona), de acordo com o grau de oxidação do oxigênio heterocíclico (Figura 1).

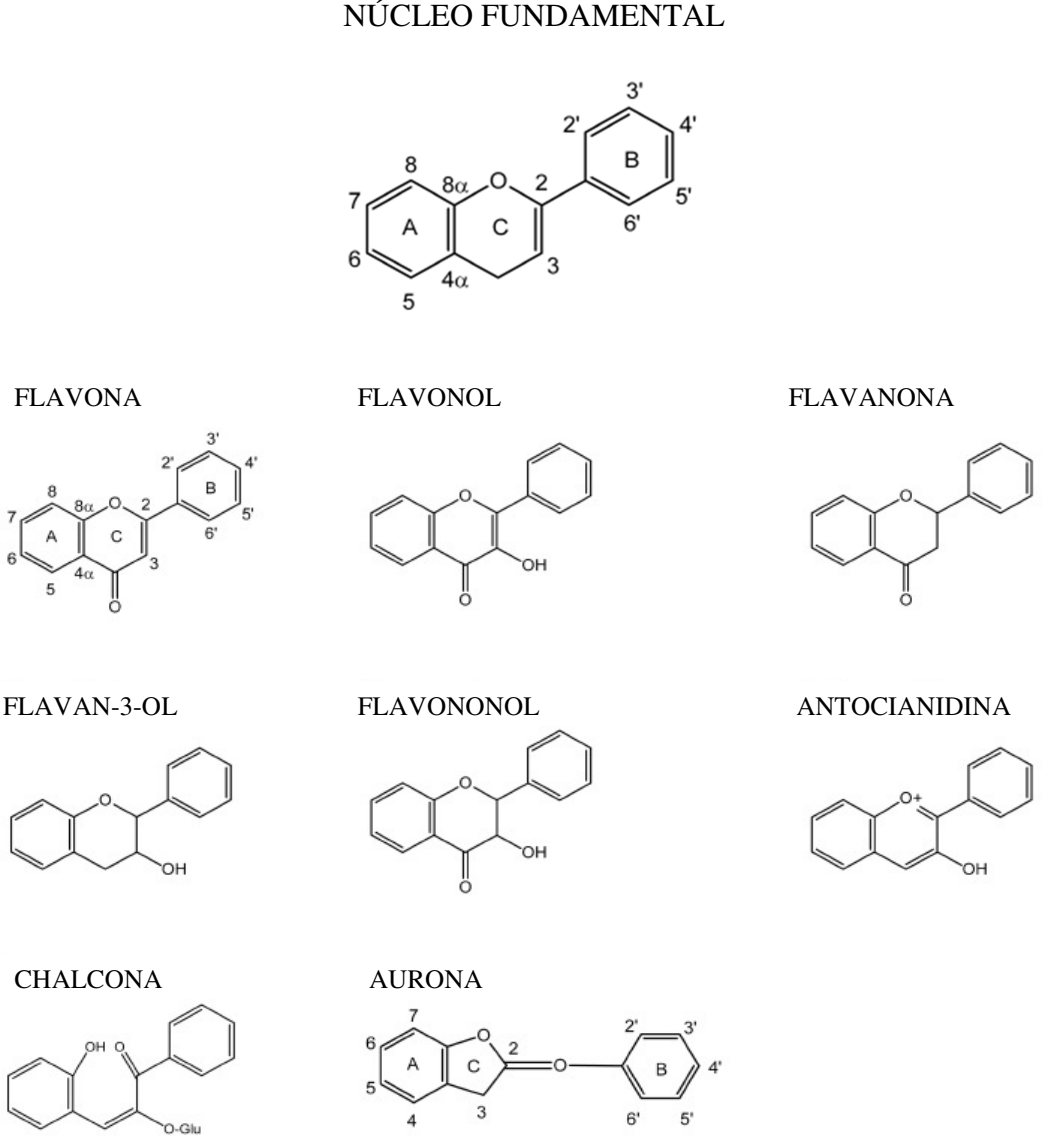
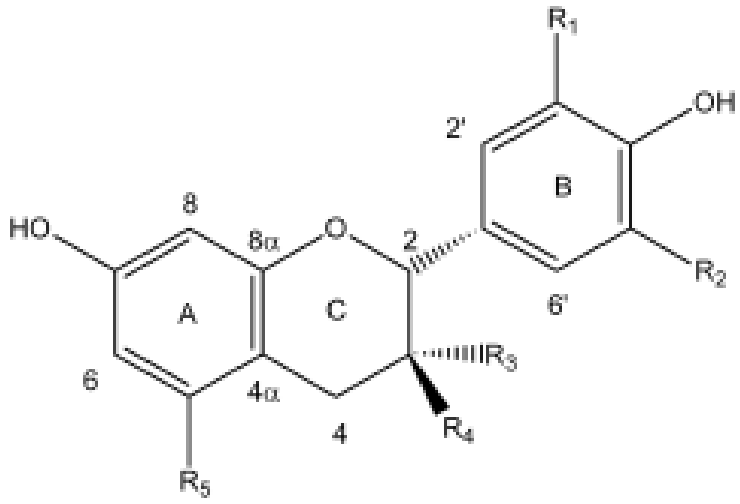


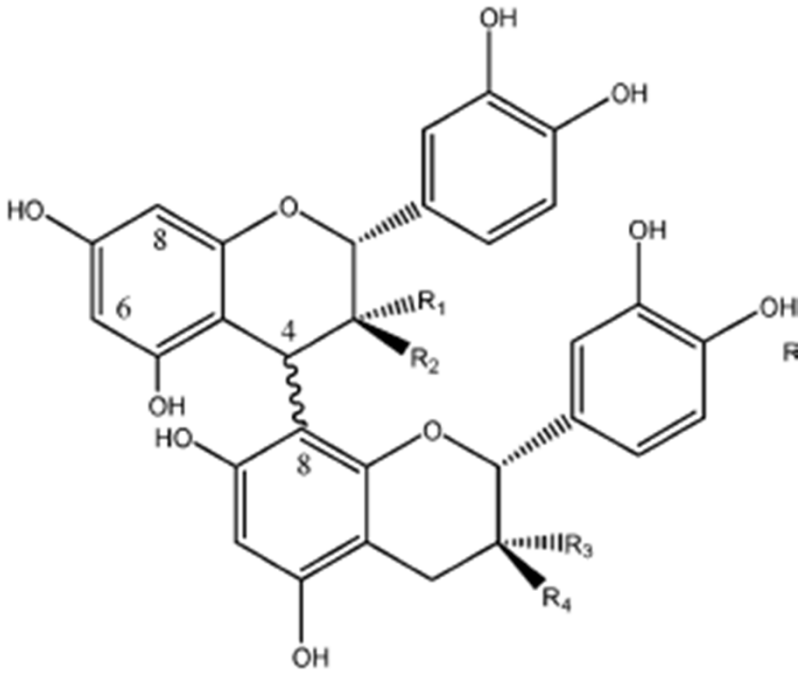
Figura 1. Núcleo fundamental e classificação dos flavonóides (Adaptado de Aron & Kennedy, 2008).

A subclasse mais comum dos flavonóides encontrada nas sementes de uva é a dos flavan-3-ols, também conhecidos como taninos. Neste grupo estão os monômeros (+)-catequina, (-)-epicatequina e (-)-epigalocatequina (Aron & Kennedy, 2008) e os dímeros de flavan-3-ols, como as protoantocianidinas B-1 ((–)-epicatequina-(4β→8)-(+)-catequina), B-2 ((-)-epicatequina-(4β→8)-(–)-epicatequina), B-3((+)-catequina-(4α→8)-(+)-catequina) e B-4 ((+)-catequina- (4α→8)-(–)-epicatequina) (Aron & Kennedy, 2008). A estrutura dos principais monômeros e dímeros de flavan-3-ols estão apresentados nas Figuras 2 e 3, respectivamente.



Monômeros de flavan-3-ols	R_1	R_2	R_3	R_4	R_5
(+)-catequina	H	OH	H	OH	OH
(-)-epicatequina	H	OH	OH	H	OH
(-)-epigalocatequina	OH	OH	OH	H	OH

Figura 2. Estrutura química dos principais monômeros de flavan-3-ols (Adaptado de Aron & Kennedy, 2008)



Dímeros de flavan-3-ols	R_1	R_2	R_3	R_4
B1	OH	H	H	OH
B2	OH	H	OH	H
B3	H	OH	H	OH
B4	H	OH	OH	H

Figura 3. Estrutura química dos principais dímeros de flavan-3-ols (Adaptado de Weber *et al*., 2007)

Entre os não flavonóides estão os ácidos fenólicos, benzóicos e cinâmicos, e outros derivados fenólicos como os estilbenos (Singleton, 1987). Nas uvas são encontrados flavonóis, antocianidinas, estilbenos, flavan-3-ols, compostos não flavonóides e ácidos fenólicos (Ribéreau-Gayon *et al*., 1972; Di Stefano, 1996; Gabetta *et al.*, 2000;; Saucier *et al*., 2001; Monagas *et al.*, 2003; Ashraf-Khorassani & Taylor,

2004; Kammerer *et al.*, 2004; Koyama *et al*., 2007; Saiko *et al*., 2008). Na casca estão presentes os flavonóis (quempferol, quercetina e miricetina), as antocianinas (cianidina, delfinidina, peonidina, petunidina, malvidina), os estilbenos (resveratrol) e os ácidos fenólicos (ácido cafeiltartárico e o ácido p-cumariltartárico) (Koyama *et al*., 2007; Saiko *et al*., 2008). Os vasos fibrovasculares são ricos em flavanóis e ácidos fenólicos do tipo benzóico (ácidos vanílico, siríngico, salicílico, protocatéquico, o gentísico, e o p-hidroxibenzóico) (Ribéreau-Gayon *et al*., 1972). Já a polpa da uva possui compostos não flavonóides (ácido ferrúlico, ácido p-cumárico e o ácido cafeico) (Di Stefano, 1996). As sementes são ricas em polifenóis, apresentando cerca de 7% de flavan-3-ols (Fuleki & Ricardo Silva, 2003), prinicipalmente, (+)-catequina, (-)-epicatequina, (-)-epicatequina 3-O-galato, oligômeros e polímeros de flavan-3-ols e o ácido gálico (Gabetta *et al.*, 2000; Saucier *et al*., 2001; Monagas *et al.*, 2003; Ashraf-Khorassani & Taylor, 2004; Kammerer *et al.*, 2004; Koyama *et al*., 2007) (Figura 4).

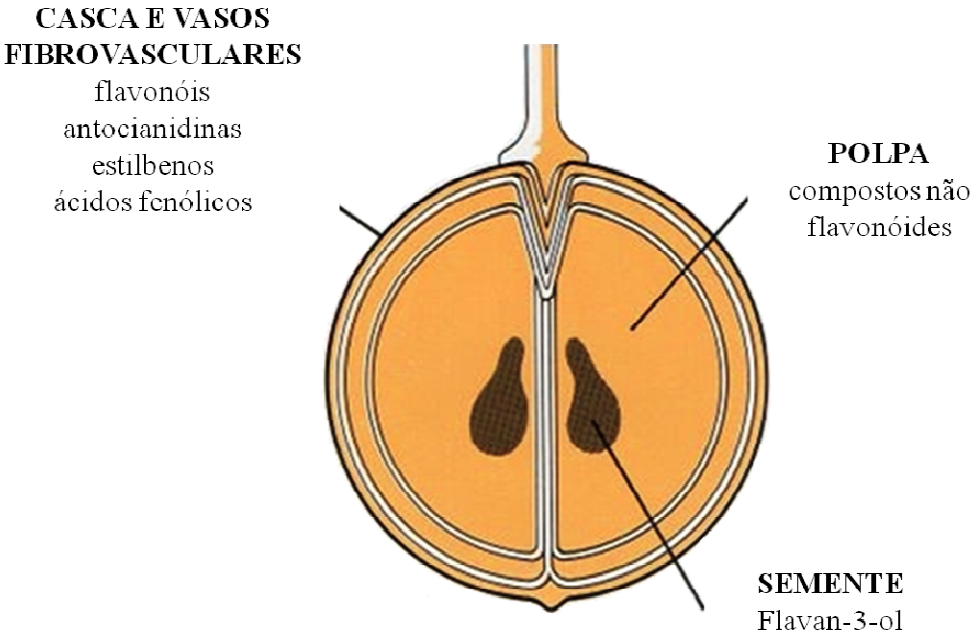


Figura 4. Distribuição dos polifenóis na uva

A atividade biológica dos polifenóis está bastante estudada. Estes compostos apresentam atividade antioxidante, anticarcinogênica, antiaterogênica, cardiopreventiva, antimicrobiana, antiviral, neuroprotetora e antiinflamatória (Gabetta *et al*., 2000; Fan & Lou, 2004; Guendez *et al*., 2005; Aron & Kennedy, 2008; Terra *et al*., 2008). Extratos de sementes de uva da espécie *V. vinifera* também tem sido alvo de inúmeros estudos (Tabela 1) e mostram, principalmente, efeitos antioxidante, antitumoral, antiaterogênico, antigenotóxico e antiinflamatório. No entanto, a maior parte dos dados refere-se a extratos obtidos a partir de solventes orgânicos, o que limita a sua utilização na indústria farmacêutica, cosmética e de alimentos. Além disso, até o momento, não existem dados sobre os efeitos de extratos de sementes de *V. labrusca*, espécie bastante utilizada em toda a América.

Extratos de semente provenientes de resíduos de vinificação de *V. vinifera (Parellada, Merlot, Garnacha),* obtidos com solventes orgânicos (acetato de etila/água, hexano, metanol/etanol/água, água/etanol) mostraram atividade antioxidante *in vitro* (Torres *et al*., 2002; Yilmaz & Toledo, 2004; Pinelo *et al*., 2005; Makris *et al*., 2007).

Tabela 1. Atividades biológicas de extratos obtidos a partir de sementes de uva (n.d. não determinado)

Uva	**Solvente**	**Atividade biológica**	**Nome comercial**	**Referência**
Vitis vinifera	etanol/água	Antitumoral	Gravinol®	Katiyar, 2008
Vitis vinifera	etanol/água	Antitumoral	Gravinol®	Nandakumar *et al*., 2008
Vitis vinifera	etanol/água	Antinociceptivo	Gravinol®	Uchida *et al*., 2008
Vitis vinifera	n.d.	Neuroprotetora	Mega Natural-AZ®	Wang *et al*., 2008

Vitis vinifera	n.d.	Neuroprotetora	Mega Natural-AZ®	Ono *et al*., 2008
Vitis vinifera	n.d.	Antioxidante	Vitisol®	Morin *et al*., 2008
Vitis vinifera	etanol/água	Antitumoral	Gravinol®	Meeran e Katiyar, 2007
Vitis vinifera	etanol/água	Antioxidante	Gravinol®	Devi *et al*., 2006
Vitis vinifera	etanol/água	Antitumoral	Gravinol®	Kaur *et al*., 2006
Vitis vinifera	etanol/água	Antitumoral	Gravinol®	Kim *et al*., 2004
Vitis vinifera	etanol/água	Antiaterogênica	Gravinol®	Yamakoshi *et al*., 1999
Vitis vinifera	etanol/água	Antioxidante	Gravinol®	Yamaguchi *et al*., 1999
n.d.	n.d.	Antiinflamatória	Vitaflavan®	Terra *et al*., 2008
n.d.	n.d.	Antiinflamatória	Vitaflavan®	Terra *et al*., 2007
n.d.	n.d.	Antiaterogênica	Grape seed extract	Polagruto *et al*., 2007a
n.d.	n.d.	Antiplaquetária e antioxidante	Oligonol™	Polagruto *et al*., 2007b
n.d.	n.d.	Antioxidante	Mega Natural-AZ®	Rababah *et al*., 2004
n.d.	n.d.	Antioxidante	AV 9090940	Shao *et al*., 2003
n.d	n.d.	Antioxidante	ChromeMate® (IH636)	Roychowdhury *et al*., 2001
Vitis vinifera	etanol/água	Antioxidante	---	Tehirli *et al*., 2008

Vitis vinifera	n.d.	Antioxidante	---	Stanković *et al*., 2008
Vitis vinifera	n.d.	Antioxidante	---	Hemmati *et al*., 2008
Vitis vinifera	etanol	Inibição dos produtos avançados de glicação	---	Farrar *et al*., 2007
Vitis vinifera	água/acetona	Antioxidante e redução de isquemia hipóxica	---	Feng *et al*., 2007
Vitis vinifera	água/acetona	Antioxidante	---	Balu *et al*., 2006
Vitis vinifera	água	Adaptogênica e nootrópica	---	Sreemantula *et al*., 2005
Vitis vinifera	água/acetona	Antioxidante e redução de isquemia hipóxica	---	Feng *et al*., 2005
Vitis vinifera	água/acetona	Antioxidante	---	Balu *et al*., 2005
Vitis vinifera	acetato de etila/metanol	Antioxidante	---	Guendez *et al*., 2005
Vitis vinifera	hexano	Antioxidante	---	Murthy *et al*., 2002
Vitis vinifera	água	Antioxidante	---	Koga *et al*., 1999
n.d.	n.d.	Antitumoral	---	Huang *et al*., 2008
n.d	acetona/água	Antioxidante e antigenotóxica	---	Fan & Lou, 2004

Além dos efeitos benéficos à saúde, a uva tem grande importância econômica para o País e região. Em 2007, o Brasil produziu 1.354.960 toneladas de uva, sendo que 47,02% foi destinada à elaboração de vinhos e sucos de uva. Apenas no Estado do Rio Grande do Sul, foram produzidos cerca de 465.591.000 litros de vinho, representando mais da metade da produção nacional (Ribeiro de Mello, 2008). Como resultado do processo de vinificação, é gerada uma grande quantidade de resíduos (cerca de 13% do peso da uva processada), que inclui casca, galhos e sementes. Normalmente, estes resíduos são descartados ou usados como adubo (Torres, *et al.,* 2002). Em cada 100 kg de resíduo úmido gerado pelas indústrias existem cerca de 10 a 12 kg de sementes (Oliveira *et al*., 2003). Apesar da possível perda de compostos bioativos durante a vinificação, ainda restam no resíduo, quantidades significativas de compostos fenólicos (Alonso *et al*., 2002; Yilmaz & Toledo, 2004; Rubilar *et al*., 2007) que podem ser utilizados na prevenção de doenças.

2.2 Avaliação da atividade antioxidante, antiinflamatória e analgésica

Diferentes metodologias têm sido desenvolvidas para obter uma medição, seja qualitativa ou quantitativa, da capacidade antioxidante de diversos compostos, tanto através de testes sem a utilização de células (testes *in vitro*) ou utilizando-se células, como por exemplo, de *Saccharomyces cerevisiae* (testes *in vivo*). Considerando que a atividade antioxidante de um composto é influenciada pelo modelo de estudo (Halliwell & Gutteridge, 2007), costuma-se empregar mais de um tipo de teste para avaliação dos compostos.

Dentre os testes *in vitro* existentes, a medida da varredura do radical livre 2,2-difenil 1-picrilhidrazil ($DPPH^{\bullet}$) vem sendo utilizada para avaliar a atividade

antioxidante de diversos compostos, sendo considerado um método colorimétrico fácil e rápido (Brand-Williams *et al*., 1985; Rice-Evans *et al*., 1995; Cheng *et al*., 2006). Além deste, a avaliação do potencial antioxidante reativo total (TRAP) e reatividade antioxidante total (TAR) têm se mostrado técnicas sensíveis e reprodutíveis, que podem ser utilizadas para determinação da atividade antioxidante em misturas complexas (Lissi *et al*., 1992; Lissi *et al*., 1995; Desmarchelier *et al.*, 1997). O ensaio TRAP avalia a diminuição da quimioluminescência (QL) induzida pelo luminol e o radical 2,2´-azobis (metilpropionamidina) diidrocloreto (AAPH), sendo que esta inibição da QL é proporcional a capacidade antioxidante (Lissi *et al*., 1995). O TAR determina o decréscimo inicial da QL calculado através da emissão inicial da QL (anteriormente a adição do antioxidante) e a intensidade de QL após a adição da amostra ou do composto de referência (Lissi *et al*., 1995).

Ensaios *in vivo* utilizando microrganismos têm se mostrado muito adequados na triagem rotineira de vários produtos, sendo testes rápidos, sensíveis, econômicos e reprodutíveis (Rabello-Gay *et al.*, 1991). A descrição do ciclo de vida de *Saccharomyces cerevisiae* e o conhecimento de suas características genéticas básicas, além da facilidade de manipulação e, principalmente, do fato deste microrganismo ser eucarioto, proporcionaram a grande difusão deste sistema biológico como metodologia experimental para estudos da atividade antioxidante (Soares *et al.*, 2003; Picada *et al.*, 2003; Lopes *et al.,* 2004, Raspor *et al.,* 2005; Spada & Salvador, 2005). Os dados obtidos nesse tipo de teste apresentam uma correlação de aproximadamente 70% em relação ao observado no homem (Rabello-Gay *et al.*, 1991).

A determinação da atividade antiinflamatória pode ser feita utilizando-se diferentes metodologias, preferencialmente em mamíferos. A indução de pleurisia por

carragenina em ratos (Ianaro *et al.,* 2000) tem sido bastante utilizada (Nardi *et al*., 2007). Um dos modelos para avaliação da atividade analgésica é o teste de formalina, em que a dor é induzida pela injeção de formalina na região subplantar da pata direita dos ratos. Os parâmetros avaliados incluem a medida do comportamento de lamber a região em um determinado tempo, conforme descrito por Hunskaar & Hole (1987).

Tendo em vista os efeitos benéficos de sementes de uva já descritos na literatura, neste trabalho buscou-se estudar as atividades antioxidante, antiinflamatória e analgésica de extratos aquosos obtidos a partir de resíduos de vinificação de *V. vinifera* e *V. labrusca,* duas espécies bastante utilizadas para a produção de vinhos e sucos em nosso País.

3. OBJETIVOS

3.1 Objetivo geral

- ✓ Determinar os principais compostos fenólicos majoritários e avaliar a atividade antioxidante, antiinflamatória e analgésica de extratos aquosos de sementes de resíduos de vinificação de *V. labrusca L.* (variedades Bordo e Isabel) e de *V. vinifera L.* (variedades Cabernet Sauvignon e Merlot).

3.2 Objetivos específicos

- ✓ Avaliar a atividade antioxidante *in vitro* dos diferentes extratos aquosos de sementes dos resíduos de vinificação;
- ✓ Avaliar a capacidade antioxidante dos diferentes extratos em células eucarióticas da levedura *Saccharomyces cerevisiae* e em fatias de fígado de ratos Wistar;
- ✓ Avaliar a atividade antiinflamatória e analgésica dos extratos em ratos Wistar;
- ✓ Quantificar os compostos fenólicos majoritários dos extratos aquosos;
- ✓ Correlacionar os teores de polifenóis com os efeitos biológicos observados.

4. RESULTADOS

4.1 CAPÍTULO 1

Grape seed extracts from winery wastes with antioxidant activity

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ABSTRACT

Grape seed extracts have already been reported to exhibit protective effects against oxidative stress, but until now there has been no data about the possible biological activity of the seeds present in winery by-products, which are considered an environmental problem. This work evaluates the main phenolic content and the antioxidant activity of aqueous extracts of seeds (GSE) from wine wastes of *Vitis labrusca* (cv. Bordo and Isabella) and *Vitis vinifera* (cv. Cabernet Sauvignon and Merlot). The main phenolic compounds found in extracts were catechin and epicatechin. All extracts showed significant *in vitro* and *in vivo* antioxidant activity that had a positive correlation with total phenolic content. These results show that it is possible to extract phenolic compounds with antioxidant activity from winery by-products using water as a solvent, which can provide an important use for the wine wastes and thus help to maintain environmental balance.

Keywords: Aqueous grape seed extract, phenolic content, *V. vinifera*, *V. labrusca*, $DPPH^{\bullet}$, *Saccharomyces cerevisiae*

1. INTRODUCTION

Worldwide wine production is around 149 million hectoliters per year (OIV, 2008). *Vitis Vinifera* is the most commonly used grape. The main varieties are Cabernet Sauvignon and Merlot. In South and North America, *Vitis labrusca*, especially the Bordo and Isabella varieties, is also used to produce wine and grape juice (Soares de Moura et al., 2002).

Wine production generates by-products composing an estimated 13% by weight of the grapes processed, which are generally used as fertilizer or simply discarded (Torres et al., 2002). These residues are sources of antioxidants (mainly phenolic compounds) that have been relatively unexploited to date, but are of increasing interest to industry. Winery residue consists of approximately 30% seeds and 70% skin (Guendez et al., 2005). Despite the transfer of phenolic compounds from grapes to wine during the winemaking process and the possible loss of some of these compounds by oxidation during the process, the seed by-products are still good sources of antioxidant compounds (Alonso et al., 2002; Torres et al., 2002; Yilmaz and Toledo, 2004; Makris et al., 2007).

Antioxidants, especially phenolic compounds, are known to exert a beneficial effect on human health, reducing the rates of cancer, atherosclerosis and age-related degenerative disorders (Ferguson, 2001). Grape seeds are rich in phenolic compounds, and studies have already reported on the antioxidant activity of grape seed extract (GSE) obtained using organic solvents (Fan et al., 2004; Yilmaz and Toledo, 2004; Guendez et al., 2005; Pinelo et al., 2005; Makris et al., 2007). Nevertheless, this extract has limited use, mainly due to the high cost of the extraction processes and the presence of used solvent residues.

This study aims to determine whether it is possible to obtain extracts with antioxidant activity from winery by-products using water as a solvent. The main phenolic content of these extracts was also analyzed.

2. MATERIALS AND METHODS

2.1 Chemicals

Procyanidins B1, B2, B3, and B4, (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, gallic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH˙) were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were purchased from Merck (Germany).

2.2 Vinification by-products

The winery by-products of *V. labrusca* (cv. Bordo and Isabella) and *V. vinifera* (cv. Cabernet Sauvignon and Merlot) were removed from the vinification tanks five days after the beginning of fermentation, in January 2006. All varieties were cultivated in the Northeast region of Serra Gaucha, Rio Grande do Sul, Brazil. Voucher specimens were identified by the Herbarium of the University of Caxias do Sul, Rio Grande do Sul, Brazil (*V. labrusca* HUCS31065-31066 and *V. vinifera* HUCS32455-32456). Seeds were manually separated from the rest of the winery by-products, dried in an air oven at 37ºC and stored at 25ºC while sheltered from light.

2.3 Preparation of the extracts

Grape seeds were pounded in a Knife (Quimis, Brazil) immediately before each assay. Different concentrations of seeds and times for extraction were analysed. *In vitro*

antioxidant activity (DPPH˙ assay) was used to choose the best extraction conditions, which were found to be five grams of seeds/100 mL of distilled water (5% w/v), under reflux (100°C), for 30 minutes. Extracts were cooled to 25ºC and then filtered in Millipore equipment (pore size, 0.45 µm; catalog number SFGS 047LS, Millipore Corp., São Paulo, Brazil). All grape seed extracts (GSE) were prepared immediately before use.

2.4 Determination of total phenolic content

Total phenolic content of the GSE was measured using Singleton and Rossi´s (1965) modification of the Folin–Ciocalteu colorimetric method. Two hundred microliters of the extracts were assayed with 1000 µL of Folin–Ciocalteu reagent and 800 µL of sodium carbonate (7.5%, w/v). The mixture was vortexed and diluted (1:10) with distilled water. After 30 minutes, the absorption was measured at 765 nm and the total phenolic content was expressed as mg/L of catechin equivalents (CTE).

2.5 Determination of phenolic compounds by HPLC

Chromatographic analyses were carried out as described by Lamuela-Raventós and Waterhouse (1994) using a HP 1100 (Palo Alto, CA) diode array UV-visible detector coupled to an HP Chem Station. A Zorbax SB C18 (250 x 4 mm), 5 µm particle size, was used for the stationary phase with a flow of 0.5 mL/min. Twenty-five microliters of GSE was injected into the HPLC system after filtration on a 0.45 µm Millipore membrane. The solvents used for the separation were as follows: solvent A (50 mM dihydrogen ammonium phosphate adjusted to pH 2.6 with orthophosphoric acid), solvent B (20% of solvent A with 80% acetonitrile) and solvent C (0.2 M

orthophosphoric acid adjusted with ammonia to pH 1.5), as described in Table 1.

Table 1. Solvent gradient conditions

	Gradient elution							
Solvent	0-5 min	5-15 min	15-25 min	25-45 min	45-50 min	50-55 min	55-60 min	60-65 min
A	100%	96%	92%	-	-	-	-	100%
B	-	4%	8%	8%	30%	40%	80%	-
C	-	-	-	92%	70%	60%	20%	-

Chromatograms were monitored at 204 nm, and identification was based on retention times relative to authentic standards ((+)-catechin, (-)-epicatechin, (-)-epigallocatechin, Procyanidin B1, B2, B3, B4 and gallic acid). Quantification was performed using the standards by establishing calibration curves for each identified compound. Results are shown in mg/L.

2.6 Antioxidant activity of GSE

In vitro antioxidant activity of GSE was measured by 2,2-diphenyl-1-picrylhydrazyl radical (DPPH˙) scavenging activity (Yamaguchi et al., 1998). The different extracts were added to Tris-HCl buffer (100 mM, pH 7.0) containing 250 µM DPPH˙ dissolved in ethanol. The tubes were kept in the dark for 20 min and absorbance was measured at 517 nm (UV-1700 spectrophotometer, Shimadzu, Kyoto, Japan). Results were calculated as IC_{50} (amount of extract necessary to scavenge 50% of DPPH˙ radical). Catechin was used as a standard.

In vivo antioxidant activity was carried out using *Saccharomyces cerevisiae* XV 185-14C (*MATα, ade 2-1, arg 4-17, his 1-7, lys 1-1, trp 1-1, trp 5-48, hom 3-10*) yeast,

provided by Dr. R. C. Von Borstel (Genetics Department, University of Alberta, Edmonton, AB, Canada). The oxidant agent was a solution of 4 mM hydrogen peroxide (H_2O_2) prepared in sterilized distilled water immediately prior to use. To evaluate antioxidant activity, 2 x 10^6 cells/mL were treated with H_2O_2 in the presence and absence of GSE. The tubes were incubated for 2 h at 28ºC. The samples were then diluted in a sodium chloride solution 0.9% (p/v), seeded into a complete culture medium (10 g/L of yeast extract, 20 g/L of peptone, 20 g/L of dextrose and 20 g/L of agar-agar) and incubated for 48 h at 28ºC. After incubation, the colonies were counted, defining the total number of colonies observed on the control plate (untreated cells) as a 100% survival rate.

2.7 Statistical analysis

All measurements were performed in triplicate, and values were averaged and reported along with the standard deviation. Data were analyzed using the Tukey´s test and Pearson correlation through an SPSS 12.0 software package (SPSS Inc., Chicago, IL).

3. RESULTS AND DISCUSSION

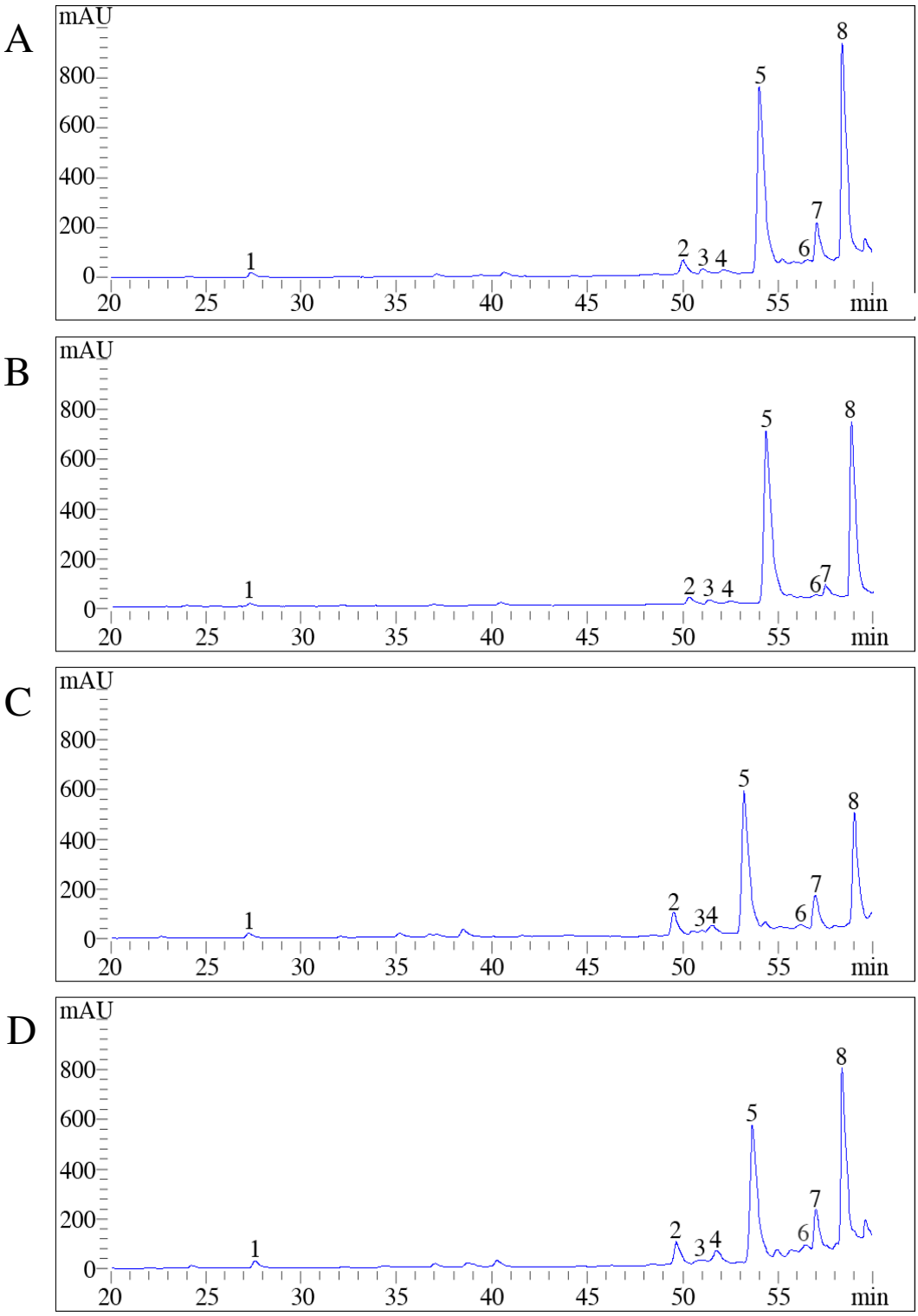
Phenolic compounds are ubiquitous in plant foods and, apart from known vitamins and minerals, may be one of the most widely marketed groups of dietary supplements. This class of plant metabolites shows antibacterial effects (Avorn et al., 1994); an ability to reduce blood pressure (Lampe, 1999) and antioxidant, anti-inflammatory, antimutagenic and/or anticarcinogenic effects, at least in *in vitro* systems (Miyazawa et al., 1999; Rodrigues et al., 2006; Saiko et al., 2008). A prospective study of 800 elderly men showed that the ingestion of flavonoids was associated with a

significant reduction in mortality from coronary heart disease (Hertog et al., 1993). Furthermore, phenolic compounds can also inhibit platelet aggregation and vascular relaxation through the production of nitric oxide (Dubick and Omaye, 2001).

The aqueous GSE studied in this work presented a total phenolic content varying from 751.38±5.30 mg/L for Merlot to 353.20±4.60 mg/L for Isabella, which showed the lowest levels of phenolic content (Table 2). To our knowledge, this is the first work concerning quantitative data on the phenolic compounds content of seed extracts of the Bordo and Isabella varieties of *V. labrusca*. Ethanol extracts of seeds from the Greece white Roditis cultivar (*Vitis vinifera* sp.) present a range from 7957±602 to 13,756±849 mg of gallic acid equivalents per 100 g of dry weight of phenolic content, depending on the type of organic solvent used (Makris et al., 2007). These different values can be attributed to the different varieties of grapes, soil, maturity and environmental growing conditions.

In the four kinds of aqueous GSE, the main phenolic compound found was catechin, followed by epicatechin (Table 2). The aqueous extracts of *V. vinifera* studied herein present a phenolic composition similar to that observed in seed extracts from the Cabernet Sauvignon and Merlot varieties prepared in ethanol (Peng et al., 2001), methanol (Monagas et al., 2003; Kammerer et al., 2004) and ethyl acetate/acetone (Delaunay et al., 2002; Jorgensen et al., 2004; Guendez et al., 2005). The Isabella extract showed the lowest levels of all the compounds assayed, except for catechin and epicatechin. It is also possible to observe other peaks in the chromatograms (Figure 1), which are probably dimers, trimers or polymers of procyanidins (Santos-Buelga et al., 1995).

Fig. 1. Chromatograms of aqueous grape seed extracts



HPLC of grape seed extracts, recorded at 204 nm. (A) Bordo, (B) Isabella, (C) Cabernet Sauvignon, (D) Merlot. (1) gallic acid, (2) procyanidin B1, (3) epigallocatechin, (4) procyanidin B3, (5) catechin, (6) procyanidin B4, (7) procyanidin B2, (8) epicatechin.

Table 2. Polyphenol content (expressed in mg/L CTE) and major compounds in aqueous grape seed extracts, as determined by HPLC (expressed in mg/L)

Variety	TPC	GA	B1	EGC	B3	CT	B4	B2	ECT
Bordo	744.89±3.13^{a}	12.98±0.54^{a}	22.42±0.51^{a}	8.96±0.05^{a}	17.45±0.01^{a}	169.26±0.92^{a}	1.85±0.12^{ab}	19.75±0.17^{a}	168.86±2.82^{a}
Isabella	353.20±4.60^{b}	6.88±0.04^{b}	8.86±0.03^{c}	5.64±0.02^{a}	9.72±0.01^{b}	135.36±0.99^{b}	1.72±0.06^{a}	3.17±3.64^{b}	112.40±0.32^{b}
Cabernet Sauvignon	715.59±5.87^{a}	11.87±0.17^{a}	26.54±1.86^{ab}	8.14±1.29^{a}	29.53±2.70^{c}	106.73±0.34^{c}	2.89±0.02^{b}	15.23±0.08^{a}	71.53±0.33^{c}
Merlot	751.38±5.30^{a}	16.42±1.15^{c}	27.80±0.82^{b}	7.49±0.97^{a}	47.16±0.45^{d}	109.57±0.20^{c}	2.87±0.19^{b}	13.73±0.17^{a}	111.08±0.05^{b}

Results reported are obtained from (5%, w/v) aqueous extracts and represent average values ± S.D. TPC, total phenolic content, GA, gallic acid, B1, procyanidin B1, EGC, epigallocatechin, B3, procyanidin B3, CT, catechin, B4, procyanidin, B4, B2, procyanidin B2, ECT, epicatechin. Different letters indicate significant differences ($p \leq 0.05$).

The *in vitro* antioxidant assay was carried out through a DPPH$^{\cdot}$ assay (Yamaguchi, 1998), a well-known method with high reproducibility (Espín et al., 2000). It was observed that all aqueous extracts showed significant antioxidant activity (Figure 2). The Bordo extract showed the best result, followed by the Cabernet Sauvignon and Merlot extracts. The Isabella extract, which showed the lowest levels of total phenolic content (Table 2), also showed the lowest antioxidant activity. A strong correlation (r^2 = 0.950, $p<0.01$) was observed between total phenolic content and *in vitro* antioxidant activity.

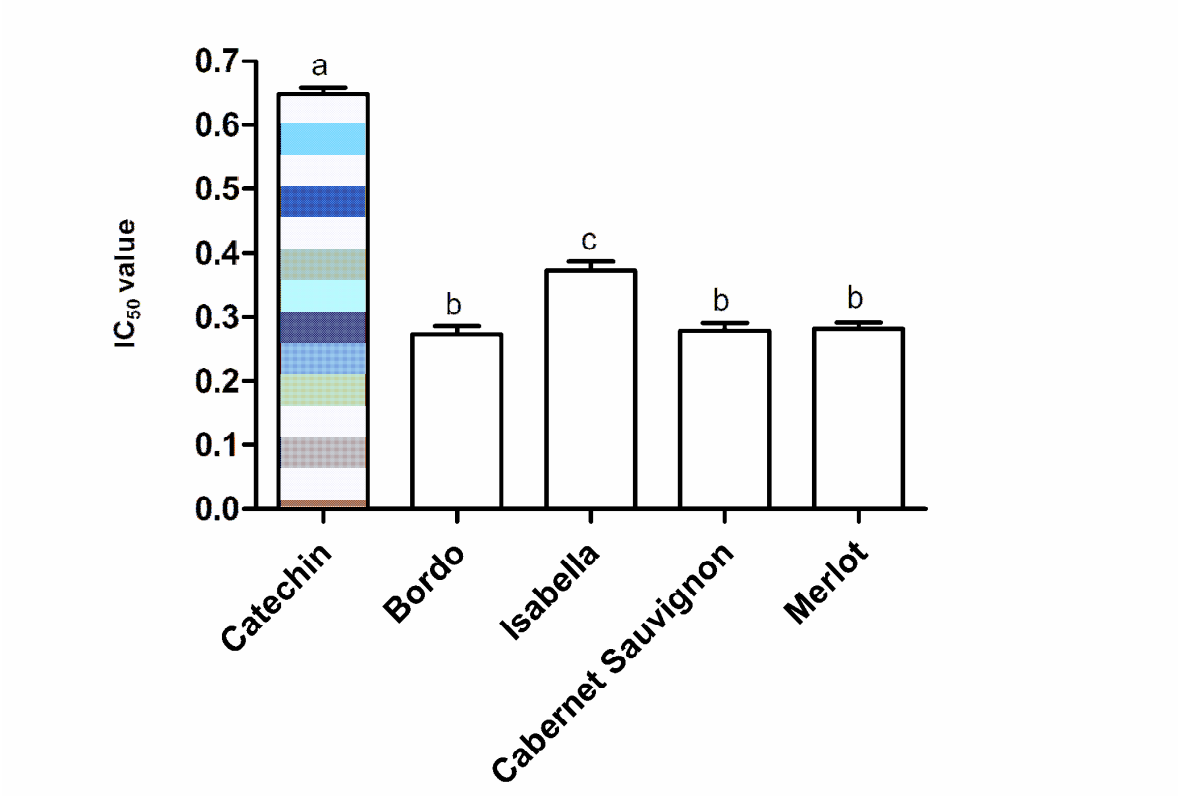


Fig. 2. *In vitro* antioxidant activity of aqueous grape seed extracts. IC_{50} (amount (%) of extracts needed to scavenge 50% of DPPH, *i.e.*, 125 μM). Control used was catechin. Different letters indicate significant differences ($p \leq 0.05$).

Until now, there have been no studies showing antioxidant activity in aqueous extracts of grape seeds from winery by-products. However, water/acetone (Fan and Lou, 2004), methanol/water (Yilmaz and Toledo, 2004), methanol/ethanol/water (Pinelo et al., 2005), ethyl acetate (Guendez et al., 2005) and ethanol (Makris et al., 2007) extracts have already shown *in vitro* antioxidant activity. Considering the low costs and the absence of possible organic solvent residues, the use of water as a solvent to obtain these extracts presents an advantage compared to traditional organic solvents.

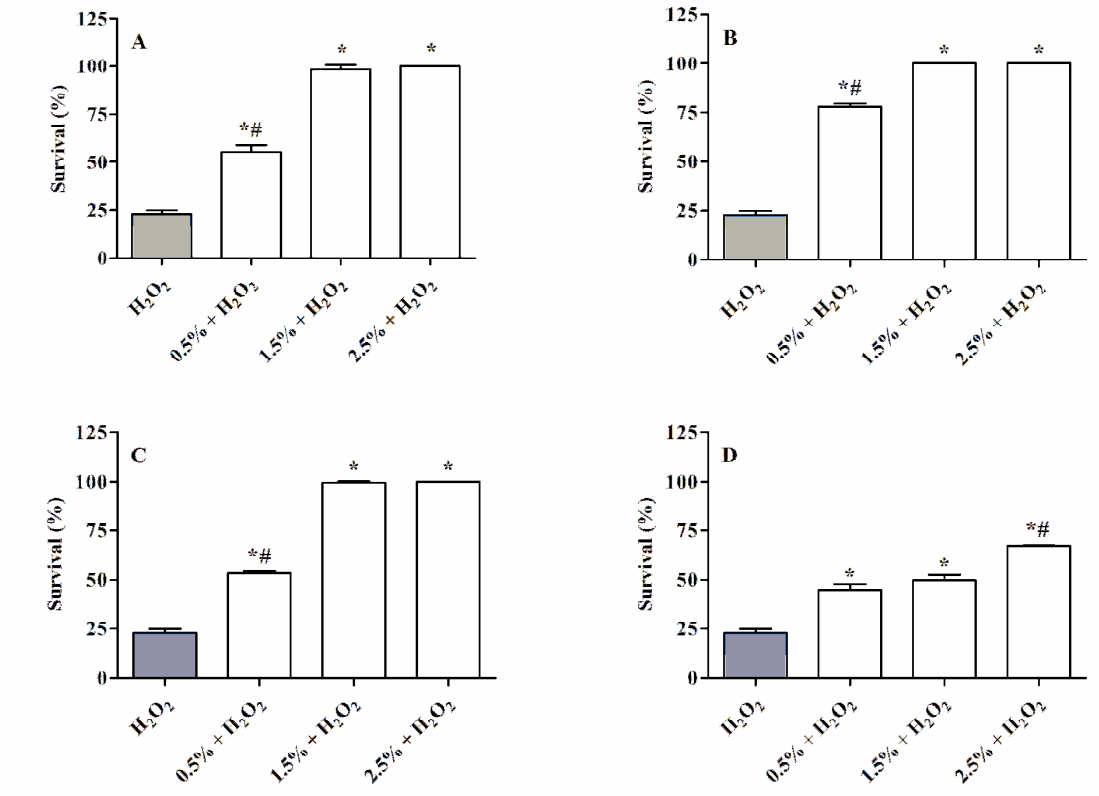


Fig. 3. Survival of *S. cerevisiae* in the presence of the different aqueous grape seed extracts and H_2O_2. (A) Bordo, (B) Isabella, (C) Cabernet Sauvignon, (D) Merlot. * significant difference from assays performed without extracts, # significant difference among different concentrations of extracts ($p>0.05$).

Measurements of *in vivo* antioxidant activity were carried out using eukaryotic cells of *Saccharomyces cerevisiae,* which have proved to be very useful in the identification of antioxidant activity (Spada and Salvador, 2005; Raspor et al., 2005). For this assay, the highest noncytotoxic concentration was chosen (2.5%, v/v) as well as 0.5% (v/v) and 1.5% (v/v) of each kind of GSE. The results (Figure 3) showed that all extracts present significant antioxidant activity, and are able to protect yeast cells against damages induced by H_2O_2. A positive correlation was observed between *in vivo* antioxidant activity and total phenolic content for each extract, as follows: Bordo (r^2 = 0.877, $p<0.05$), Isabella (r^2 = 0.847, $p<0.05$), Cabernet Sauvignon (r^2 = 0.867, $p<0.05$) and Merlot (r^2 = 0.935, $p<0.05$).

H_2O_2, a common product formed during normal cellular aerobic metabolism, and/or under certain pathophysiological conditions (Das and Maulik, 2003), is able to induce damages in proteins and lipids as well as in DNA, mainly by the generation of hydroxyl radicals via the Haber-Weiss/Fenton reaction (Fan and Lou, 2004; Spada and Salvador, 2005). The mechanisms of the antioxidant action of phenolic compounds are complex and are still being studied. In a general manner, they can avoid reactive species formation either by inhibition of enzymes or by chelation of trace elements involved in free radical production, they can scavenge reactive species, and they can upregulate or protect antioxidant defense (Halliwell and Gutteridge, 2007). Some compounds can also act similarly to enzymatic defenses, since they are able to neutralize reactive species such as superoxide anions and hydrogen peroxide (Silalahi, 2002). Epidemiological studies suggest that high green tea consumption in the Japanese population (Imai et al., 1997) and moderate wine consumption in the French population (Renaud and Lorgeril, 1992) may be beneficial for the prevention of cancer and heart diseases. Similarly, the extensive exposure of populations in Asia to flavonoids from a soya-based diet is

believed to be involved in the lowered incidence of estrogen-dependent breast and prostate cancer, and in the reduction of menopausal symptoms (Ferguson, 2001).

The data obtained in this study show that aqueous extracts rich in phenolic compounds, with significant antioxidant activity, can be obtained from seeds of winery by-products. These extracts could be used in the cosmetic and pharmaceutical industries and could help maintain environmental balance, reducing storage, transformation and disposal problems in the wine industry. The utilization of renewable sources and the design of processes based on the integral exploitation of natural products are important especially at this time, when there is continuous population growth and natural resources are limited.

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4.2 CAPÍTULO 2

Antioxidant and anti-inflammatory of *V. vinifera* and *V. labrusca* grape seed extracts

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ABSTRACT

Winery by-products are rich in phenolic compounds, known by their several health beneficial effects. The aim of this work was to evaluate the antioxidant, anti-inflammatory, and analgesic activities of aqueous extracts of seeds (GSE) from wine wastes of *Vitis labrusca* (cv. Bordo and Isabella) and *Vitis vinifera* (cv. Cabernet Sauvignon and Merlot). All extracts showed significant antioxidant activity, which was positively correlated with total phenolic content, suggesting that these compounds might be the major contributors to the antioxidant activity of these extracts. Grape seed extracts also showed an important reduction in lymphocyte influx to the inflammatory site. No analgesic activity was observed. These results show that it is possible to extract phenolic compounds with antioxidant and anti-inflammatory activities from winery by-products using water as a solvent, which would configure an important use for wine wastes and, thus, help maintain environmental balance.

Keywords: Antioxidant, anti-inflammatory, grape seed, *V. vinifera*, *V. Labrusca*.

INTRODUCTION

Efficient and environmentally rational utilization of agri-food industry wastes is of undisputed importance for higher profitability and minimal environmental impact. One of the higher value options is the recovery of bioactive plant food constituents, which could be used in the pharmaceutical, cosmetics, and food industries (*1*).

Worldwide wine production is around 149 million hectoliters per year (*2*) and it generates around 13 % of the weight of the processed grapes as by-products, which are generally used as fertilizer or simply discarded (*3*). Grape seeds are rich in phenolic compounds, known by its several health beneficial effects, such as antioxidant, anticarcinogen, cardiopreventive, antimicrobial, antiviral, and neuroprotective (*4*).

Grape seed extracts (GSE) obtained using organic solvents were already studied. (*1, 5-8*). However, these extracts have limited use due to the high cost of the extraction processes and the presence of solvent residues.

The aim of this work was to obtain an effective aqueous grape seed extract from winery by-products of *Vitis vinifera* (Cabernet Sauvignon and Merlot), the most commonly used grape in the world (*9*) and of *Vitis labrusca* (Bordo and Isabella), also used to produce wine and grape juice in South and North America (*10*). Assays of the antioxidant, anti-inflammatory, and analgesic activities of these extracts were conducted in *in vitro*, *ex vivo* and *in vivo* systems.

MATERIALS AND METHODS

Chemical Reagents

The 2,2-diphenyl-picrylhydrazyl ($DPPH^{\bullet}$), thiobarbituric acid (TBA), luminol (3-aminophthalhydrazide), *tert*-butyl hydroperoxide (*t*-BOOH), bovine serum albumin (BSA), carrageenan, and formalin were purchased from Sigma-Aldrich (St. Louis, MO).

The 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Aldrich Chemical (Milwaukee, WI). Acetic acid and glycine were purchased from Nuclear (Diadema, SP, Brazil). Trichloroacetic acid (TCA) and sodium carbonate were purchased from Synth (Diadema, SP, Brazil). The lactate dehydrogenase (LDH) commercial kit was purchased from Labtest, Brazil.

Plant material

The winery by-products of *V. labrusca* (cv. Bordo and Isabella) and *V. vinifera* (cv. Cabernet Sauvignon and Merlot) were removed from the vinification tanks five days after the beginning of fermentation, in January 2006. All varieties were cultivated in the northeastern region of the Serra Gaucha, Rio Grande do Sul, Brazil. Voucher specimens were identified by the herbarium of the University of Caxias do Sul, Rio Grande do Sul, Brazil (*V. labrusca* HUCS31065-31066 and *V. vinifera* HUCS32455-32456). Seeds were manually separated from the rest of the winery by-products, dried in an air oven at 37 ºC and stored at 25 ºC while sheltered from light.

Preparation of the extracts

Grape seeds were pounded in a knife (Quimis, Brazil) immediately before each assay. The extracts were made with 5 g seeds/100 mL distilled water (5% w/v), under reflux (100 °C), for 30 minutes. Extracts were cooled to 25 ºC and then filtered in Millipore equipment (pore size 0.45 µm; catalog number SFGS 047LS, Millipore Corp., Sao Paulo, Brazil). The extracts were freeze-dried (Edward) at -60 °C, 10^{-2} bar, and were stored at -20 °C. All grape seed extracts were solubilized in distilled water immediately before use. Total phenolic compounds and the major constituents of the extracts are shown in Table 1.

Table 1. Total polyphenol content (mg/L of catechin) and major compounds (mg/L) in aqueous grape seed extracts.

Variety	TPC	GA	B1	EGC	B3	CT	B4	B2	ECT
Bordo	732,19±1,44[a]	11.03±0.23[a]	16.58±0.16[a]	6.66±0.16[a]	13.46±0.87[a]	134.05±1.08[a]	2.24±0.14[ab]	15.27±0.04[a]	130.53±0.89[a]
Isabella	364,37±1,06[b]	4.21±0.10[b]	6.66±0.03[b]	4.42±0.02[b]	7.15±0.08[b]	103.35±0.16[b]	2.11±0.13[ab]	4.98±0.16[b]	85.70±0.05[b]
Cabernet Sauvignon	679,79±1,02[c]	6.72±0.27[c]	10.92±0.43[c]	2.16±0.16[c]	19.11±0.08[c]	56.92±0.10[c]	1.65±0.30[b]	5.76±0.30[c]	33.50±0.17[c]
Merlot	782,16±4,23[a]	11.78±0.38[a]	23.32±1.59[d]	6.80±0.76[a]	43.88±0.16[d]	96.68±0.87[d]	2.75±0.02[a]	12.04±0.02[d]	87.68±0.86[b]

Results represent average values ± S.D. TPC, total phenolic content; GA, gallic acid; B1, procyanidin B1; EGC, epigallocatechin; B3, procyanidin B3; CT, catechin; B4, procyanidin B4; B2, procyanidin B2; ECT, epicatechin. Different letters indicate significant differences ($p \leq 0.05$)

Animals

Three month Wistar rats (250–350 g) were obtained from our own breeding colony. They were caged in groups of five with free access to food and water and were maintained on a 12-h light–dark cycle (7:00–19:00 h) at 23±1°C. All experimental procedures were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1985) and were carried out according to the determinations of the Brazilian College of Animal Experimentation, COBEA.

Antioxidant activity

The *in vitro* antioxidant activity of the GSE was assayed by the 2,2-diphenyl-picrylhydrazyl radical ($DPPH^{\bullet}$) scavenging activity, the total reactive antioxidant potential (TRAP), and the total antioxidant reactivity (TAR). The $DPPH^{\bullet}$ scavenging activity was measured according to Yamaguchi et al. (*11*). GSE were added to Tris-HCl buffer (100 mM, pH 7.0) containing 250 μM $DPPH^{\cdot}$ dissolved in ethanol. The tubes were kept in the dark for 20 min and absorbance was measured at 517 nm (UV-1700 spectrophotometer, Shimadzu, Kyoto, Japan). Results were calculated as IC_{50} (amount of extract necessary to scavenge 50% of the $DPPH^{\cdot}$ radical). Catechin was used as a standard. The total reactive antioxidant potential (TRAP) and total antioxidant reactivity (TAR) assays were done as previously described (*12-14*). Briefly, the reaction mixture (4 mL, pH 8.6) containing AAPH (10 mM) and luminol (4 mM) in glycine buffer (0.1 M) was incubated at 21 °C for 2 h. AAPH is a source of peroxyl radicals that react with luminol yielding chemiluminescence (CL). The system was calibrated using the α-tocopherol hydrophilic analogue, Trolox. The addition of 10 μL of the extracts (final concentration 2.5 μg/mL) or of 10 μL of Trolox (final concentration 200 nM) decreases

the CL proportionally to its antioxidant potential. The TRAP profile was obtained measuring CL emission in a liquid scintillation counter (Wallac 1409)–operating in the "out of coincidence" mode–as counts per minute (CPM). CL intensity was monitored for 50 min after the addition of the extracts or Trolox. The results were expressed calculating the area under the curve (AUC) of the CL profile. TAR index was determined by measuring the initial decrease of luminol luminescence calculated as the ratio Io/I, where Io is the initial emission of CL (before the addition of the antioxidant) and I is the instantaneous CL intensity after addition of an aliquot of the sample or the reference compound (Trolox).

Antioxidant activity of GSE was also evaluated in an *ex vivo* assay using *t*-BOOH (*tert*-butyl hydroperoxide) as oxidative-stress inducer. Rat liver slices (400 μm) were preincubated with the extracts (final concentration 2.5 μg/mL) for 30 min at 37 °C under 95 % O_2/5 % CO_2 in a shaking water bath (60 oscillations/min) in a medium of oxygen-equilibrated Krebs-Ringer phosphate buffer (10 mM glucose, pH 7.4). After this incubation with the extracts, 0.5 mM *t*-BOOH was added to different samples of liver slices. After incubation, rat liver slices were removed and the medium was centrifuged at 12000 g for 10 min. The supernatant portion was used to measure lactate dehydrogenase activity. The rat liver slices were homogenized with phosphate buffer (pH 7.4) and used to determine lipid peroxidation, through a thiobarbituric acid-reactive species (TBARS) assay described by Draper and Hadley (*15*). First, 600 μL of 15 % trichloroacetic acid were added to 300 μL of the liver slice homogenates and centrifuged at 7000 g for 10 min. Then, 500 μL of supernatant were mixed with 500 μL of 0.67 % thiobarbituric acid. The reaction mixture was incubated in a boiling water bath for 20 min, cooled to room temperature and the absorbance read at 532 nm. The results were normalized by protein content and were expressed as malondialdehyde

(MDA) equivalents. Protein concentration was measured by Lowry et al. method, using bovine serum albumin as standard (*16*).

Anti-inflammatory and analgesic activities

Wistar rats (300-350g, n = 6) were treated intraperitoneally with saline or the different GSE (10 mg/kg) 30 minutes before the induction of pleurisy by injection of 0.2 mL of carrageenan 1% (*17*). Four hours after the induction of inflammation, animals were sacrificed and the pleural exudates from each animal were harvested by washing the pleural cavity with 2 mL of sterile saline solution for measuring lactate dehydrogenase activity, total protein, and total and differential cell count.

The total cells in the pleural exudate were enumerated for total leukocyte counts using Neubauer chamber. For cytology of inflammatory cells, the pleural exudate was centrifuged at 500 rpm for 5 minutes and the supernatant was analyzed compared with the cell morphology counted previously in the microscope (*18*). Protein concentration in pleural exudate was measured by Lowry et al. method, using bovine serum albumin as standard (*16*). Lactate dehydrogenase activity was performed using a commercial kit.

The analgesic activity of GSE was determined using the formalin method. Pain was induced by injecting 20 µL formalin 2.5% in the subplantar region of the right foreleg. The licking behavior on the region was observed for 5, 15, and 30 minutes after formalin injection (*19*).

Statistical analysis

All measurements were performed in triplicate and values were averaged and reported along with the standard deviation. The data were analyzed through Tukey´s test

and Pearson correlation using the SPSS 12.0 software package (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Antioxidant activity

In a DPPH$^{\cdot}$ assay (Figure 1A), which evaluates the ability of one compound to donate electrons to the stable radical DPPH$^{\cdot}$, all aqueous extracts showed antioxidant activity higher than the standard catechin. Merlot, which showed the highest levels of total phenolic content (Table 1), also showed the highest antioxidant activity. In fact, a positive correlation (r^2 = 0.626, $p<0.01$) between total phenolic content and DPPH$^{\bullet}$ antioxidant activity was observed.

The total reactive antioxidant potential (TRAP) was determined using a method based on the quenching of luminol-enhanced chemiluminescence derived from the thermolysis of AAPH, used as a reliable and quantifiable source of alkyl peroxyl radicals (*12-13*). All GSE (Figure 1B) showed the ability of reducing the luminol-enhanced chemiluminescence, indicating the presence of compounds with peroxyl scavenging properties.

The TAR index was obtained from the initial decrease in the luminescence associated with the additive incorporation of the sample to the ABAP–luminol system, and it indicates the initial reactivity of the sample when compared to Trolox. The results of TAR index (Figure 1C) indicated that the four kinds of GSE show antioxidant reactivity. This effect was higher in Merlot GSE. A strong correlation (r^2 = 0.920, $p<0.01$) between total phenolic content and TAR index was found, suggesting that the phenolic compounds might be the major contributors to the antioxidant activities of these extracts.

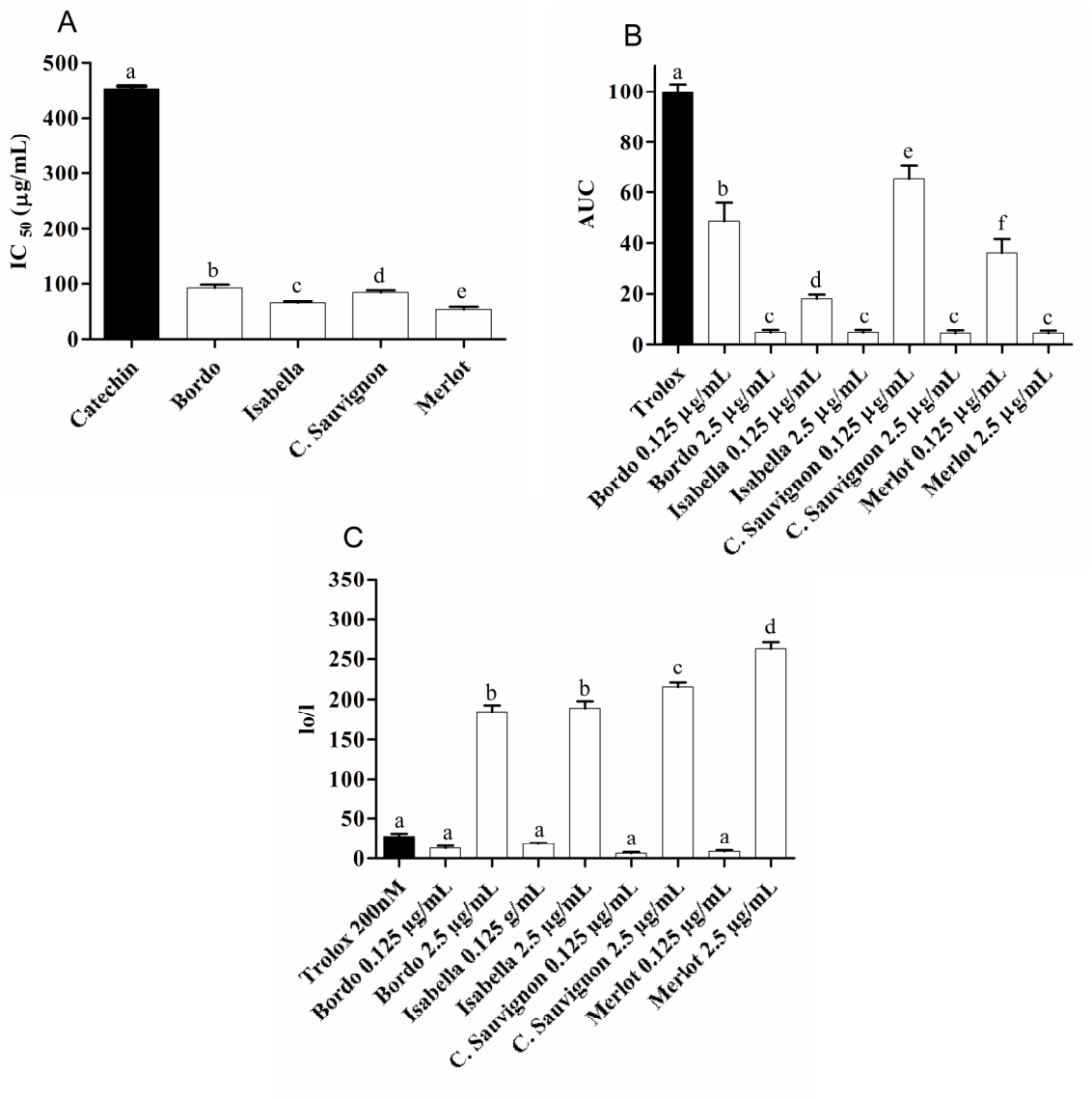

Figure 1. *In vitro* antioxidant activity of grape seed extracts**. (A)** IC_{50} (amount of extracts needed to scavenge 50% of DPPH, *i.e.*, 125 *µM*). **(B)** Percentual of CPM (calculated from area under the curve – AUC) measured after addition of 10 µL of Trolox (final concentration 200 nM) and the extracts (final concentration 0.125 and 2.5 µg/ml) to 4 mL of glycine buffer (0.1 M), pH 8.6, containing luminol (4 mM) and AAPH (10 mM) at 21 °C. **(C)** TAR index (Io/I where Io is the initial emission of CL, before the addition of the antioxidant, and I is the instantaneous CL intensity after addition of an aliquot of the sample or the reference compound, Trolox) of aqueous grape seed extracts. Catechin and Trolox were used as a control. Different letters indicate significant differences ($p \leq 0.05$).

In order to evaluate the *ex vivo* antioxidant effect of the four kinds of GSE at the lowest concentration found as significant in the TRAP assay, oxidative stress was induced in rat liver slices using 0.5 mM *tert*-butyl hydroperoxide (*t*-BOOH) in the incubation medium. The results showed (Figure 2) that incubation of rat liver slices in the presence of *t*-BOOH induced a significant increase ($p \leq 0.05$) in the MDA equivalents content (when compared to the basal liver homogenate) and LDH activity (data not shown). Interestingly, this effect was higher when GSE were added to *t*-BOOH. Treatments only with GSE did not increase the MDA equivalents (data not shown).

The organic hydroperoxide, *t*-BOOH induces oxidative stress by the acceleration of lipid peroxidation chain reactions and alterations of the intracellular calcium homeostasis resulting from glutathione and protein thiol depletion (*20*). Although the mechanism is not known, it was observed that U937 cells treated with *t*-BOOH and resveratrol showed an increase in DNA oxidative damage beyond that induced by t-BOOH itself (*21*). Further studies are needed to clarify the interaction between t-BOOH and some antioxidants.

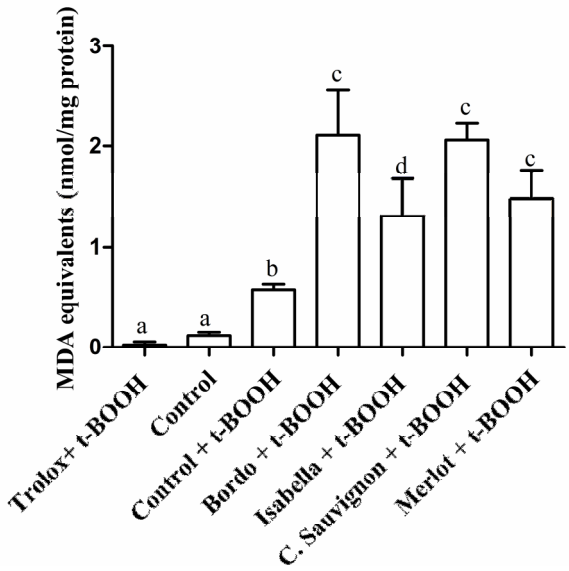

Figure 2. TBARS levels in rat slices treated with grape seed extract and *t*-BOOH. Results are expressed as means ± S.D. from two independent experiments. Different letters indicate significant differences ($p \leq 0.05$).

Anti-inflammatory and analgesic activities

The intraperitoneal injection of 0.2 mL of 1 % carrageenan into the pleural cavity of rats induced an inflammatory reaction characterized by exudates formation and cell migration when compared to the control group (Figure 3). Treatments with GSE showed no significant reduction of the total cell number (Figure 3A) or of polymorphonuclear migration (Figure 3B). However, an important reduction in the migration of lymphocytes to the inflammatory site (Figure 3C) was observed. Treatments with GSE showed no effects on TNF-alpha levels, LDH, and total proteins in the exudate (data not shown). There was also no reduction of licking behavior after formalin injection, suggesting absence of analgesic activities from GSE (data not shown).

Inflammatory diseases are accompanied by the chronic release of cytokines and reactive oxygen and nitrogen species, which may be involved in increased tissue injury (*22*). Some studies have demonstrated the participation of ROS in models of inflammation such as carrageenan-induced pleurisy in rats (*23-25*). In acute and chronic inflammation, high concentrations of ROS are produced (mainly O_2^-), which generate an oxidative imbalance and decrease the capacity of the endogenous antioxidant enzymes (*24*).

Plant-derived compounds have historically been valued as a source of anti-inflammatory agents (*26*). Flavonoids are powerful antioxidants and exert anti-inflammatory activities *in vitro* (*27*). Recent studies have shown important anti-inflammatory effects of procyanidins on experimental inflammation in rats and mice (*28-29*). However, until now, the anti-inflammatory action mechanisms of polyphenols remain poorly understood and could be associated to oxygen free radical scavenging, inhibition of lipid peroxidation, inhibition of the formation of inflammatory cytokines,

alterations in cell membrane receptors, intracellular signaling pathway proteins, and modulation of gene expression (*27*).

The data obtained in this study show that aqueous extracts rich in phenolic compounds, with significant antioxidant and anti-inflammatory activity, can be obtained from seeds of winery by-products. These extracts could be used in the cosmetic and pharmaceutical industries and help maintain environmental balance, reducing storage, transformation, and waste disposal problems in the wine industry.

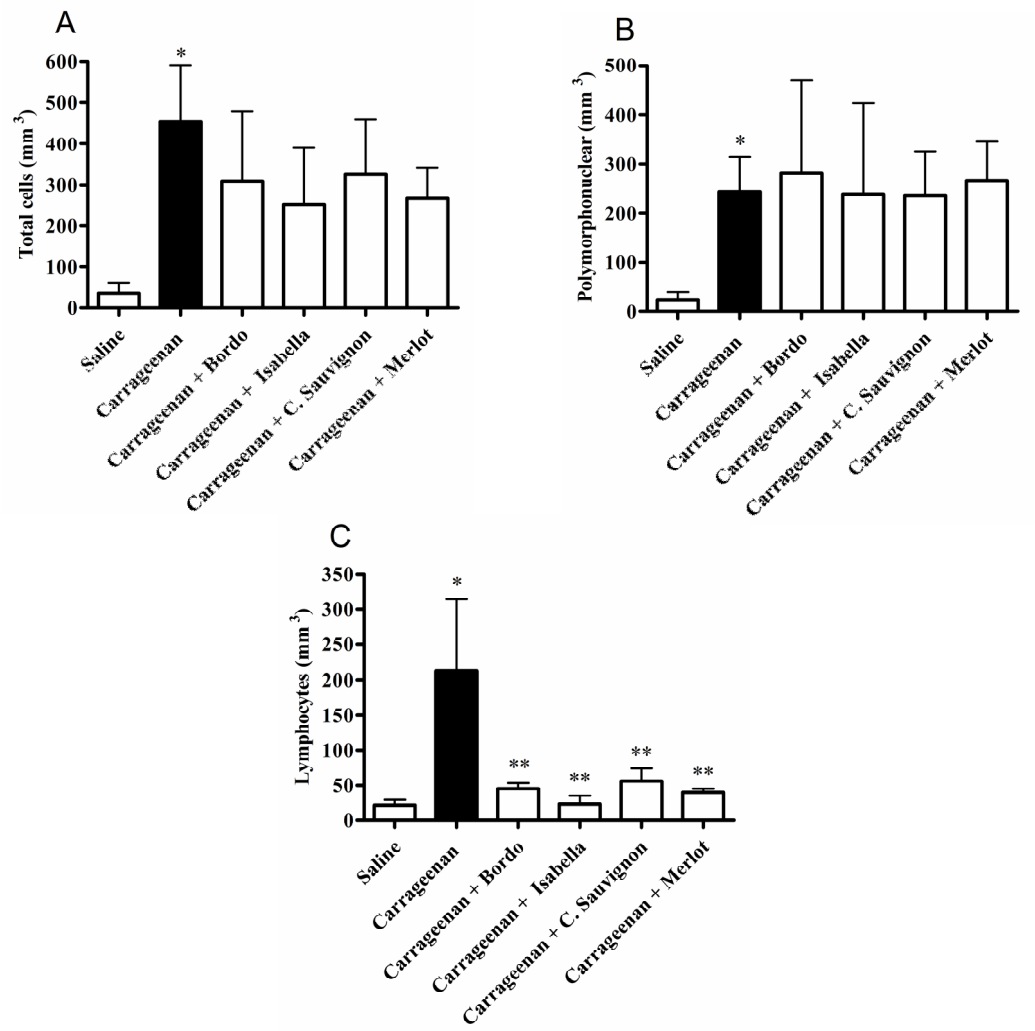

Figure 3. Effect of different aqueous extracts (10 mg/Kg body wt., i.p.) plus carrageenan on total cells migration (A), on polymorphonuclear migration (B), and on lymphocytes migration (C) into pleural cavity of rats. * Significant differences from saline ($p \leq 0.05$). ** Significant differences from carrageenan ($p \leq 0.05$).

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5. DISCUSSÃO GERAL

Estudos epidemiológicos têm mostrado que o consumo de polifenóis está associado com a redução de diversas doenças, incluindo câncer, doenças cardiovasculares e neurodegenerativas (para revisão, ver Ferguson, 2001; Ferguson e Philpott, 2008; Aron & Kennedy, 2008). Sementes de uva são ricas em polifenóis (Gabetta *et al.*, 2000; Saucier *et al*., 2001; Monagas *et al.*, 2003; Fuleki & Ricardo Silva, 2003; Ashraf-Khorassani & Taylor, 2004; Kammerer *et al.*, 2004; Koyama *et al*., 2007), especialmente os flavan-3-ols, (+)-catequina, (-)-epicatequina, (-)-epicatequina 3-O-galato, seus oligômeros e polímeros e ácido gálico (Gabetta *et al.*, 2000; Saucier *et al*., 2001; Monagas *et al.*, 2003; Ashraf-Khorassani & Taylor, 2004; Kammerer *et al.*, 2004; Koyama *et al*., 2007).

Tanto *V. vinifera* quanto *V. labrusca* têm sido tradicionalmente utilizadas para a obtenção de vinhos finos e de mesa, respectivamente. Em 2007, a produção brasileira de *V. vinifera* foi de 72.152 t (14.489,153 t de Cabernet Sauvignon e 11.399,362 t de Merlot) e de 498.383,917 t de *V. labrusca* (76.907,674 t da variedade Bordo e 228.558,808 t de Isabel) (Embrapa, 2008).

Parte considerável dessas uvas é destinada a elaboração de vinhos e sucos gerando grandes quantidades de resíduos (casca, sementes e galhos). Baseado nisto, esse trabalho teve como objetivo avaliar a possibilidade de obtenção de extratos aquosos de

sementes de resíduos de vinificação de *V. vinifera L.* (variedades Cabernet Sauvignon e Merlot) e *V. labrusca L.* (variedades Bordo e Isabel) com propriedades biológicas (atividade antioxidante, antiinflamatória e analgésica).

Os resíduos da vinificação foram fornecidos por uma indústria vinícola da Serra Gaúcha e retirados dos tanques de vinificação após 5 dias a partir do início do processo fermentativo, tempo normalmente utilizado para a separação das cascas e engaço do mosto de uva. Considerando a diversidade na composição dos resíduos obtidos (galhos, cascas e sementes), optou-se por separar manualmente as sementes do restante do resíduo. Testes preliminares mostraram, no entanto, que é possível obter-se extratos aquosos com atividade antioxidante a partir da totalidade do resíduo (dados não mostrados), o que pode ser uma opção econômica para a indústria vinícola.

Para determinação das melhores condições de extração foram preparados extratos a 5, 10 e 15% (p/v), por decocção com e sem condensador, durante 30 e 60 minutos, a uma temperatura de 100°C. A determinação da atividade antioxidante *in vitro* ($DPPH^{\bullet}$) foi utilizada como critério para avaliação dos resultados. Verificou-se que a maior atividade antioxidante foi obtida nos extratos preparados a 5% (p/v), a partir de decocção com condensador por 30 minutos (dados não mostrados). Desta forma, este método de extração foi utilizado para obtenção dos extratos utilizados neste trabalho.

A avaliação da atividade antioxidante foi realizada através de testes *in vitro* ($DPPH^{\bullet}$) e *in vivo* (células eucarióticas de *Saccharomyces cerevisiae*). Os quatro extratos estudados mostraram importante atividade antioxidante, tanto *in vitro* como *in vivo.* Observou-se uma correlação positiva entre a atividade antioxidante *in vitro* com o conteúdo de polifenóis totais ($r^2 = 0,950$, $p \leq 0,01$) e entre a atividade antioxidante *in vivo* e o conteúdo polifenólico total de cada extrato [Bordo ($r^2 = 0,877$, $p \leq 0,05$), Isabel ($r^2 =$

0,847, p≤0,05), Cabernet Sauvignon (r^2 = 0,867, p≤0,05) e Merlot (r^2 = 0,935, p≤0,05)], sugerindo que os polifenóis são responsáveis, ao menos em parte, pela atividade antioxidante observada como descrito para outros extratos de plantas ricos em polifenóis (Shan *et al*., 2005; Zainol *et al*., 2003; Silva *et al*., 2007; Kappel *et al*., 2008).

Considerando a instabilidade das soluções aquosas de polifenóis, os extratos obtidos a partir das 4 variedades de *Vitis* foram liofilizados a -60°C e pressão de 10^{-2} bar. A Tabela 2 apresenta os valores de compostos fenólicos nos extratos recém-preparados e após sua liofilização. Embora tenham sido observadas pequenas alterações no conteúdo de polifenóis totais antes e após a liofilização, estas diferenças não foram estatisticamente significativas pelo teste *t*, indicando que a liofilização não ocasionou perda de compostos fenólicos.

Tabela 2. Teores de polifenóis totais dos extratos recém preparados e liofilizados

Conteúdo de polifenóis totais (mg/L)		
Variedades	Extratos recém preparados	Extratos liofilizados
Bordo	744,89±3,13[a]	732,19±1,44[a]
Isabel	353,29±4,60[b]	364,37±1,06[b]
Cabernet Sauvignon	715,59±5,87[a]	679,79±1,02[c]
Merlot	751,38±5,30[a]	782,16±4,23[a]

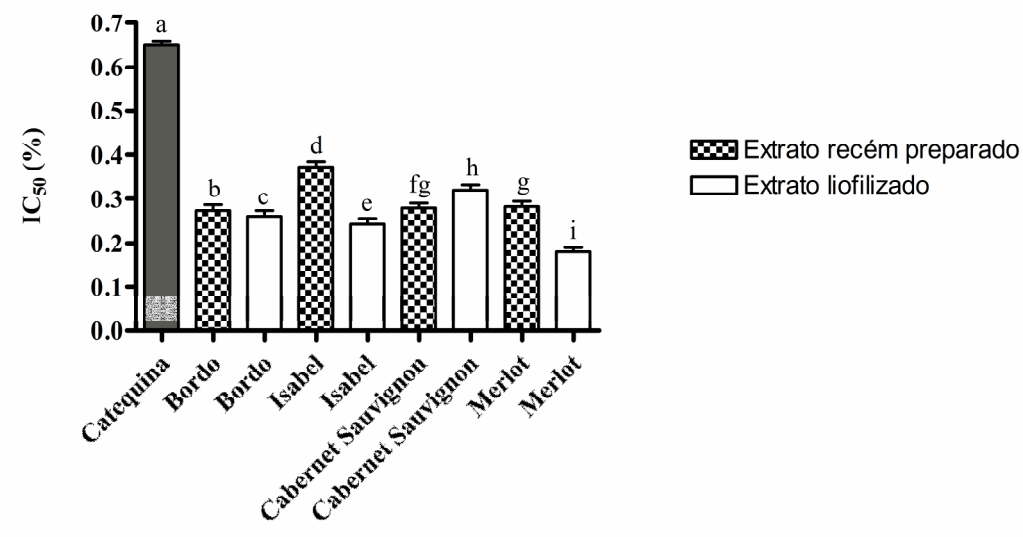
Letras distintas diferem significativamente, em cada grupo, pelo teste de Tukey, para p≤0,05.

A quantificação dos polifenóis majoritários nos extratos aquosos recém preparados e liofilizados foi realizada por cromatografia líquida de alta eficiência - CLAE. Foram identificados oito polifenóis majoritários, o ácido gálico, (+)-catequina,

(-)-epicatequina, (-)-epigalocatequina e as proantocianidinas B1, B2, B3 e B4, tanto nos extratos recém preparados quanto nos liofilizados (Tabela 3). Os extratos recém preparados apresentaram maiores quantidades de (+)-catequina, (-)-epicatequina, (-)-epigalocatequina, proantocianidinas B1 e B3, e ácido gálico, quando comparados com os respectivos extratos liofilizados. Apesar disso, o perfil de polifenóis foi o mesmo antes e após o processo de liofilização.

Curiosamente, com exceção da variedade Cabernet Sauvignon, os demais extratos liofilizados apresentaram maior atividade antioxidante do que os recém preparados, no ensaio da capacidade de varredura do radical livre DPPH$^{\bullet}$ (Figura 5). Considerando que a atividade antioxidante depende, entre outros fatores, do ensaio utilizado (Yamaguchi *et al*., 1998), seria interessante realizar outros testes a fim de confirmar estes resultados.

Figura 5. Atividade antioxidante *in vitro* dos extratos de sementes de resíduos de vinificação.



IC_{50} (quantidade necessária do extrato para seqüestrar 50% do radical DPPH$^{\bullet}$). *Letras distintas diferem significativamente pelo teste de Tukey, para $p \leq 0,05$.

Tabela 3. Compostos majoritários (mg/L) dos extratos recém preparados e liofilizados

Extratos	AG	B1	EGC	B3	CT	B4	B2	ECT
Bordo fresco	12,98±0,54$^{a\#*}$	22,42±0,51^{d}	8,96±0,05^{a}	17,45±0,01^{cd}	169,26±0,92^{a}	1,85±0,012^{ab}	19,75±0,17^{a}	168,86±2,82^{a}
Bordo liofilizado	11,03±0,23^{b}	16,58±0,16^{c}	6,66±0,16^{abc}	13,46±0,87^{bc}	134,05±0,16^{b}	2,24±0,14^{bc}	15,27±0,04^{ab}	130,53±0,89^{b}
Isabel fresco	6,88±0,04^{c}	8,86±0,03^{ab}	5,64±0,02^{bc}	9,72±0,01^{ab}	135,36±0,99^{b}	1,72±0,06^{ab}	3,17±3,64^{d}	112,40±0,32^{c}
Isabel liofilizado	4,21±0,00^{d}	6,66±0,03^{a}	4,42±0,02^{cd}	7,15±0,08^{a}	103,35±1,16^{c}	2,11±0,13^{ab}	4,98±0,16^{d}	85,70±0,05^{d}
Cabernet Sauvignon fresco	11,87±0,17^{ab}	26,54±1,86^{ef}	8,14±1,29^{ab}	29,53±2,70^{e}	106,73±0,34^{d}	2,89±0,02^{d}	15,23±0,08^{ab}	71,53±0,33^{e}
Cabernet Sauvignon liofilizado	6,72±0,27^{c}	10,92±0,43^{b}	2,16±0,16^{d}	19,11±0,08^{d}	56,92±0,00^{e}	1,65±0,30^{a}	5,76±0,30^{d}	33,50±0,17^{f}
Merlot fresco	16,42±1,15^{e}	27,80±0,82^{g}	7,49±0,97^{ab}	47,16±0,45^{f}	109,57±0,20^{f}	2,87±0,19^{d}	13,73±0,17^{c}	111,08±0,05^{c}
Merlot liofilizado	11,78±0,38^{ab}	23,32±1,59^{de}	6,80±0,76^{abc}	43,88±0,16^{f}	96,68±0,87^{g}	2,75±0,02^{cd}	12,04±0,02^{c}	87,68±0,86^{d}

AG, ácido gálico, B1, procianidina B1, EGC, epigalocatequina, B3, procianidina B3, CT, catequina, B4, procianidina B4, B2, procianidina B2, ECT, epicatequina. #DP=desvio padrão. *Letras distintas diferem significativamente pelo teste Tukey para p≤0,05.

A procura por novas drogas e/ou extratos de plantas que efetivamente interfiram no processo inflamatório tem ganhado destaque (Calixto et al., 2003; Passos *et al*., 2007). O modelo mais utilizado para avaliação deste efeito é a indução de resposta inflamatória por carragenina em ratos. Neste tipo de ensaio, observa-se liberação de histamina, serotonina e bradiquinina (Passos, *et al*., 2007) e aumento na produção de citocinas pró-inflamatórias, particularmente IL-1β e TNFα (Pinheiro e Calixto, 2002). Além destes mediadores, a resposta inflamatória induzida por carragenina aumenta a produção de prostaglandinas, ativando as ciclooxigenase COX-1 e COX-2 e elevando a concentração de óxido nítrico (Simmons *et al*., 2004; Passos, *et al*., 2007). Apesar de ter sido observada uma diminuição na migração dos linfócitos para o sítio inflamatório, em ratos tratados com carragenina em presença dos diferentes extratos (Capítulo 2), não foram visualizadas diferenças nos níveis de TNFα no líquido pleural dos animais. Outros estudos são necessários para confirmar a possível atividade antiinflamatória dos extratos estudados neste trabalho.

O processo inflamatório é caracterizado por calor, rubor, tumor e dor (Santos Júnior, 2003), sendo que alguns compostos antioxidantes extraídos de plantas mostraram-se capazes de diminuir a dor (Thabrew *et al*., 2001). Em vista disso, avaliou-se a ação analgésica dos diferentes extratos em ratos tratados com formalina (Hunskar & Hole, 1987). Neste modelo, o comportamento de lamber a região aonde foi injetado a formalina é um reflexo da ativação das fibras nervosas sensoriais (Uchida *et al*., 2008). Ao contrário do esperado, os extratos de sementes de resíduos de vinificação não foram capazes de reduzir o comportamento de lamber, sugerindo ausência de poder analgésico (dado não mostrado).

Em resumo, este trabalho mostra, pela primeira vez, que é possível preparar extratos aquosos de sementes de resíduos de vinificação com importante atividade biológica. A ausência de solventes e custo reduzido (em relação a extratos obtidos com

solventes orgânicos) pode facilitar o aproveitamento dos resíduos das vinícolas, transformando-os em produtos passíveis de utilização na indústria cosmética, farmacêutica e de alimentos. Estes resultados podem contribuir, ainda, para o aumento do rendimento econômico do setor vitivinícola e a manutenção do equilíbrio ambiental, diminuindo problemas de estocagem, transformação ou eliminação destes resíduos pelas vinícolas.

6. CONCLUSÕES

Os dados obtidos neste estudo permitem concluir que:

6.1 É possível obter-se extratos aquosos de sementes de resíduos de vinificação (cv. Bordo, Isabel, Cabernet Sauvignon e Merlot) com quantidades significativas de polifenóis. Os compostos majoritários encontrados nestes extratos foram o ácido gálico, (+)-catequina, (-)-epicatequina, (-)-epigalocatequina e as procianidinas B1, B2, B3 e B4.

6.2 Todos os extratos apresentaram significativa atividade antioxidante *in vitro* ($DPPH^{\bullet}$, TRAP e TAR) e *in vivo* (células eucarióticas de *Saccharomyces cerevisiae*). Observou-se uma correlação positiva entre o teor de polifenóis e a atividade antioxidante, tanto nos ensaios *in vitro* quanto *in vivo*.

6.3 Observou-se uma diminuição da migração linfocitária no exudato pleural de ratos tratados com carragenina em presença dos 4 extratos de resíduos de vinificação. Nenhum dos extratos mostrou atividade analgésica em ratos tratados com formalina.

7. PERSPECTIVAS

7.1 Avaliar a atividade antioxidante, anticonvulsivante e comportamental dos extratos de sementes de resíduos em tecidos de ratos Wistar.

7.2 Determinar a possível atividade antimutagênica, antigenotóxica e quimiopreventiva dos extratos em ratos Wistar e linhagens celulares de mamíferos.

7.3 Estudar o mecanismo responsável pela modulação inflamatória observada, avaliando a atividade e expressão de COX-1 e COX-2, interleucinas, nitritos/nitratos e das enzimas antioxidantes em ratos.

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9. ADENDOS COMPLEMENTARES

Gustavo Scola
Curriculum Vitae

Dados Pessoais

Nome Gustavo Scola
Nascimento 29/09/1980 - Caxias do Sul/RS - Brasil
CPF 81169248004

Formação Acadêmica/Titulação

2006 - Mestrado em Biotecnologia.
Universidade de Caxias do Sul, UCS, Caxias Do Sul, Brasil
Título: Avaliação da atividade antioxidante, antiinflamatória e analgésica de resíduos de vinificação
Orientador: Mirian Salvador
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

2000 - 2005 Graduação em Fisioterapia.
Centro Universitário Feevale, FEEVALE, Brasil
Título: O atendimento fisioterapêutico no estresse oxidativo de pacientes com comprometimento neurológico grave internados em uma Unidade de Terapia Intensiva em um Hospital do Vale dos Sinos
Orientador: Cristiane Saraiva

Formação complementar

2002 - 2002 Extensão universitária em Crochetagem mio-aponeurótica.
Centro Universitário Feevale, FEEVALE, Novo Hamburgo, Brasil

2003 - 2003 Curso de curta duração em Osteopatia aplicada nas algias da coluna.
JOPEF Fisioterapia, JOPEF, Brasil

2003 - 2003 Curso de curta duração em Abordagens hidrocinesiterapêuticas no paciente.
Centro Universitário Feevale, FEEVALE, Novo Hamburgo, Brasil

2003 - 2003 Curso de curta duração em I Jornada das disfunções motoras-espasticidade.
Centro Universitário Feevale, FEEVALE, Novo Hamburgo, Brasil

2003 - 2003 Curso de curta duração em Integração Sensório Motora.
Centro Universitário Feevale, FEEVALE, Novo Hamburgo, Brasil

2003 - 2003 Curso de curta duração em Hidrocinesioterapia na reabilitação neurológica.
JOPEF Fisioterapia, JOPEF, Brasil

2003 - 2003 Curso de curta duração em Método Kabat.
JOPEF Fisioterapia, JOPEF, Brasil

2004 - 2004 Estágio voluntário de fisioterapia.

Centro de Saúde Escola Murialdo, CSEM, Brasil

2005 - 2005 Curso de curta duração em physiotherapy, manual therapy and medical training.
Praxis für Physiotherapie Melaine Bröckelmann, BRÖCKELMANN, Alemanha

2005 - 2005 Estágio voluntário de fisioterapia Hospitalar/UTI.
Centro Universitário Feevale, FEEVALE, Novo Hamburgo, Brasil

2005 - 2005 Curso de curta duração em Surgery, orthopaedics and neurology physiotherapy.
Praxis für Physiotherapie Frithjof Rothacker, FRITJOF ROTHACKE, Alemanha

Atuação profissional

1. Universidade de Caxias do Sul - UCS

Vínculo institucional

2006 - Vínculo: Mestrando , Enquadramento funcional: Aluno de Mestrado , Carga horária: 40, Regime: Dedicação Exclusiva

Atividades

10/2006 - Atual Pesquisa e Desenvolvimento, Centro de Ciências Biológicas e da Saúde, Instituto de Biotecnologia
Linhas de Pesquisa:
Estresse Oxidativo e Antioxidantes

10/2006 - Atual Projetos de pesquisa, Centro de Ciências Biológicas e da Saúde, Instituto de Biotecnologia
Participação em projetos:
Avaliação da atividade antioxidante, antiinflamatória e analgésica de sementes de resíduos de vinificação

2. Universidade Federal do Rio Grande do Sul - UFRGS

Vínculo institucional

2005 - 2005 Vínculo: Voluntário de Iniciação Cientí , Enquadramento funcional: Voluntário de Iniciação Científica , Carga horária: 20, Regime: Parcial

2003 - 2004 Vínculo: Bolsista FAPERGS , Enquadramento funcional: Bolsista de Iniciação Científica , Carga horária: 20, Regime: Parcial

2000 - 2002 Vínculo: Bolsa FAPERGS , Enquadramento funcional: Bolsista de Iniciação Científica , Carga horária: 20, Regime: Parcial

Atividades

07/2000 - 08/2005 Projetos de pesquisa, Instituto de Biociências, Departamento de Fisiologia

Participação em projetos:
Atividade da ATPase de S-conjugados de glutationa (GS-X/MRP) e da expressão de proteínas de choque térmico em linfócitos: perspectivas terapêuticas. , Metabolismo do colesterol em monócitos de ratos hipertensos: , Tratamento de animais portadores de aterosclerose com prostaglandinas ciclopentenônicas (CP-PGs) , Fisiologia do fenômeno de transferência intercelular de lípides , Estresse celular no sistema cardiovascular

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Linhas de pesquisa

1. Estresse celular no sistema cardiovascular

2. Exercício Físico aplicado a pacientes HIV/AIDS

3. Fisiologia da ATPase de S-conjugados de glutationa (bomba GS-X/MRP) no câncer

4. Metabolismo lipídico na aterosclerose

5. Prostaglandinas ciclopentenônicas no sistema cardiovascular: potencial terapêutico por citoproteção e redirecionamento do metabolismo lipídico

6. Estresse Oxidativo e Antioxidantes

Objetivos:Estudar os efeitos biológicos causados pelas espécies reativas em células eucarióticas e analisar a proteção conferida por diferentes antioxidantes a estes danos.

Projetos

2006 - 2008 Avaliação da atividade antioxidante, antiinflamatória e analgésica de sementes de resíduos de vinificação

Descrição: Perspectivas terapêuticas sobre o aproveitamento dos resíduos de vinificação, transformando-os em produtos passíveis de utilização na indústria farmacêutica e de alimentos. Os resultados deste estudo podem contribuir, ainda, para o aumento do rendimento econômico do setor vitivinícola e a manutenção do equilíbrio ambiental.
Situação: Concluído Natureza: Pesquisa
Alunos envolvidos: Graduação (1); Especialização (0); Mestrado acadêmico (1); Mestrado profissionalizante (0); Doutorado (0);
Integrantes: Gustavo ScolaMirian Salvador (Responsável)
Financiador(es): Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq, Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul-FAPERGS
Número de produções C,T & A: 4/

2000 - 2005 Metabolismo do colesterol em monócitos de ratos hipertensos:

Descrição: Efeito da angiotensina II sobre o metabolismo lipídico e apoptose de monócitos.
Situação: Concluído Natureza: Pesquisa
Alunos envolvidos: Graduação (4); Especialização (0); Mestrado acadêmico (0); Mestrado profissionalizante (0); Doutorado (1);
Integrantes: Gustavo ScolaAlexandre Maslinkiewicz; Denise Lagranha; Paulo Ivo Homem de Bittencourt Jr (Responsável); Lisiane Baldissera
Financiador(es): Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP, Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq, Fundação de Amparo à Pesquisa

do Estado do Rio Grande do Sul-FAPERGS
Número de produções C,T & A: 1/

2000 - 2005 Fisiologia do fenômeno de transferência intercelular de lípides

Descrição: Implicações da transferência intercelular de lípides na fisiopatologia da aterosclerose, diabetes, hipertensão arterial, envelhecimento e câncer.
Situação: Concluído Natureza: Pesquisa
Alunos envolvidos: Graduação (8); Especialização (0); Mestrado acadêmico (1); Mestrado profissionalizante (0); Doutorado (4);
Integrantes: Gustavo ScolaAlexandre Maslinkiewicz; Lisiane Paula Baldissera; Lavínia Almeida Cruz; Angela kolberg; Denise Jacques Lagranha; Paulo Ivo Homem de Bittencourt Jr (Responsável); Minéia Taise Puhl; Daiane da Rocha Janner; João Bonatto Costa; Karen Lacerda Pires; Lino Pinto de Oliveira; Lucila Ludmila Paula Gutierrez; Luis Felipe Dupont da Silva
Financiador(es): Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul-FAPERGS

2000 - 2005 Estresse celular no sistema cardiovascular

Descrição: Estresse celular no sistema cardiovascular: estudo da produção de radicais livres, espécies ativas do oxigênio, proteínas de choque térmico e suas implicações metabólicas durante o processo de isquemia/reperfusão, hipertensão arterial e aterosclerose.
Situação: Concluído Natureza: Pesquisa
Alunos envolvidos: Graduação (6); Especialização (0); Mestrado acadêmico (1); Mestrado profissionalizante (0); Doutorado (2);
Integrantes: Gustavo ScolaAlexandre Maslinkiewicz; Rui Curi; Lisiane Paula Baldissera; Lavínia Almeida Cruz; Angela kolberg; Carolina Kolberg; Denise Jacques Lagranha; Paulo Ivo Homem de Bittencourt Jr (Responsável); Minéia Taise Puhl; Daiane da Rocha Janner; Augusto Reinaldo pimentel Guimarães; João Bonatto Costa
Financiador(es): Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul-FAPERGS

2000 - 2005 Atividade da ATPase de S-conjugados de glutationa (GS-X/MRP) e da expressão de proteínas de choque térmico em linfócitos: perspectivas terapêuticas.

Descrição: Desenvolvimento de protocolo para prognóstico de imunossupressão no câncer pelo estudo da capacidade de exportação de prostaglandinas ciclopentenônicas através da ATPase bomba GS-X/MRP e da expressão de proteínas de choque térmico em linfócitos: perspectivas terapêuticas.
Situação: Concluído Natureza: Pesquisa
Alunos envolvidos: Graduação (5); Especialização (0); Mestrado acadêmico (0); Mestrado profissionalizante (0); Doutorado (1);
Integrantes: Gustavo ScolaTatiana Gomes Rosa; Paulo Ivo Homem de Bittencourt Jr (Responsável); Minéia Taise Puhl; Juliane da Silva Rossato; Bibiana Sgorla de Almeida
Financiador(es): Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq, Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP, Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul-FAPERGS
Número de produções C,T & A: 1/

2000 - 2005 Tratamento de animais portadores de aterosclerose com prostaglandinas ciclopentenônicas (CP-PGs)

Descrição: Estudos de nosso grupo mostraram que prostaglandinas ciclopentenônicas (CP-PGs) desviam o metabolismo lipídico de macrófagos inflamatórios e macrófagos-foam cells no sentido da síntese de fosfolípides, em detrimento da síntese de colesterol e ésteres de colesterol. Isso levou ao desenvolvimento de uma terapia à base de CP-PGs direcionadas especificamente ao endotélio vascular doente que leva à redução total do acúmulo de lípides nos vasos de camundongos ateroscleróticos.
Situação: Em Andamento Natureza: Pesquisa
Alunos envolvidos: Graduação (4); Especialização (0); Mestrado acadêmico (0); Mestrado profissionalizante (0); Doutorado (1);
Integrantes: Gustavo ScolaAlexandre Maslinkiewicz; Denise Lagranha; Paulo Ivo Homem de Bittencourt Jr (Responsável); Lisiane Baldissera
Financiador(es): Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP, Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq, Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul-FAPERGS

Número de produções C,T & A: 1/

Produção em C, T& A

Produção bibliográfica
Artigos completos publicados em periódicos

1. Kappel, VD, Costa, Geison M., SCOLA, G., Silva, Francilene A., Landell, Melissa F., Valente, Patr?cia, Souza, Daiana G., Vanz, Danielli C., Reginatto, Flávio H., Moreira, José C.F.
Phenolic Content and Antioxidant and Antimicrobial Properties of Fruits of L. var. at Different Maturity Stages. Journal of Medicinal Food. , v.11, p.267 - 274, 2008.

2. HOMEMDEBITTENCOURTJR, P, LAGRANHA, D, MASLINKIEWICZ, A, SENNA, S, TAVARES, A, BALDISSERA, L, JANNER, D, PERALTA, J, BOCK, P, GUTIERREZ, L, Scola, Gustavo
LipoCardium: Endothelium-directed cyclopentenone prostaglandin-based liposome formulation that completely reverses atherosclerotic lesions. Atherosclerosis (Amsterdam). , v.193, p.245 - 258, 2007.

3. Kolberg, Angela, ROSA, Tatiana Gomes, Puhl, Minéia Taíse, Scola, Gustavo, JANNER, Daiane da Rocha, MASLINKIEWICZ, Alexandre, LAGRANHA, Denise Jacques, HECK, Thiago Gomes, CURI, Rui, de Bittencourt, Paulo Ivo Homem
Low expression ofMRP1/GS-X pump ATPase in lymphocytes of Walker 256 tumour-bearing rats is associated with cyclopentenone prostaglandin accumulation and cancer immunodeficiency. Cell Biochemistry and Function. , v.24, p.23 - 39, 2006.

Trabalhos publicados em anais de eventos (completo)

1. SCOLA, G, PUHL, Minéia Taise, KOLBERG, Angela, KOLBERG, Carolina, ROSA, Tatiana, BALDISSERA, Lisiane Paula, CURI, Rui
Atividade da ATPase de S-conjugados de Glutationa (bomba GS - X/MRP) em linfócitos e a sensibilidade a prostaglandinas antiproliferativas no câncer In: XVI Salão de Iniciação Científica, 2002, Porto Alegre.

2. SCOLA, G
Efeitos da combinação entre alcoolismo e tabagismo sobre o metabolismo antioxidante de ratos In: XIV Salão de Iniciação Científica, 2002, Porto Alegre.

3. SCOLA, G, TAVARES, Angela Maria Vicente, JANNER, Daiane, BALDISSERA, Lisiane Paula, BITTENCOURT JUNIOR, Paulo Ivo Homem de, CURI, Rui
Influência das prostaglandinas no acúmulo e exportação de lípides em macrófagos na aterosclerose In: V Mostra de Iniciação Científica da Ulbra, 2002, Cachoeria do Sul.

4. SCOLA, G, BALDISSERA, Lisiane Paula, CURI, Rui
Metabolismo do colesterol em monócitos de ratos hipertensos: imlicações para o desenvolvimento da aterosclerose In: XVI Salão de Iniciação Científica, 2002, Porto Alegre.

5. SCOLA, G
Metabolismo do Colesterol em monócitos de ratos hipertensos: Implicações para o desenvolvimento da Aterosclerose In: Feira de Iniciação Científica, 2002, Novo Hamburgo.

6. SCOLA, G, BALDISSERA, Lisiane Paula, CURI, Rui
Tratamento de animais portadores de aterosclerose com prostaglandinas ciclopentenônicas In: XVI Salão de Iniciação Científica, 2002, Porto Alegre.

7. SCOLA, G
Tratamento de animais portadores de aterosclerose com prostaglandinas ciclopentenônicas In: Feira de Iniciação Científica, 2002, Novo Hamburgo.

8. SCOLA, G, BALDISSERA, Lisiane Paula, PUHL, M. T., ROSA, Tatiana Gomes, KOLBERG, Angela, KOLBERG, Carolina, MASLINKIEWICZ, Alexandre, LAGRANHA, Denise Jacques, SENNA, Sueli Moreno, HOMEM de BITENCOURT JR, P. I., CURI, Rui
Atividade da ATPase de S- conjugados de glutationa (bomba GS - X/MRP) em linfócitos e a sensibilidade a prostaglandinas antiproliferativas no câncer In: 21 Semana Científica do HCPA, 2001, Porto Alegre.

9. SCOLA, G, PUHL, M. T., KOLBERG, Angela, KOLBERG, Carolina, ROSA, Tatiana Gomes, BALDISSERA, Lisiane Paula, LAGRANHA, Denise Jacques, MASLINKIEWICZ, Alexandre, SENNA, Sueli Moreno, CURI, Rui, Homem de Bittencourt Jr Paulo Ivo Homem de
Atividade da ATPase de S- conjugados de glutationa (BOMBA GS -X MRP) em linfócitos e a sensibilidade a prostaglandinas antiproliferativas no câncer In: XIII Salão de Iniciação Científica, 2002, Porto Alegre.

10. SCOLA, G, BALDISSERA, Lisiane Paula, LAGRANHA, Denise Jacques, MASLINKIEWICZ, Alexandre, PUHL, M. T., ROSA, T. G., SENNA, Sueli Moreno, CURI, Rui, HOMEM de BITENCOURT JR, P. I., KOLBERG, Carolina, KOLBERG, Angela
Atividade da ATPase de S- conjugados de glutationa (Bomba GS-X/MRP) em linfócitos e a sensibilidade a prostaglandinas antiproliferativas no câncer In: II Salão de Iniciação Científica, 2001, Guaíba.

11. SCOLA, G, BALDISSERA, Lisiane Paula, LAGRANHA, Denise, MASLINKIEWICZ, Alexandre, CRUZ, Lavínia Almeida, SENNA, Sueli Moreno, CURI, Rui, HOMEM de BITENCOURT JR, P. I.
Metabolismo do colesterol em monócitos circulantes de ratos Hipertensos: implicações para o desenvolvimento da aterosclerose In: 21 Semana Científica do HCPA, 2001, Porto Alegre.

12. SCOLA, G, BALDISSERA, Lisiane Paula, MASLINKIEWICZ, Alexandre, LAGRANHA, Denise Jacques, CRUZ, Lavínia Almeida, SENNA, Sueli Moreno, CURI, Rui, Homem de Bittencourt Jr Paulo Ivo Homem de
Metabolismo do colesterol em monócitos de ratos hipertensos: implicaçoes para o desenvolvimento da aterosclerose In: XIII Salão de Iniciação Científica, 2001, Porto Alegre.

13. SCOLA, G, BALDISSERA, Lisiane Paula, LAGRANHA, Denise, MASLINKIEWICZ, Alexandre, CRUZ, L., SENNA, S., CURI, Rui, Homem de Bittencourt Jr Paulo Ivo Homem de
Metabolismo do colesterol em monócitos de ratos hipertensos: implicações para o desenvolvimento da aterosclerose In: II Salão de Iniciação Científica, 2001, Porto Alegre..

14. SCOLA, G, HOMEM de BITTENCOURT JR, P. I.., BALDISSERA, Lisiane P, LAGRANHA, D. J., MASLINKIEWICZ, Alexandre, CRUZ, L. A., SENNA, S. M., CURI, Rui
Metabolismo do colesterol em monócitos de ratos hipertensos: implicações para o desenvolvimento da aterosclerose. In: II Salão de Iniciação Científica, 2001, Porto Alegre.

15. SCOLA, G, BALDISSERA, Lisiane Paula, LAGRANHA, Denise Jacques, MASLINKIEWICZ, Alexandre, SENNA, Sueli Moreno, CURI, Rui, Homem de Bittencourt Jr Paulo Ivo Homem de
Tratamento de animais portadores de aterosclerose com prostaglandinas ciclopentenônicas In: X Feira de Iniciação científica, 2001, Porto Alegre.

16. SCOLA, G, BALDISSERA, Lisiane Paula, LAGRANHA, Denise, MASLINKIEWICZ, Alexandre, SENNA, Sueli Moreno, CURI, Rui, HOMEM de BITENCOURT JR, P. I.
Tratamento de animais portadores de aterosclerose com prostaglandinas ciclopentenônicas In: II Salão de Iniciação Científica, 2001, Guaíba.

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Biological activities and main compounds of fruits

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Abstract

Many studies have shown that the consumption of fruits and vegetables is associated with a reduced risk of many diseases, including cancer, atherosclerosis, and neurovegetative diseases, which are related to elevated levels of oxidative stress. Antioxidant compounds can decrease oxidative stress, minimizing the incidence of these diseases. Fruits supply several antioxidant compounds, as for example vitamin C, carotenes, and/or polyphenols. On the other hand, some compounds present in fruits have themselves been identified as being mutagenic. This chapter reviews the major compounds and their corresponding biological activities of 23 fruits commonly consumed in the world.

Keywords: fruits, main compounds, biological activities.

In the last years, there has been a growing interest in nutraceuticals and functional foods. Plants, including food plants (fruits and vegetables), synthesize a vast array of secondary chemical compounds that, although not involved in primary metabolism, are important for a variety of ecologic functions that enhance the plant's ability to survive. Interestingly, these compounds may be responsible for the multitude of beneficial effects that have been reported for fruits with an array of health-related bioactivities (Joseph *et al*., 2005). Many studies (Joseph *et al*., 1999; Joseph *et al*., 1998; Prior *et al*., 1998; Cao *et al*., 1996; Wang *et al*., 1996) have suggested that the most important benefits of such compounds may be derived from their antioxidant, antimutagenic, anticarcinogenic, and anti-inflammatory properties.

Fruits present a large spectrum of constituents. Besides carbohydrates, lipids, and proteins (for review see Spada *et al*., 2008), carotenoids, vitamins and polyphenols are the most widely and best-studied compounds of fruits (Table 1).

Many fruits present high levels of carotenoids, for example acerola, mango, papaya and Surinam cherry (Table 1). About fifty to sixty different carotenoids are typically present in the human diet, and the most abundant forms found in plasma are β-carotene (precursor of vitamin A), lycopene, lutein, β-cryptoxanthin and zeaxanthin (Halsted, 2003). The biological effects of carotenoids are related to their antioxidant properties (Faulks and Southon, 2001), which can prevent the appearance of serious diseases such as cancer, pulmonary disorders, cataract (Tapiero, 2004) and atherogenesis (Faulks and Southon, 2001; Voutilainen *et al*., 2006).

Vitamins, mainly C and E, can also be found in fruits (Table 1). Vitamin C or ascorbic acid is ubiquitous in fruits. This compound is an important antioxidant (Fenech and Ferguson, 2001), antimutagenic (Kojima *et al*., 1992; Guha and Khuda-Bukhsh, 2002) and a regulator of DNA-repair enzymes (Cooke *et al*., 1998; Lunec *et al*., 2002). It is also involved in wound healing, tyrosine metabolism, conversion of folic acid to folinic

acid, carbohydrate metabolism, synthesis of lipids and protein, iron metabolism, and resistance to infections (Suntornsuk *et al*., 2002, Saffi *et al*., 2006).

Vitamin E can be found in cashew apple, mango, red grapes, and peaches (Table 1). This vitamin is able to donate its hydrogen to free radicals, thereby forming a stable species (Rimbach *et al*., 2002). Vitamin E radical can be regenerated by ascorbate, resulting in the formation of an ascorbyl radical (Rimbach *et al*., 2002). There is epidemiologic and clinical evidence that high intake of vitamin E may be associated with a decreased risk of coronary heart disease (Diaz *et al*., 1997; Kohlmeier *et al*., 1997). Chronic oral administration of vitamin E prevented the loss of mitochondrial function and reduced ROS-induced damage in aging mice (Navarro *et al*., 2005). These beneficial effects were paralleled by an increased lifespan, better neurological performance and higher exploratory activity (Panetta *et al*., 2004).

Phenols (hydroxybenzenes) and especially polyphenols (containing two or more phenol groups) are ubiquitous in plant foods and, apart from known vitamins and minerals, may be one of the most widely marketed groups of dietary supplements. This class of plant metabolites contains more than 8000 known compounds, ranging from simple phenols such as phenol itself through to materials of complex and variable composition such as tannins (Bravo, 1998). Phenolic compounds in fruits (Table 1) include flavonoids (mainly quercetin, hesperidin, anthocyanins, catechins, and kaempherol), phenolic acids (salicylic acid), hydroxycinnamic acids (coumaric and caffeic), and stilbenes (resveratrol).

Much of the literature on polyphenolic compounds concerned about the deleterious effects associated with the ability of certain phenols to bind and precipitate macromolecules including protein and carbohydrates, thereby reducing the digestibility of foods (Singleton and Rossi, 1983). More recently, interest has been rekindled in the recognition that many polyphenols, although non-nutrients, show antibacterial effects

(Avorn, 1994), ability to reduce blood pressure (Lampe, 1999), antioxidant, anti-inflammatory, antimutagenic and/or anticarcinogenic effects, at least in *in vitro* systems (Saiko *et al*., 2008; Rodrigues *et al*., 2006; Sairam *et al*. 2003; Miyazawa *et al*. 1999; Bravo, 1998). A prospective study of 800 elderly men showed that the ingestion of flavonoids, mainly in tea, onions, and apples, was associated with significant reduction in mortality from coronary heart disease (Hertog *et al*., 1993). In addition, polyphenols can also inhibit platelet aggregation and vascular relaxation through the production of nitric oxide (Dubick *et al*., 2001).

Almost all the fruits present in this review show antioxidant activity (Table 2), which can be associated with the presence of carotenoids, vitamins, and mainly, polyphenols. The mechanisms of the antioxidant action of polyphenols are complex and they are still been studied. In a general way, they can avoid reactive species formation either by inhibition of enzymes or by chelation of trace elements involved in free radical production, scavenging reactive species, and up-regulating or protecting antioxidant defense (Halliwell and Gutteridge, 1999). Some compounds can also act in a similar way to the enzymatic defenses, since they are able to neutralize reactive species such as superoxide anion and hydrogen peroxide (Silalahi, 2002).

Many fruits (Table 2) can also present antimutagenic activity. There are a number of different mechanisms, which have been implicated in the antimutagenic effects of polyphenols. Some of these are non-specific as for example, polyphenols can exert an antioxidant action (Hartman and Shankel, 1990; Hoensch and Kirch, 2005; Anisimov *et al*., 2006; Valcheva-Kuzmanova and Belcheva, 2006, Srinivasan, 2007) or inhibit the uptake of mutagens such as benzo[a]pyrene (Hatch *et al*., 2000). Different polyphenols may act to upregulate the activity of glutathione S-transferase and/or may directly interfere with DNA adduct formation (Ferguson, 2001).

Although many polyphenols can present antimutagenic effects, some of them can act as a weak mutagenic agent (Ferguson, 2001). The exact reason why a polyphenol can be a mutagenic or an antimutagenic compound is not known, but structure-activity relationships among the flavonoids suggested that bacterial mutagenicity required a double bond between positions 2 and 3 and a hydroxyl group at position 3 (Nagao *et al*., 1981). It is also known that a number of polyphenols, including quercetin, can bind to DNA (Alvi *et al*., 1986) and this direct interaction may be an important mechanism of bacterial mutagenicity. Interestingly, some fruits (cashew apple, coconut and kiwi fruit) can present both mutagenic and antimutagenic activities (Table 2). It is known that high concentrations of ascorbic acid (Franke *et al*., 2005) and some kinds of polyphenols can induce mutagenic effects (De Flora, 1998; De Flora *et al*., 2001) depending on factors such as pH and the presence of Cu(II) and Fe(III) in the media (Wang *et al*., 1996; Ferguson, 2001; De Flora, 1998).

Intensive research conducted over the last few years has shown that polyphenols, carotenoids, and vitamins derived from fruits interfere with tumor progression by acting directly on tumor cells as well as by modifying the tumor's microenvironment (stroma) and creating physiological conditions that are hostile to tumor growth (Béliveau and Gingras, 2007). Anticarcinogenic activity can also be related to the antioxidant effect (Béliveau and Gingras, 2007). Some fruits, like coconut, kiwi fruit, lemon, mango, and red grape can present anticarcinogenic activities *in vivo* assays (Table 2).

Various plant polyphenols have profound effects on the function of immune and inflammatory cells (Middleton Jr. *et al*., 1992). Polyphenols present in green tea (mainly epigallocatechin gallate) can inhibit the inducible nitric oxide (NO) synthase and block NO-associated DNA damage (Bartsch *et al*., 1996). Acai, black mulberry, mango, and raspberry have shown important anti-inflammatory effects (Table 2).

Plants have developed sophisticated active defense systems against pathogens, among them the production of antibiotic compounds. Centuries of folk wisdom have identified certain fruits or vegetables as having antibacterial potential (Lampe, 1999). Cashew apple, red grape, red guava, lemon, mango and papaya present antibacterial and/or antifungal actions (Table 2) acting against *Bacillus subtilis, Enterobacter cloacae, Escherichia coli, Salmonella typhi, Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa,* and *Klebsiella pneumonia* (Sairam *et al*., 2003; Osato *et al*., 1993). The microbial activity of fruits is related to the presence of different types of polyphenols, mainly procyanidins (Taguri *et al*., 2004).

Briefly, this review compiles data from some studies about biological activity and the main secondary compounds of fruits, reinforcing the idea that a diet rich in fruits could be used to prevent many kinds of pathologies, providing a genuine beneficial effect on human populations.

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Table 1. **Compounds with potential biological activity in different fruits**

Fruit	Compounds with potential biological activities	References
Acai (*Euterpe oleracea* L.)	Vitamin C[1], carotenoids[1], polyphenols (cyanidin[2,4,5], procyanidin[2,3], peonidin[2], pelargonidin[2], catechin[2], epicatechin[2,3], resveratrol[2], protocatechuic acid[3])	[1]Spada *et al*., 2008; [2]Rocha *et al*., 2007; [3]Rodrigues *et al*., 2006; [4]Lichtenthaler *et al*., 2005; [5]Del Pozo-Insfran *et al*., 2004.
Acerola (*Malpighia glabra* L.)	Vitamin C[1], carotenoids[1], polyphenols[1,2]	[1]Spada *et al*., 2008; [2]Mezadri *et al*. 2006.
Apple (*Malus domestica* B.)	Vitamin C[1,5], polyphenols (procyanidins[2,3], anthocyanins[3], catechin[3,4], epicatechin[4], quercetin[5])	[1]Spada *et al*., 2008; [2]Kahle *et al*., 2005; [3]Vrhovsek *et al*., 2004; [4]Sanoner *et al*., 1999; [5]Ballot *et al*., 1987.
Black mulberry (*Morus nigra* M.)	Vitamin C[1], carotenoids[1], polyphenols (coumaric acid[2], salicylic acid[2], caffeic acids[2])	[1]Spada *et al*., 2008; [2]Zadernowski *et al*., 2005.
Cashew apple (*Anacardium occidentale* L.)	Vitamin C[1], vitamin E[2], carotenoids[1], polyphenols[3] (quercetin, procyanidin, anacardic acid)	[1]Spada *et al*., 2008; [2]Ryan *et al*. 2006; [3]Melo Cavalcante *et al*., 2003.
Coconut (*Cocos nucifera* L.)	Vitamin C [1,3], polyphenols (catechin[4], phenolic acids[2])	[1]Spada *et al*., 2008; [2]Dey *et al*., 2005; [3]Mantena *et al*., 2003; [4]Kirszberg *et al*., 2003.
Cupuacu (*Theobroma grandiflorum* W.)	Vitamin C[1], polyphenols (catechin[2], epicatechin[2], quercetin[2], kaempferol[2])	[1]Spada *et al*., 2008; Yang *et al*., 2003.
Kiwi fruit (*Actinidia chinensis* P.)	Vitamin C[1,3], carotenoids[1], polyphenols[1,2]	[1]Spada *et al*., 2008; [2]Chang and Case, 2005; [3]Kvesitatdze *et al*., 2001.
Lemon (*Citrus limon* B.)	Vitamin C[1,2], polyphenols (eriocitrin[2,3], hesperidin[2,3], diosmetin[2,3], diosmin[2,3], diosmetin[2,3,4], quercetin[4], apigenin[4], hesperetin[4], homoeriodictyol[4])	[1]Spada *et al*., 2008; [2]González-Molina *et al*., 2008; [3]Miyake *et al*., 2007; [4]Gil-Izquierdo *et al*., 2004.
Mango *Mangifera indica* L.	Vitamin C[1,2,10], vitamin E[3], carotenoids[1,3,4,5,8]; polyphenols (quercetin[4,8], rhamnetin[5,8], gallotannins[6], flavonols[8], kaempferol[8], xanthone[8], isomangiferin[8], galloyl derivatives[8], mangiferin[8,9], catechin[9], epicatechin[9], tannic acid[7], caffeic acid[7], gallic acid[9], benzoic acid[9])	[1]Spada *et al*., 2008; [2]Ribeiro *et al*., 2007; [3]Ornelas-Paz Jde *et al*., 2007; [4]Berardini *et al*., 2005; [5]Chen *et al*., 2004; [6]Berardini *et al*., 2004; [7]Singh *et al*. 2004; [8]Schieber *et al*., 2003; [9]Nunez Selles *et al*., 2002; [10]Ballot *et al*., 1987.
Melon (*Cucumis melo* L.)	Vitamin C[1,3,4], carotenoids[1,2], polyphenols[1]	[1]Spada *et al*., 2008; [2]Portnoy *et al*., 2008; [3]Lester, 2008; [4]Gil *et al*., 2006.
Orange (*Citrus aurantium* L.)	Vitamin C[1], carotenoids[1], polyphenols[2] (neoeriocitrin, narirutin, naringin, hesperidin, neohesperidin, naringenin, hesperetin)	[1]Spada *et al*., 2008; [2]Pellati *et al*., 2004.

Papaya (*Carica papaya* L.)	Vitamin C[1,4], carotenoids[1-3], polyphenols[1]	[1]Spada *et al*., 2008; [2]Mutsuga *et al*., 2001; [3]Cano *et al*., 1996; [4]Osato *et al*., 1993.
Passion Fruit (*Passiflora alata* L.)	Vitamin C[1], carotenoids[1,2]	[1]Spada *et al*., 2008; [2]Mourvaki *et al*., 2005.
Peach (*Prunus persica* L.)	Vitamin C[1,3,4,5], vitamin E[3], carotenoids[1], polyphenols (catechin[2], epicatechin[2], quercetin[2], eriodictyol[2], naringenin[2], kaempferol[2], isorhamnetin[2], protocatechuic acid[2], vanillic acid[2], coumaric acid[2])	[1]Spada *et al*., 2008; [2]Wijeratne *et al*., 2006; [3]Carbonaro *et al*., 2002; [4]Gil *et al*., 2002; [5]Ballot *et al*., 1987.
Pineapple (*Ananas ssp*)	Vitamin C[1], carotenoids[1,3], polyphenols[1,2]	[1]Spada *et al*., 2008; [2]Wen *et al*., 1999; [3]Ballot *et al*., 1987.
Raspberry (*Rubus idaeus* L.)	Vitamin C[1], carotenoids[1], polyphenols (anthocyanins[2-4], procyanidins[3], ellagitannins[3,4], kaempferol[4], quercetin[4], ellagic acid[4])	[1]Spada *et al*., 2008; [2]Fang Chen *et al*., 2007; [3]Beekwilder *et al*., 2005; [4]Mullen *et al*., 2002.
Red grape (*Vitis vinifera* L.)	Vitamin C[1], vitamin E([2)3], tocotrienol[2)3], carotenoids[1], polyphenols (malvidin[2], quercetin[4,5,6], catechin [4,5,6], epicatechin[4,5,6], resveratrol[2,4,5,6], delphinidin[2,5], cyanidin [2,5], petunidin[4], peonidin[2,5], malvidin[5], procyanidin[5], epicatechin gallate[5], *trans*-polydatin[5], isorhamnetin[5], kaempferol[5], gallic acid[5], protocatechuic acid[5], caftaric acid[5], *p*-hydroxybenzoic acid[5], caffeic acid[5], *p*-coumaric acid[5], ferulic acid[5], ellagic acid[6])	[1]Spada *et al*., 2008; [2]Dani *et al*., 2008; [3]Horvatha *et al*., 2006, [4]Chafer *et al*., 2005; [5]Kammerer *et al*., 2004; [6]Yilmaz and Toledo, 2004.
Red guava (*Psidium guajava* L.)	Vitamin C[1,5], carotenoids[1,4], polyphenols (guajadial[2], quercetin[3], myricetin[3], kaempferol[3], apigenin[3])	[1]Spada *et al*., 2008; [2]Carasek *et al*., 2006; [3]Miean *et al*. 2001; [4]Mercadante *et al*., 1999; [5]Ballot *et al*., 1987.
Soursop (*Annona muricata* L.)	Polyphenols[1,2]	[1]Spada *et al*., 2008; [2]Augusto *et al*., 2000.
Strawberry (*Fragaria vesca* L.)	Vitamin C[1,3], carotenoids[1,2], polyphenols[1,2]	[1]Spada *et al*., 2008; [2]Kiselova *et al*., 2006; [3]Ballot *et al*., 1987.
Surinam cherry (*Eugenia uniflora* O.)	Polyphenols[1,2]	[1]Spada *et al*., 2008; [2]Almeida *et al*., 1995.
Tangerine (*Citrus reticulata* L.)	Vitamin C[1], carotenoids[1,2,4], polyphenols[1,3]	[1]Spada *et al*., 2008; [2]Ricón *et al*., 2005; [3]Gil-Izquierdo *et al*., 2004; [4]Nogata *et al*., 2003.

Table 2. Biological activities of fruits

Fruits	Biological activities	References
Acai	Antioxidant activity[1-5] Vasodilatory activity[2] Anti-inflammatory effect[3] Mutagenic activity[1]	[1]Spada *et al*., 2008; [2]Rocha *et al*., 2007; [3]Rodrigues *et al*., 2006; [4]Schauss *et al*., 2006; [5]Lichtenthaler *et al*. 2005.
Acerola	Antioxidant activity[1,2]	[1]Spada *et al*., 2008, [2]Hanamura *et al*., 2005.
Apple	Antioxidant activity[1,2]	[1]Spada *et al*., 2008, [2]Leu *et al*., 2006
Black mulberry	Antioxidant activity[1,2] Anti-inflammatory effect[2]	**[1]Spada *et al*., 2008, [2]Kim and Park, 2006.**
Cashew apple	**Antioxidant activity [1-3]** **Mutagenic/comutagenic activities [1,4,5]** Antibacterial activity[2] Antimutagenic activity [4,5]	[1]Spada *et al*., 2008, [2]Green *et al*., 2007; [3]Konan *et al*., 2006; [4]Melo Cavalcante *et al*., 2003; [5]Trevisan *et al*., 2006
Coconut	Antioxidant activity[1,5,6] Mutagenic activity[2-4] Antimutagenic activity[3,5] Anticarcinogenic activity[5]	[1]Spada *et al*., 2008, [2]Sandhya and Rajamohan, 2006; [3]Petta *et al*., 2004; [4]Narasimhamurthy *et al*., 1999; [5]Nalini *et al*., 1997; [6]Bell and Kamens, 1990.
Cupuacu	**Antioxidant activity[1,2]** **Antimutagenicity[1]**	[1]Spada *et al*., 2008; [2]Yang *et al*., 2003.
Kiwi fruit	**Comutagenic activity; low antimutagenic and mutagenic effects[1-3]** **Anticarcinogenic activity[4]**	[1]Spada *et al*., 2008; [2]Deters *et al*., 2005; [3]Tang and Edenharder, 1997; [4]Edenharder *et al*., 1994.
Lemon	Antifungal activity[1] Antimutagenic activity[2-4] Anticarcinogenic activity[5]	[1]Ben-Yehoshua *et al*., 2008; [2]Spada *et al*., 2008; [3]Higashimoto *et al*., 1998; [4]Bala and Grover, 1989; [5]National Toxicology Program, 1990.
Mango	Antioxidant activity[1,3-5] Anti-inflammatory activity[2] Anticarcinogenic activity[4] Antimutagenic activity[1,6] Antidiarrhoeal activity[7] Antibacterial activity[7]	[1]Spada *et al*., 2008; [2]Knödler *et al*. 2007; [3]Mahattanatawee *et al*. 2006; [4]Rodriguez *et al*. 2006; [5]Percival *et al*. 2006; [6]Pardo-Andreu *et al*. 2006; [7]Sairam *et al*. 2003
Melon	Antioxidant activity[1-4]	[1]Spada *et al*., 2008; [2]Lester, 2008; [3]Vouldoukis *et al*. 2004; [4]Lester *et al*., 2004.
Orange	Antioxidant activity[1,3,5,6] Adrenergic activity[2] Antigenotoxic activity[4] Antimutagenic activity[1,7]	[1]Spada *et al*., 2008; [2]Nelson *et al*., 2007; [3]Jayaprakasha *et al*., 2007 ; [4]Franke *et al*., 2006; [5]Deyhim *et al*., 2006 ; [6]Hosseinimehr and Karami, 2005 ; [7]Miyazawa *et al*. 1999;
Papaya	Antioxidant activity[1,2,4,5,8,14] Antibacterial activity[3,11,14] Inhibitory effect on sperm motility[6]	[1]Spada *et al*., 2008; [2]Lohiya *et al*., 2008; [3]Nayak *et al*., 2007 ; [4]Gambera *et al*., 2007 ; [5]Mehdipour *et al*., 2006 ; [6]Rahmat *et al*., 2004 ; [7]Lohiya *et*

	Antifertility activity[7,9,10,12,15] Androgenic activity[13]	*al*., 1999 ; [8]Imao *et al*., 1998; [9]Lohiya *et al*., 1994 ; [10]Chinoy *et al*., 1994, [11]Osato *et al*., 1993 ; [12]Lohiya and Goyal, 1992; [13]Chinoy and Ranga Geetha, 1984 ; [14]Emeruwa, 1982 ; [15]Gopalakrishnan and Rajasekharasetty, 1978.
Passion Fruit	Antioxidant activity[1,2] Anticonvulsant effect[2]	[1]Spada *et al*., 2008; [2]Nassiri-Asl *et al*., 2007.
Peach	Antioxidant and antimutagenic activities[1]	[1]Spada *et al*., 2008
Pineapple	Antioxidant activity[1-3] Antifertility activity[4]	[1]Spada *et al*., 2008; [2]Herraiz and Galisteo, 2003; [3]Sun *et al*., 2002 ; [4]Garg *et al*., 1970
Raspberry	Antioxidant activity[1,2-6] Antimutagenic activity[1] Vasodilatory activity[3]	[1]Spada *et al*., 2008; [2]Viljanen *et al*., 2004, [3]Wada and Ou, 2002; [4]Mullen *et al*., 2002; [5]Wang and Jiao, 2000; [6]Kalt *et al*., 1999.
Red grape	Antioxidant activity[1,2,4,6,8,9,13,14] Antimutagenic activity[1] Antibacterial activity[3] Anticarcinogenic activity[5,9] Antiarrhythmic and cytoprotective effects[7] Protective effects against ischemia-reperfusion[10,15] Radioprotective effects[14] Antiexudative and capillaritonic effects[16] Vasodilatory activity[11,12]	[1]Spada *et al*., 2008; [2]Kedage *et al*., 2007; [3]Thimothe *et al*., 2007; [4]El-Ashmawy *et al*., 2007; [5]Lala *et al*., 2006 ; [6]Devi *et al*., 2006 ; [7]Al-Makdessi *et al*., 2006 ; [8]Janisch *et al*., 2006; [9]Stagos *et al*., 2005; [10]Nakagawa *et al*., 2005; [11]Madeira *et al*., 2005; [12]Soares *et al*., 2004, [13]Shafiee *et al*., 2003; [14]Castillo *et al*., 2000; [15]Maffei Facinó *et al*., 1996; [16]Zafirov *et al*., 1990.
Red guava	Antioxidant activity[1,5] Antibacterial activity[2,4] Antimutagenic effect[1,6] Hypoglycemic effects[3,7]	[1]Spada *et al*., 2008; [2]Pelegrini *et al*., 2008; [3]Rai *et al*., 2007; [4]Abdelrahim *et al*., 2002; [5]Jime'nez-Escrig *et al*., 2001; [6]Grover and Bala, 1993; [7]Cheng and Yang, 1983.
Strawberry	Antioxidant activity[1-4] Mutagenic activity[1]	[1]Spada *et al*., 2008; [2]Kiselova *et al*. 2006; [3]Rababah *et al*., 2005; [4]Kahkonen *et al*., 2001.
Soursop	Antioxidant and antimutagenic activities[1]	[1]Spada *et al*., 2008.
Tangerine	Antioxidant and antimutagenic activities[1]	[1]Spada *et al*., 2008.

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Phenolic Content and Antioxidant and Antimicrobial Properties of Fruits of *Capsicum baccatum* L. var. *pendulum* at Different Maturity Stages

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ABSTRACT The phenolic content, antioxidant potential, and antimicrobial activity of extracts of different parts of the fruit from *Capsicum baccatum* L. var. *pendulum* were investigated. The analysis of phenolic content was performed by the Folin-Ciocalteu method and reversed-phase high-performance liquid chromatography. The *in vitro* antioxidant activity was assessed by the total reactive antioxidant potential and total antioxidant reactivity index. The antioxidant activity was positively correlated with the amount of phenolics found in each sample. The *ex vivo* antioxidant potential was assessed using the rat liver slice model. The antimicrobial activity was screened using Gram-positive and Gram-negative bacteria and fungi. All the extracts revealed antioxidant activity and a weak antimicrobial activity.

KEY WORDS: • *antioxidant potential* • *antimicrobial activity* • Capsicum • *maturity* • *phenolics*

INTRODUCTION

MANY EPIDEMIOLOGICAL STUDIES have indicated an inverse association between a plant-based diet and the risk of development of chronic pathological processes associated with oxidative stress, including cancer, cardiovascular diseases, and other age-related degenerative disorders.[1–3] The presence of antioxidant compounds in fruits and vegetables could be associated with these beneficial health effects, protecting biomolecules from oxidative damage. Regarding plant components, phenolic compounds, or polyphenols, are an important group of secondary metabolites that have been widely investigated concerning their antioxidant potential.[4–7] These phytochemicals may act as antioxidants because they demonstrate redox properties, which allow them to scavenge free radicals and inactivate other pro-oxidants.[8] Furthermore, the antimicrobial activity of these compounds also has been reported.[9,10]

Recently, several researchers have investigated the chemical composition and antioxidant activity of different fruits and vegetables.[11–13] Peppers—*Capsicum* species (Family Solanaceae)—are native plants of America and are important vegetables popularly used as spices, foods, and external medicine. Pepper fruits are a remarkable source of antioxidant compounds, like vitamins,[14,15] carotenoids,[16–18] capsaicinoids,[19–22] and phenolic compounds,[23–25] but levels of these phytochemicals may vary by genotype, stage of maturity, and plant part consumed. Thus, it becomes pertinent to study these variations in different parts of the fruit from different genotypes during maturity to select the best for health benefits.

Capsicum baccatum L. var. *pendulum* (Cambuci) is widely consumed in South Brazil, and there are few studies in literature concerning its chemical composition and biological properties. The objectives of this study were (1) to determine the total phenolic content of extracts of three different parts of *C. baccatum L.* var. *pendulum* immature and mature fruits, (2) to evaluate and compare total antioxidant capacity and phenolic content of these extracts, (3) to establish the relationship between antioxidant activity and phenolic compounds of extracts, (4) to identify the phenolic profile of the extracts by reversed phase (RP)-high-performance liquid chromatography (HPLC), and (5) to determine the antimicrobial activity of these extracts.

MATERIALS AND METHODS

Chemicals

Thiobarbituric acid (TBA), luminol (3-aminophthalhydrazide), *tert*-butyl hydroperoxide (*t*-BOOH), bovine

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serum albumin, and standard chemicals of capsaicin, galic, chlorogenic and caffeic acids, and rutin were purchased from Sigma Chemical Co. (St. Louis, MO). 2,2′-Azobis(2-methylpropionamidine)dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Aldrich Chemical (Milwaukee, WI). Acetonitrile, acetic acid, methanol, and glycine were purchased from Nuclear (Diadema, SP, Brazil). Folin-Ciocalteu reagent was purchased from Merck (Darmstadt, Germany). Trichloroacetic acid and sodium carbonate were purchased from Synth (Diadema).

Plant material

The fruits of *C. baccatum* L. var. *pendulum* were collected in Passo Fundo, RS, Brazil, in January 2006. Voucher specimens were identified by Prof. M.S. Branca Severo from the University of Passo Fundo and are on deposit in the Herbarium of the Museu Zoobotânico Augusto Ruschi of the University of Passo Fundo (voucher number RSPF 10449).

Preparation of extracts

The fresh fruits of *C. baccatum* were divided based on their color into two maturity stages (immature, green; mature, red) and separated in three different parts (pericarp, seeds, and placenta). Ten grams of each structure were separately extracted using 100 mL of 40% ethanol (plant:solvent, 1:10, wt/vol) under reflux (80°C) for 30 minutes. After cooling, the extracts were filtered, evaporated under reduced pressure to dryness, and stored at −20°C until use. During the preparation and analysis, the extracts were protected against light.

Determination of total phenolic content

Total phenolic content of the hydroethanolic extracts was determined by the Folin-Ciocalteu assay, and chlorogenic acid was used as the standard.[26] Briefly, a 100-μL aliquot of extracts was assayed with 100 μL of Folin-Ciocalteu reagent and 200 μL of sodium carbonate (25%, wt/vol). The mixture was vortex-mixed and diluted with distilled water to a final volume of 2 mL. After 2 hours, the absorption was measured at 726 nm, and the total phenolic content was expressed as chlorogenic acid equivalents (CAE) in mg/100 g of dry weight.

HPLC analysis

The qualitative analysis of some phenolic compounds and capsaicin from extracts was done by HPLC using a Shimadzu (Columbia, MD) liquid chromatography apparatus composed of two LC-10AD pumps, a SPD-10AV ultraviolet/visible detector, an SCL-10A controller, and a Rheodyne (Rohnert Park, CA) injector (20-μL sample loop). The data acquisition system was a Class-VP software. The separation was achieved on a Waters (Milford, MA) Spherisorb ODS-2 column (RP-C18, 5 μm particle size, 150 × 4.6 mm i.d.). A linear gradient with eluents A (methanol) and B (water/acetic acid, 99:1, vol/vol) as the mobile phase was used as follows: 0 minutes, 15% A; 15 minutes, 40% A; and 25 minutes, 50% A. The mobile phase was prepared daily and degassed by sonication before use. The flow rate was 1 mL/minute, and the chromatograms were recorded at 280 nm. The phenolic compounds were identified by comparing the retention time with reference standards and by co-injection of standards by adding them to the extracts. For this, 500 μL of mobile phase or aqueous solution containing chlorogenic or caffeic acid or rutin (all 200 μg/mL) was added to 500 μL of the crude extract solution (1,000 μg/mL). Capsaicin analyses were conducted by the same HPLC apparatus, but the mobile phase was isocratic, consisting of 65% solvent A (methanol) and 35% B (acetic acid 1%), at a flow rate of 1 mL/minute and detection at 280 nm. The presence of capsaicin was determined based on the retention time of a standard solution.

Antioxidant activity

The *in vitro* antioxidant activity of the extracts was estimated by the total reactive antioxidant potential (TRAP) and the total antioxidant reactivity (TAR), as previously described.[27–29] Briefly, the reaction mixture (4 mL), containing AAPH (10 m*M*) and luminol (4 m*M*) in glycine buffer (0.1 *M*, pH 8.6), was incubated at 21°C for 2 hours. AAPH is a source of peroxyl radicals that react with luminol yielding chemiluminescence (CL). The system was calibrated using the α-tocopherol synthetic analogue, Trolox. Addition of 10 μL of the extracts (final concentration 2.5 μg/mL) or of 10 μL of Trolox (final concentration 200 n*M*) decreases the CL proportionally to its antioxidant potential. The TRAP profile was obtained measuring the CL emission in a liquid scintillation counter (model 1409, Wallac, Turku, Finland), operating in the "out of coincidence" mode, as counts per minute (CPM). The CL intensity was monitored for 50 minutes after the addition of the extracts or Trolox. The TAR index was determined by measuring the initial decrease of luminol luminescence calculated as the ratio I_o/I, where I_o is the initial emission of CL (before the addition of the antioxidant) and I is the instantaneous CL intensity after addition of an aliquot of the sample or the reference compound (Trolox).

Ex vivo *assay*

The antioxidant activity of *C. baccatum* parts extracts was also evaluated in an *ex vivo* assay using *t*-BOOH as the oxidative stress inducer. Rat liver slices (400 μm) were preincubated with the extracts for 30 minutes at 37°C under 95% O_2/5% CO_2 in a shaking water bath (60 oscillations/minute) in a medium of oxygen-equilibrated Krebs-Ringer phosphate buffer (10 m*M* glucose, pH 7.4). After this incubation with the treatments, 0.5 m*M* *t*-BOOH was added to different liver slice samples. After incubation, rat liver slices were

removed, and the medium was centrifuged at 12,000 *g* for 10 minutes. The supernatant portion was used to measure lactate dehydrogenase (LDH) activity using a commercial kit (LDH Liquiform™, Labtest Diagnostica, Lagoa Santa, MG, Brazil). For lipid peroxidation assay, the rat liver slices were homogenized with phosphate buffer, pH 7.4, and kept at –75°C prior to analysis.

Lipid peroxidation

TBA-reactive species (TBARS) formation was used to evaluate lipid peroxidation.[30] First, 600 μL of 15% trichloroacetic acid was added to 300 μL of the liver slice homogenates and centrifuged at 7,000 *g* for 10 minutes. Then, 500 μL of supernatant was mixed with 500 μL of 0.67% TBA. The reaction mixture was incubated in a boiling water bath for 20 minutes and cooled to room temperature, and the absorbance was read at 532 nm. The results were normalized by protein content and were expressed as malondialdehyde (MDA) equivalents (nmol/mg of protein). Protein concentration in rat liver slice homogenates was measured by the method of Lowry *et al.*,[31] using bovine serum albumin as standard.

Antimicrobial assays

The disk diffusion method was used as screening test for antimicrobial activity.[32] Filter paper disks (6 mm in diameter) impregnated with extract solutions were placed on Mueller-Hinton agar plates (Merck), which were inoculated with test organisms according to the standard protocol described by the National Committee of Clinical Laboratory Standards.[33] The filter paper disks were impregnated with 20 μL of the extract solutions in order to obtain final concentrations of 2,000, 1,000, and 200 μg of extract in the disks. The microorganisms used for the biological evaluation were *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecalis* (ATCC 14506), *Enterococcus faecium* (ATCC 10541), *Candida glabrata* (NRRL Y-55), *Candida albicans* (ATCC 18804), *Candida krusei* (IMUFERJ 50149), *Candida dubliniensis* (NRRL Y-17841), *Candida parapsilosis* (ATCC 22019), *Cryptococcus neoforms* (ATCC 24065B), and two clinic-isolated strains, *C. albicans* and *C. parapsilosis*. The plates were incubated at 35°C (± 1°C), and after 18 hours the diameters of the inhibition zones were measured. Filter paper disks containing sterile water without any test compound were used as the negative control, and no inhibition was observed. Standard antibiotic disks were selected according to the sensitivity of the bacteria or fungi tested. Thus, ampicillin (10 μg), chloramphenicol (30 μg), ceftazidin (30 μg), and fluconazole (25 μg) were used.

Statistical analysis

Data were expressed as mean ± SD values of triplicates from two independent experiments. Differences between treatments were compared by one-way analysis of variance, followed by Tukey's test for approximately normally distributed variables. Pearson's correlation coefficient was used to test correlation between total phenolic content (CAE) and TAR index. Data analyses were performed using the SPSS version 13.0 software package (SPSS Inc., Chicago, IL), and the statistical significance was set at the .05 level (two-tailed).

RESULTS AND DISCUSSION

Total phenol content, antioxidant capacity, and relationship between TAR and CAE

Many studies have established that polyphenols present in foods inhibit oxidative stress because of their free radical scavenging activities *in vitro* and *in vivo*.[34,35] This way, the quantification of phenolics in fruits and vegetables is necessary to increase functional properties of these foods. Total phenolic content was quantified by using the Folin-Ciocalteu phenol reagent. Although the Folin-Ciocalteu method could overestimate total phenolics, it is so far the only single and widely used method for estimating total phenolics.[36] The results of the total phenolic content of immature and mature parts of *C. baccatum* fruits range from 1,523.9 to 7,688.1 mg of CAE/100 g of dry weight (Table 1).

The phenol content of *C. baccatum* parts presented similar or higher content of total phenolics than other *Capsicum* species.[23,25,36,37] An overview of the results shows that the total phenolic content of all parts is higher in immature fruits than in mature fruits. This trend in the total phenolic content for *C. baccatum* with maturity stage is in agreement with the trend reported by Marín *et al.*[24] for *Capsicum annuum* L. cv. Vergasa (a variety of sweet pepper), where the green pepper had higher total phenol content than the red pepper. However, some studies have observed an increasing trend in total phenolics during maturity stage in other *Capsicum* cultivars.[23,36]

In addition, we observed a change in the location of the highest phenolic content during the maturing process, as we noticed that the highest level of phenolics in the immature

TABLE 1. TOTAL PHENOLIC CONTENT IN DIFFERENT PARTS OF *C. BACCATUM* FRUITS AT DIFFERENT MATURITY STAGES

Maturity stage	*Structure extract*	*Total phenolic*
Immature	Pericarp	1,920.0 ± 24.0^{e}
	Seed	5,449.8 ± 261.6^{c}
	Placenta	7,688.1 ± 89.4^{a}
Mature	Pericarp	1,523.9 ± 64.9^{f}
	Seed	5,847.2 ± 121.9^{b}
	Placenta	3511.4 ± 114.7^{d}

Data are mean ± SD values, expressed as mg of CAE/100 g of dry weight ($n = 6$).

Values with the same letters indicate no significant differences ($P < .05$).

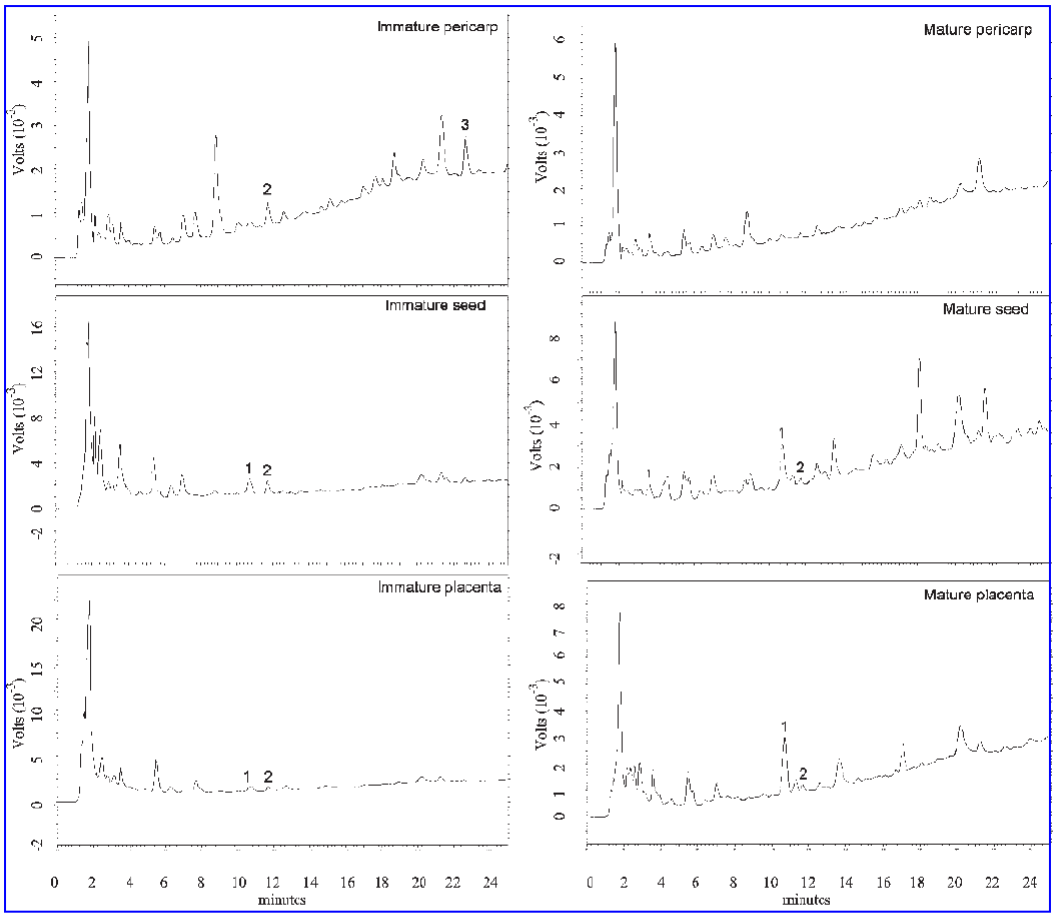

FIG. 1. HPLC profiles (280 nm) of extracts from parts of *C. baccatum* fruits. **1**, chlorogenic acid; **2**, caffeic acid; **3**, rutin.

parts was found in the placenta (7,688.1 mg of CAE/100 g) and in the mature parts in the seeds (5,847.2 mg of CAE/100 g). Thus, we have verified that the seeds are the parts that contribute most to the total phenolic content in the mature fruits of *C. baccatum* (Table 1). Seeds from various plant sources have been shown to be rich in phenolics and to contribute significantly to high phenolic content in the maturity stage. This was observed in one cultivar of *C. annuum* from which seeds had been removed and the total phenolic level decreased with maturity.[13,23] Thus, our results suggest a clear understanding of metabolic changes in phenolics during the maturation process, and a characterization of the phenolic profile of all separate parts of the fruit is necessary.

The phenolic compounds in the extracts under study were analyzed using RP-HPLC by comparison with authentic phenolic standards. The different co-injections—with extract + standards, extract + mobile phase, and standards + mobile phase—allowed us to observe an increase in the peak area concerning the chlorogenic and caffeic acids and rutin, suggesting that these compounds are present in *C. baccatum* L. var. *pendulum* fruits. Figure 1 displays HPLC profiles of all extracts studied. The analyses of pericarp extracts revealed different chemical profiles since only the immature pericarp presented phenolic compounds (caffeic acid and rutin). The profile of the other parts analyzed indicates the presence of chlorogenic and caffeic acids in all of them.

However, capsaicin was not detected in any extracts analyzed, indicating that *C. baccatum* L. var. *pendulum* is a species that can be considered a "sweet" pepper, and this characteristic flavor makes it suitable for culinary preparation.

The TRAP was determined using a method based on the quenching of luminol-enhanced CL derived from the thermolysis of a water-soluble azo compound, AAPH, used as a reliable and quantifiable source of alkyl peroxyl radicals.[27,28] This widely used assay has proved to be a simple, sensitive, and reproducible method that can be used to determine the antioxidant capacity in complex mixtures such as plant extracts.[29] On the other hand, the TAR index was obtained from the initial decrease in the luminescence associated with the additive incorporation of the sample to the AAPH–luminol system, and it indicates the initial reactivity of the sample when compared to Trolox.[27–29] Figure 2 shows the CL profile obtained after addition of 10 μL of Trolox (final concentration 200 n*M*), used as a standard, and the different extracts (final concentration 2.5 μg/mL). This allowed us to compare the TRAP profiles, and we observed that CL decrease following addition of the plant extracts is qualitatively different to that obtained when Trolox is used. These differences can be the result of efficient and inefficient antioxidants in the extracts. All the extracts studied were active in reducing the luminol-enhanced CL, indicating the presence of compounds with peroxyl scavenging properties. However, immature placenta and mature seed extracts showed the highest antioxidant activity, as observed in the instantaneous reduction of CL and maintenance of this activity during the period analyzed. The results of TAR index indicated that all extracts have antioxidant reactivity (Fig. 3), but the same extracts that showed better activity on TRAP profile revealed a significantly higher TAR index when compared to other parts in the same maturity stage. Figure 3 shows that TAR indexes of immature placenta and mature seeds were not statistically different ($P < .05$) from the TAR index of Trolox.

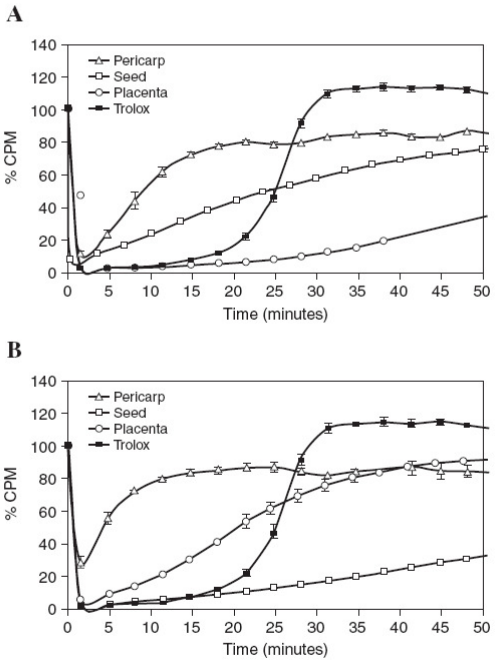

FIG. 2. TRAP profile of (**A**) immature parts and (**B**) mature parts of *C. baccatum* fruits. CL intensity (percentage of CPM) was measured after addition of 10 μL of Trolox (final concentration 200 n*M*) and the extracts (final concentration 2.5 μg/mL) to 4 mL of glycine buffer (0.1 *M*, pH 8.6) containing luminol (4 m*M*) and AAPH (10 m*M*) at 21°C. Values are means ± SD of two determinations ($n = 6$).

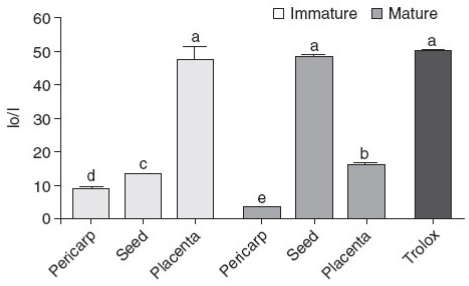

FIG. 3. TAR index (I_o/I) of parts of *C. baccatum* fruits during different maturity stages. Data are mean ± SD values ($n = 6$). The values with the same letters indicate no significant differences by Tukey's test ($P < .05$).

The results of total phenolic content basically coincided with those of total antioxidant capacity. In other words, the extracts that had high antioxidant activity showed a tendency towards high phenolic content. Thus, as in other studies that evaluated the correlations between the antioxidant capacity and phenolic profile of fruits and vegetables,[13,37–39] we found a positive significant correlation ($r = 0.863$, $P < .05$) between total phenolic content and antioxidant capacity (TAR index) in all analyzed extracts (Fig. 4), indicating that the phenolic compounds might be the major contributors to the antioxidant activities of these extracts.

The marked difference observed in the total phenolic content between the immature and mature parts of *C. baccatum* fruits could be attributed to the fact that peppers undergo profound physiological and biochemical changes during the course of maturation, with the conversion of some phytochemicals, and this influences the antioxidant activity of these fruits.[24,25,40]

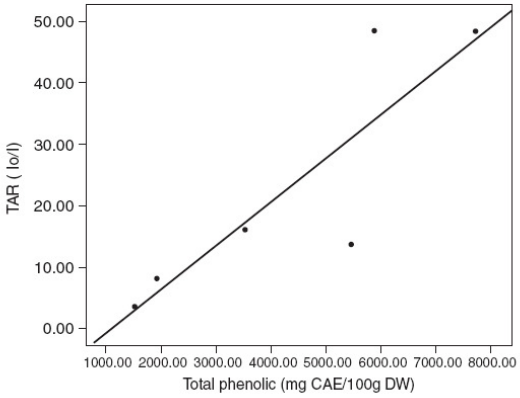

FIG. 4. Correlation between total phenolic content (CAE) and TAR index (I_o/I) of extracts from parts of *C. baccatum* fruits ($r = 0.863$, $P < .05$).

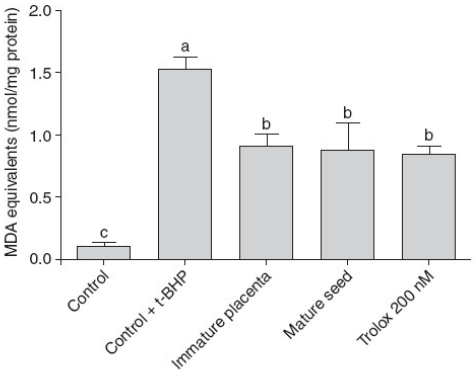

FIG. 5. Effect of immature placenta and mature seed extracts (final concentration 2.5 μg/mL) on TBARS levels. Results are expressed as mean ± SD values of triplicates from two independent experiments. Values with the same letters indicate no significant differences by Tukey's test ($P < .05$).

Ex vivo *assay*

In our *ex vivo* model, we used rat liver slices, which represent a complete and heterogeneous system close to the physiological system, to study the peroxidative damage. The incubation of physiologically active liver slices with the inducer *t*-BOOH substantially increased cell death and the generation of TBARS. The peroxyl radicals are important agents that mediate lipid peroxidation, causing damage to cell membranes.[41] In order to evaluate if the extracts with the highest *in vitro* antioxidant activity (immature placenta and mature seeds) were capable of inhibiting *ex vivo* oxidative stress, lipid peroxidation was assessed by means of an assay that determines the production of MDA and related compounds in rat liver homogenates. The results obtained are shown in Figure 5. The results clearly show that incubation of rat liver slices in the presence of 0.5 m*M t*-BOOH caused a significant increase ($P < .05$) in the MDA equivalents content of the liver homogenate when compared to the basal liver homogenate. However, the extracts tested (fi-

TABLE 2. ANTIMICROBIAL ACTIVITY OF THE EXTRACT OF IMMATURE SEEDS OF *C. BACCATUM* FRUIT ($N = 3$)

	Inhibition zone[a]						
	Dose (μg) of immature seed extract			*Antibiotics*			
Microorganism	*2,000*	*1,000*	*200*	*AP*	*CP*	*CZ*	*FC*
S. aureus	NA	NA	NA	28	NT	NT	NT
E. coli	NA	NA	NA	NT	25	NT	NT
P. aeruginosa	NA	NA	NA	NT	NT	25	NT
E. faecalis	NA	NA	NA	28	NT	NT	NT
E. faecium	NA	NA	NA	29	NT	NT	NT
C. glabrata	13	NA	NA	NT	NT	NT	30
C. albicans	10	NA	NA	NT	NT	NT	28
C. krusei	10	NA	NA	NT	NT	NT	30
C. dubliniensis	NA	NA	NA	NT	NT	NT	29
C. parapsilosis	12	11	NA	NT	NT	NT	33
C. neoforms	12	NA	NA	NT	NT	NT	30
C. albicans[b]	10	NA	NA	NT	NT	NT	30
C. parapsilosis[b]	10	NA	NA	NT	NT	NT	29

AP, ampicillin; CP, chloramphenicol; CZ, ceftazidin; FC, fluconazole; NA, not active; NT, not tested.
[a]Diameter of zone of inhibition (in mm) including disk diameter of 6 mm.
[b]Clinic-isolated.

nal concentration 2.5 μg/mL) were significantly effective in reducing the MDA levels. Whereas the addition of an oxidative stress inducer (0.5 m*M* *t*-BOOH) in the incubation medium substantially increased cell death, the pretreatment with immature placenta and mature seeds extracts prevented the increase in cell death, evidenced by decreased LDH leakage when compared to control slices (data not shown). In accordance with our study, Oboh *et al*.[40] demonstrated that other *Capsicum* species (*C. annuum* and *Capsicum chinese*) prevent lipid peroxidation in rat brain and that this effect is probably due to its higher total phenolic content. In addition, several studies investigating the antioxidant activity of extracts rich in polyphenols have demonstrated protective effects against lipid peroxidation.[42,43]

Antimicrobial activity

The antimicrobial screening of all extracts was carried out by the disk diffusion method. The extracts did not show antibacterial activity. This could be related to the absence of capsaicin in the extracts, since Molina-Torres *et al*.[44] demonstrated the antimicrobial activity of this compound. The immature seed extract was the only extract that showed antifungal activity (Table 2). Some studies demonstrated antimicrobial activity of compounds isolated from seeds of *C. annuum* L.[45,46]

In conclusion, our study indicates that the fruit extracts of *C. baccatum* L. var. *pendulum* have *in vitro* and *ex vivo* antioxidant activity, which is related to total phenolic content, fruit part, and maturity stage. Therefore, this pepper could be considered a new source of natural antioxidants, but further studies are needed to examine the potential use of these extracts in the prevention of pathologies.

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LipoCardium: Endothelium-directed cyclopentenone prostaglandin-based liposome formulation that completely reverses atherosclerotic lesions

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Abstract

Atherosclerosis is a multifactorial inflammatory disease of blood vessels which decimates one in every three people in industrialized world. Despite the important newest clinical approaches, currently available strategies (e.g. nutritional, pharmacological and surgical) may only restrain the worsening of vascular disease. Since antiproliferative cyclopentenone prostaglandins (CP-PGs) are powerful anti-inflammatory agents, we developed a negatively charged liposome-based pharmaceutical formulation (LipoCardium) that specifically direct CP-PGs towards the injured arterial wall cells of atherosclerotic mice. In the blood stream, LipoCardium delivers its CP-PG contents only into activated arterial wall lining cells due to the presence of antibodies raised against vascular cell adhesion molecule-1 (VCAM-1), which is strongly expressed upon inflammation by endothelial cells and macrophage-foam cells as well. After 4 months in a high-lipid diet, all low-density lipoprotein receptor-deficient adult control mice died from myocardium infarction or stroke in less than 2 weeks, whereas LipoCardium-treated (2 weeks) animals (still under high-lipid diet) completely recovered from vascular injuries. In vitro studies using macrophage-foam cells suggested a tetravalent pattern for LipoCardium action: anti-inflammatory, antiproliferative (and pro-apoptotic only to foam cells), antilipogenic and cytoprotector (via heat-shock protein induction). These astonishing cellular effects were accompanied by a marked reduction in arterial wall thickness, neointimal hyperplasia and lipid accumulation, while guaranteed lifespan to be extended to the elderly age. Our findings suggest that LipoCardium may be safely tested in humans in a near future and may have conceptual implications in atherosclerosis therapy.

Keywords: Atherosclerosis; Cyclopentenone prostaglandins; Leukocytes; Macrophages; Lipid metabolism; Endothelium; Adhesion molecules

1. Introduction

Since the first observations by Ross et al. [1] which described atherosclerotic lesions as a response to localized injury to the lining of the arteries (reviewed in ref. [2]), it has become apparent that atherosclerosis is a multifactorial inflammatory disease, where the major stimuli for the atheroma plaque formation [namely, hypertension, high plasma levels of low-density lipoproteins (LDL), dyslipidemias, diabetes and obesity] are implicated with vascular wall inflammation, while inflammatory biomarkers are

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correlated with the risk for the first manifestation of cardiovascular disease [3,4].

Although atherogenesis may involve several blood cell types, monocytes are the primary actors in the initiation of lesions within vascular wall and, once recruited by injured endothelial cells to the subendothelial space, they differentiate into macrophages which, in turn, secrete many different cytokines, reactive oxygen and nitrogen species, and ingest increasing amounts of lipids thus becoming foam cells (or foamy macrophages), as they are referred to due to the characteristic accumulation of cytoplasmic lipid droplets [4–6]. Foamy macrophages are proliferative inflammatory cells that, besides their usual inflammatory attributes, have an extremely active lipid metabolism (taking up, building and exporting lipids [7]), and release proteases, growth factors, interleukins, prostaglandins (PGs) and other mediators that activate endothelial and smooth muscle cells in their surroundings [8], rendering them secretory and proliferating cells [5,9–11]. All these interactions exacerbate the lesion, specially because atheromata is not a common inflamed tissue, but a chronic inflammation, where physiological feedback mechanisms are overcome by the constant noxious stimuli determined by the risk factors stated above which, contrarily, feed forward the arterial wall lesion.

A main feature of atherosclerosis initiation as an inflammatory disease is the activation, within endothelial cells, of nuclear factor κB (NF-κB) by different endothelial stressors [12,13]. NF-κB activation, in turn, leads to the expression of key-mediators of inflammatory and immune responses, as well as intercellular adhesion molecules [12,14]. By switching NF-κB, endothelial cells express selectins (which mediate rolling and the initial steps of leukocyte attachment to the endothelium [4,15]) and vascular cell adhesion molecule-1 (VCAM-1 also known as CD106), responsible for the firm arrest of monocytes to the arterial wall rendering them prone to transendothelial migration [4,16]. Moreover, growth factors produced by injured endothelial cells induce VCAM-1 expression in monocytes/macrophages as well [17]. Hence, the NF-κB-dependent expression of VCAM-1 (as well as other leukocyte-endothelium adhesion molecules) in response to endothelial assault in certain territories may trigger atherogenesis. Also, oxidative stress induced by activated macrophages and endothelial cells mediates the activation of NF-κB [18] thus resulting in augmented leukocyte adhesion to the endothelium [19] and perpetuation of such an inflammatory response.

After the striking discovery, in 1997, by Maria Gabriella Santoro's group in Italy, that PGA_1, a cyclopentenone prostaglandin (CP-PG), inhibits NF-κB activation [20], it has become clear that CP-PGs should be studied as potential pharmacological tools against atherosclerosis. CP-PGs (Fig. 1) are naturally occurring 20-carbon antiproliferative fatty acids derived from essential fatty acids, such as arachidonic (the most abundant in humans) that, differently from other PGs (e.g. PGE_2) which act via extracellular membrane receptors, are transported to the intracellular compartment where they promote strong biological effects. CP-PGs are characterized by an α,β-unsaturated cyclopentane ring which is obligatory to all their biological activity (reviewed in ref. [21]). The powerful anti-inflammatory activity of CP-PGs was further demonstrated by the Santoro's group which found that these PGs directly inhibit IKKβ catalytic subunit of IKK complex [22] thus blocking NF-κB activation. These findings led to the concept of CP-PGs as the physiological molecules for the "resolution of inflammation", now well accepted. CP-PGs also stimulate heat shock protein (hsp) biochemical pathway, which is *sine qua non* for their biological actions [23] and promotes tissue repair, since the expression of the chaperone hsp70 confers cytoprotection [24]. Inasmuch as our previous results have shown that the CP-PG PGA_2 has a potent suppressing effect on cholesterol metabolism of inflammatory macrophages [25], we started to investigate the effect of CP-PGs as well as other non-cyclopentenone ring-possessing PGs on foamy macrophages in order to

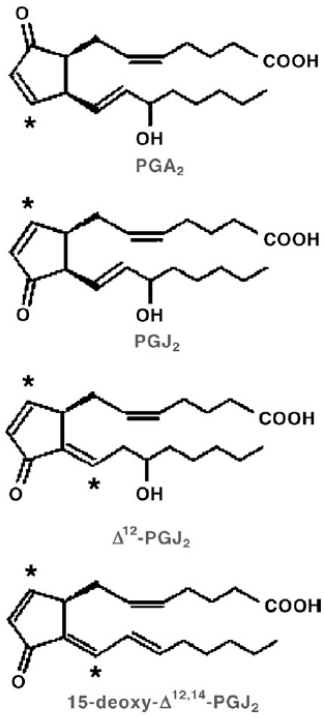

Fig. 1. Structures of CP-PGs of A_2 and J_2 families. Asterisks denote the electrophilic carbons that are susceptible to Michael addition reaction with nucleophiles, such as reactive sulfhydryls present in GSH molecules and cysteine residues of cellular proteins: C-11 in PGA_2 ring, C-9 in PGJ_2 ring, and C-9 and C-13 in both Δ^{12}-PGJ_2 and 15-d-PGJ_2 molecules.

evaluate the feasibility of a CP-PG-based therapeutic approach.

2. Materials and methods

2.1. Animals

Male LDL receptor knockout ($ldlr^{-/-}$) mice (B6129-SLDLr-$^{tm1\text{-}Her}$) and the $ldlr^{+/+}$ counterparts (B6129SF2) were purchased from The Jackson Laboratories. Since B6129SF2 mice present a number of genetic differences as compared to B6129SLDLr-$^{tm1\text{-}Her}$ mice, these $ldlr^{+/+}$ counterparts were used only in studies performed to assess LipoCardium toxicity. Male Wistar rats (utilized to obtain peritoneal macrophages) were from The Institute of Basic Health Sciences Animal Care Facility-UFRGS. Animals (five per cage) were maintained at 23 ± 2 °C in an environment illuminated from 7:00 to 19:00 h at The Institute of Basic Health Sciences Animal Care Facility. Both rats and mice were fed *ad libitum* with a Purina standard chow for rodents having free access to water until they complete 3 months when they were used in the experiments. At the age of 3 months, $ldlr^{-/-}$ mice were fed *ad libitum* with a pelleted high-lipid chow consisting of (w/w) 52.5% Purina Rodent Chow (52% carbohydrate, 21% protein and 4% lipid), casein (Sigma, 20%), powered bone mineral supplement (VETNIL Veterinary Products, São Paulo, SP; 1.5%), amino acid and vitamin complement Aminomix® (VETNIL, 10%), cholesterol 95%-pure (Sigma, 1%) and commercial lard (15%). LipoCardium experiments were conducted after mice have completed 4 months under the above hyperlipemic diet. After treatments, tissues were used for histological studies; mice were manipulated and killed by cervical dislocation under combined ketamine (90 mg/kg body weight)–xylazine (10 mg/kg body weight). Animal handling and protocols were performed as approved by the UFRGS Institute of Basic Health Sciences Committee for Animal Care and Experimentation.

2.2. Cells

Rat peritoneal macrophages were obtained as previously described [26] whereas U937 cells were from Rio de Janeiro Cell Bank. After seeding, cells (2×10^6 cells/well in 24-well plates, 1 mL final volume) were left to adhere for 1 h in RPMI1640 culture medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM glutamine, 50 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B (Gibco), except if otherwise stated. The cells were transformed into foamy macrophages by incubation (18 h, 37 °C) in the presence of 20 μg/mL mildly oxidized LDL (see below) in culture medium. Afterwards, foamy macrophages (or control untransformed cells) were washed three times with phosphate-buffered saline (PBS, pH 7.4) and treated with test PGs (or control ethanol diluent, 0.1% final concentration, by volume) and oxLDL that was freshly re-added to the medium 15 min after PG addition to avoid PG sequestration by the serum proteins. Also, when PGs were to be tested, FBS was added only after 15 min of PG treatments. Carbaprostacyclin ($cPGI_2$, Sigma), and PGA_2, PGE_2 and 15-d-PGJ_2 (Cayman) were stored as 10 mg/mL solutions at −20 °C in absolute ethanol until use and were diluted to appropriate concentrations in ethanol only at the moment of experiments. Cell viability was always assessed by trypan blue exclusion and was found to be higher than 95% after 72 h in culture.

2.3. Liposomes and LipoCardium

In order to avoid systemic action of CP-PGs employed in this study, different endothelium-directed CP-PG-based liposome (EDCPL) formulations were prepared. The best results were found when negatively charged liposomes containing anti-VCAM-1 antibodies and PGA_2 (at various concentrations) were used. Then EDCPL patent (LipoCardium) was drawn by Federal University of Rio Grande do Sul (UFRGS). Details on the preparation, types of CP-PGs, concentrations utilized and other disclosures of claims may be obtained in the LipoCardium patent documentation (see Acknowledgments and Supplementary Material).

2.4. Oxidized LDL preparation

Commercial human LDL (Sigma) was extensively dialyzed, diluted to 1 mg/mL with PBS and then mildly oxidized for 3 h at 37 °C in the presence of 5 μM $CuSO_4$ in PBS as previously described [27]. Afterwards, oxLDL was HPLC-purified (PRP-X500 Hamilton ionic exchange column, [28]) and its integrity verified by dot blot (for apoB100; apoA-1 and Lp(a) were also probed with negative results) and 5–15% gradient SDS-PAGE followed by Western blot for apoB-100. The extent of oxidation was evaluated by the thiobarbituric acid-reactive substances (TBARS) method [29] by using malondialdehyde (MDA) as standard. The values, in nmols of MDA/mg protein, were 19.5 ± 2.0 for oxLDL versus 1.0 ± 0.1 for the controls.

2.5. Labeling procedures

In order to assess lipogenesis rates, after foam cell transformation cells were pulsed with [1-^{14}C]acetate (sodium salt, 52.8 mCi/mmol, Amersham-GE) at a final concentration of 2 mM (0.4 μCi/mL). In order to label macrophage intracellular cholesterol pool and to assess cholesteryl ester metabolism, cells were cultivated with either TLC-pre-purified 0.1 μCi/mL [4-^{14}C]cholesterol (57.1 mCi/mmol, Amersham-GE) or TLC-pre-purified 1.0 μCi/mL [2,3-^{3}H]cholesterol oleate (50.0 Ci/mmol, Amersham-GE) dissolved in ethanol (0.05% final concentration, by volume)

in culture medium. The cells were cultured for additional 24 h with each radiotracer in the presence or absence of test PGs (see figure legends). In exporting experiments, cells were 24-h pulsed without test PGs and then washed three times with PBS. Therefore, fresh culture medium supplemented with test PGs was added and the cells were cultivated for additional 24 h. After labeling, the cells and supernatant fractions were lipid-extracted and prepared to be TLC-analyzed. Cholesterol and cholesteryl ester contents (mass) were spectrophotometrically assayed by the cholesterol oxidase method in the presence or absence of cholesteryl ester hydrolase (LabTest Brazil, in-factory specially customized kit). All the above procedures were carried out as previously described [26]. [^{3}H]PGA_2 was obtained by dehydrating 125 pmol (25 μCi) of [5,6,8,11,12,14,15-^{3}H(N)]PGE_2 (NEN-Perkin-Elmer, NET428025UC, 200 Ci/mmol) 70% (v/v) ethanol solution in the presence of 10N HCl in a final incubation volume of 1.1 mL for 2 h at 37 °C. After completion, the reaction mixture was twice-extracted with 1-mL volumes of chloroform/methanol (2:1, by volume) and lipid fraction was then re-extracted with three volumes of 50 μL of chloroform/methanol (2:1, by volume), purified by TLC (F254 250-μm plates, Sigma) and re-extracted with 50 μL ethyl acetate in the presence of cold PGA_2 as described in ref. [30]. PGA_2 identity was confirmed by HPLC (Shimadzu) after Sep-PakC18 (Millipore) column pre-purification [31]. [^{3}H]PGA_2 was added to EDCPL preparations and injected in the animals to be used as a tracer.

2.6. RT-PCR

Total RNA (2 μg) was treated with 1 U DNAse (Invitrogen) for 25 min at 25 °C and inactivated with 2.5 mM EDTA. Afterwards, the cDNA was synthesized using oligo $(dT)_{12–18}$ in a 20-μL reaction containing 1 mM of each dNTP, 10 mM DTT, and 200 Units SuperScript™ II Rnase H$^-$ reverse transcriptase (Invitrogen) at 42 °C for 50 min according to manufacturer's instructions. The reaction was inactivated by heating at 70 °C for 15 min. PCR reactions were performed in a total volume of 50 μL, containing 5 μL of DNA polymerase enzyme buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 9.0), 10 pmol of the primers, 200 μM of each nucleotide (dATP, dCTP, dGTP, and dTTP) and 2.5 U of Taq DNA polymerase (Amersham-GE), and 2 μL of cDNA. The reverse-transcriptase and PCR reactions were carried out in a Techne Touchgene Gradient (model FTGRAD2SD, Cambridge, UK). Primer sequences and RT-PCR protocols and product analyses were carried out as described earlier [32]: *HMG-CoA reductase* (annealing temperature 56 °C), sense 5′-CAAGCCTAGAGACATAATCATC-3′, antisense 5′-TACCATGTCAGGGGTACGTC-3′; *PPARγ* (annealing temperature 59 °C), sense 5′-GCATTATGAGACATCCCCAC-3′, antisense 5′-TCTCTCCGTAATGGAAGACCA-3′; *CD36* (annealing temperature 55 °C), sense 5′-GAGAACTGTTATGGGGCTAT-3′, antisense 5′-TTTCAACTGGAGAGGCAAAGGC-3′; *Caspase-3* (annealing temperature 58 °C), sense 5′-TGTTTCAGCATGGCACAAAGCG-3′, antisense 5′-GTCGATGCAGCAAACCTCAGGG-3′; *p53* (annealing temperature 60 °C), sense 5′-GCACAAACACGCACCTCAAAGC-3′, antisense 5′-CTTGCATTCTGGGACAGCCAAG-3′; *Bcl-x$_L$* (annealing temperature 59 °C), sense 5′-GGTCAGTGTCTGGTCATTTCCG-3′, antisense 5′-CATGGCAGCAGTAAAGCAAGC-3′; *β-actin* (annealing temperature 55 °C), sense 5′-GTGGGGCGCCCCAGGCACCA-3′, antisense 5′-CTCCTTAATGTCACGCACGATTTC-3′. Images were analyzed by using the software Kodak Digital Science 1D Image Analysis (Life Technologies) and the results were normalized in terms of β-actin expression.

2.7. SDS-PAGE and immunoblotting for hsp70

After experiments, foamy macrophages were processed and prepared for SDS-PAGE and immunoblot analysis of hsp70 expression exactly as described in ref. [33]. Sigma H5147 monoclonal anti-hsp70 antibody (which recognizes both the 73-kDa constitutive HSC70 and the 72-kDa inducible hsp70 isoforms), anti-β-actin (A5441) and horseradish peroxidase-labelled secondary antibodies (A9044) were used.

2.8. Histology and morphological analyses

After treatments and cultures, foamy macrophages were washed three times with 1-mL volumes of PBS and freshly processed for morphological *in situ* analyses by the Oil Red O, Sudan III or Sudan Black techniques [34]. All the dyes were from Sigma. Cells were then directly observed under phase contrast light microscope coupled to interference filter and camera (Olympus BX60 system). For histological tissue examinations [35], the mice were ketamine/xylazine anesthetized (see above), heparinized (50 U in 100 μL PBS) and intracardiacly perfused with PBS (10 mL) followed by a 4% paraformaldehyde solution in PBS (10 mL). Tissues (see results) were surgically excised, fixed, serially cut (50 μm, Leitz 1720 cryostat, Wetzlar, Germany), mounted on slides, treated with hematoxylin–eosin (Sigma) [34] or prepared for Sudan Black [35] to be photographed under light microscopy. Color intensities and cells structures were evaluated by VDS equipment (Amersham-GE) and Olympus MIC-D digital microscope followed by quantitation via ImageMaster (Amersham-GE) software.

2.9. Statistical analysis

As demanded in each case, one-way ANOVA followed by Bonferroni's post test for multiple comparisons, and unpaired bitailed Student's *t*-test were used throughout. Only those differences where the probability α for type I errors were $P < 0.05$ (see the text and figure captions for individual P values) were considered significant. Analyses were performed by using GraphPad InStat (v. 3.06).

3. Results

3.1. PG effects on lipid metabolism in foam cells

In order to assess the effects of PGs on foamy macrophage lipogenesis (the *de novo* lipid synthesis from acetyl-CoA), the cells were pulsed with [1-^{14}C]acetate for 24 h in the presence or absence of the synthetic analog of prostacyclin, carbaprostacyclin (cPGI$_2$, 1 μM), prostaglandin E$_2$ (PGE$_2$, 1 μM) and the CP-PGs PGA$_2$ (1 or 20 μM) and 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ (15-d-PGJ$_2$, 1.5 μM) to check for the fates of acetyl unit as schematized in Fig. 2L. OxLDL-dependent transformation of macrophages into foam cells decreased total (cellular + supernatant fractions) phospholipids synthesis by 40%, mainly by decreasing the export of synthesized phospholipids (46%-decrease, $P = 0.0293$; Fig. 2A), which constitute the major fate for foamy macrophage phospholipids produced via *de novo* synthesis. Among PGs tested, only CP-PGs enhanced total phospholipids synthesis: PGA$_2$ by up to 70% and 15-d-PGJ$_2$ by 73%. This was due basically to augmented phospholipid export (PGA$_2$, at 1 μM, by 92%, $P = 0.0173$ and 15-d-PGJ$_2$ by 71%, $P = 0.0256$). Contrarily, all the PGs repressed cholesterol synthesis (which was 2.9-fold higher in foamy macrophages as compared to control cells in terms of total synthesis, $P = 0.0001$; Fig. 2B). Although cholesterol exported from foamy macrophages was found to be roughly the same in all the groups, the effects of PGs on the synthesis of cholesterol maintained within the cells was dramatic. While oxLDL treatment enhanced intracellular cholesterol by 23.6-fold ($P = 0.0008$), all the PGs reduced it by *ca.* 90% (at least $P < 0.002$). As shown in Fig. 2C, fatty acid fates were not affected by either foam cell transformation or PG treatment, although PGA$_2$ (1 μM) seemed to lead to a tendency of raise ($P = 0.0809$). Total (intracellular + exported) triacylglycerol rate of *de novo* synthesis was greatly enhanced by foam cell transformation (a 2.7-fold increase relative to control cells), which reflected the 15-fold rise in the rate of triacylglycerol found within the cells, $P = 0.0001$; Fig. 2D). In this case, while 15-d-PGJ$_2$ enhanced it by 48% (due to a 56%-rise, $P = 0.0122$), PGA$_2$ (1 μM) caused a 38% decrease ($P = 0.0269$) in the rate of triacylglycerol accumulation but no effect was found in the total values. Intriguingly, at the highest dose, PGA$_2$ also tended to increase total triacylglycerol synthesis (by 26%, $P > 0.05$). Remaining PGs did not affect incorporations into triacylglycerols. Except for the fact that the total rate of cholesteryl ester *de novo* synthesis from [1-^{14}C]acetate was not affected by foam cell transformation, the influence of PGs on cholesteryl ester accumulation was similar to that observed for triacylglycerols. As depicted in Fig. 2E, PGA$_2$ at the lowest concentration did not affect the results whereas both PGA$_2$ (20 μM) and 15-d-PGJ$_2$ (1.5 μM) significantly enhanced total cholesteryl ester synthesis by 60% ($P = 0.010$) and 68% ($P = 0.050$) respectively, which was found to be due to their enhancing effects on cholesteryl esters found in the cellular fraction (20 μM PGA$_2$ by 71%, $P = 0.0099$, and 15-d-PGJ$_2$ by 100%, $P = 0.040$).

Besides an enhanced cholesterol synthesis, uptake and export rates of both cholesterol and cholesteryl esters may be of importance in leading to lipid accumulation in foamy macrophages. Hence, we addressed these points by labeling foamy macrophages for 24 h with either [4-^{14}C]cholesterol or [2,3-^{3}H]cholestery oleate in the presence or the absence of the above mentioned PGs, which gives a scenario of the cholesterol and cholesteryl ester taken up by the cells. Additionally, in order to evaluate PG effects on the exporting rates of cholesterol derivatives, cells were 24-h pulsed with the radiotracers (without any PG addition) and, after washing, foamy macrophages were treated for additional 24 h with PGs. As shown in Fig. 2F, only PGA$_2$ at 1 μM interfered in cholesterol uptake, by reducing total (free + esterified) cholesterol incorporation into foamy macrophages ($P = 0.0342$), which was a consequence of a decrease in the amount stocked in the free form ($P = 0.0410$) and a decline ($P = 0.0001$) in cholesteryl esters synthesized from the cholesterol added to the culture medium. Moreover, PGA$_2$ also enhanced total cholesterol export towards the extracellular space (Fig. 2G) by 65% (at 1 μM, $P = 0.00269$) and 87% (at 20 μM, $P = 0.0294$). Although the export of cholesteryl esters synthesized from the cholesterol given to the cells was negligible relative to free cholesterol exported (more than 95% of the total cholesterol exported), oxLDL transformation reduced the rate of cholesteryl ester exported in 24 h by 45% (in relation to control, $P = 0.0120$) while CP-PG treatment reversed this effect: PGA$_2$ (1 μM) a 100% rise ($P = 0.0725$), PGA$_2$ (20 μM) a 583% rise ($P = 0.0157$), and 15-d-PGJ$_2$ a 143% rise ($P = 0.0001$). Even though predictable pathways for intracellular distribution and final fates of [4-^{14}C]cholesterol should be as described in Fig. 2M, the profile of [4-^{14}C]cholesterol moieties was also checked in the cells after the 24-h period of export towards the extracellular space. The results (Fig. 2H) showed, that among the PGs tested, only CP-PGs reduced the amount of total cholesterol remaining within foamy macrophages after the exporting period. This was due to a decreasing effect on free cholesterol (roughly by 40%; P at least 0.0134), since no alteration in the remaining cholesteryl ester pool was observed. Hence, these data approximately match those from exporting rates, where only PGA$_2$ significantly enhanced the exit of cholesterol from the cells.

As depicted in Fig. 2N, it is expected that cholesteryl esters (whose fates were traced by using [2,3-^{3}H]cholesterol-oleate herein) after entering cells, be (a) returned to the extracellular space in the intact form, (b) be hydrolyzed by the action of intracellular cholesteryl ester hydrolases to fatty acid and free [2,3-^{3}H]cholesterol which may be followed, or (c) be re-esterified with the same or other fatty acyl-CoA via acyl-CoA:cholesterol acyl transferase (ACAT) forming a radiolabeled cholesteryl ester that may be measured inside or outside the cells. As shown in Fig. 2I, foam cell transformation enhanced cholesteryl ester uptake by 87% ($P = 0.0001$), while CP-PGs reversed this feature by decreasing radioac-

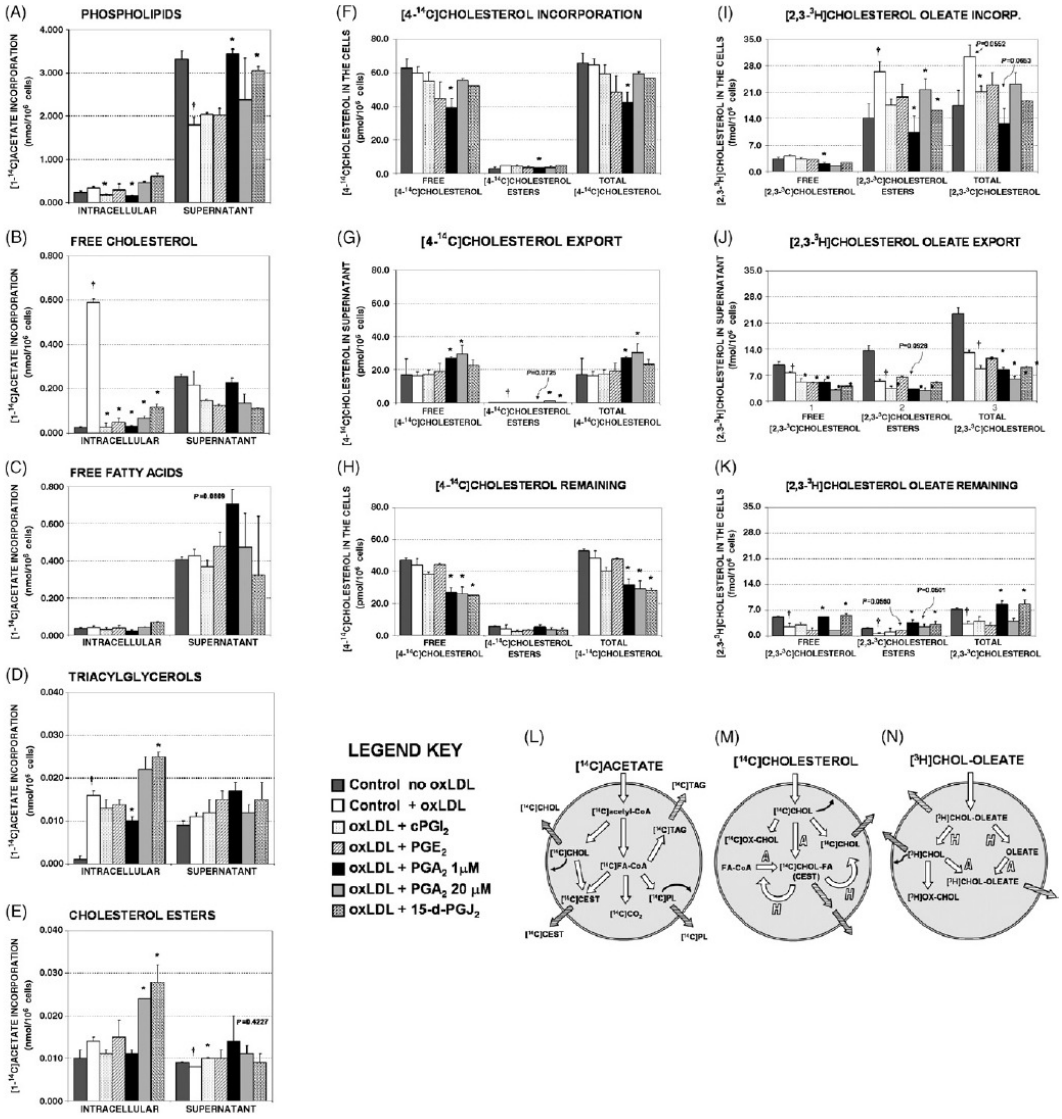

Fig. 2. PG effects on lipogenesis and cholesterol metabolism in oxLDL-elicited foamy macrophages. [1-^{14}C]acetate incorporations (24 h) into both cell (lipogenesis) and supernatant (exporting rates) lipid fractions were assessed in the presence or absence of test PGs: (A) phospholipids, (B) free cholesterol, (C) free fatty acids, (D) triacylgycerols and (E) cholesteryl esters. (F) [4-^{14}C]Cholesterol uptake and intracellular distribution into free cholesterol or cholesteryl esters. The influence of PGs on cholesterol export towards the extracellular space (G) in [4-^{14}C]cholesterol-labeled cells was assessed after washing and further cultivating cells for additional 24 h; (H), cholesterol moieties remaining in the cells. (I) [2,3-^{3}H]cholesterol oleate uptake and distribution (hydrolysis and re-esterification). The effects of PGs on exporting capacity and fates of cholesteryl esters were assessed by measuring [2,3-^{3}H]cholesterol moieties exported (J) from or remaining (K) in the cells cultured for additional 24 h. Data are the means ± S.E.M. of three different preparations, each in double. Significant differences: (*) between each test group and the oxLDL controls by the Student's *t*-test; (†) between control (untransformed) and oxLDL controls (one-way ANOVA, Bonferroni's test); *P* values are in the text. Expected routes for: (L) [1-^{14}C]acetate-derived acetyl units, (M) [4-^{14}C]cholesterol and (N) [2,3-^{3}H]cholesterol fluxes. In this inset: black arrows represent lipid incorporation into cell membrane; *A* and *H* letters indicate reactions catalyzed by ACAT and cholesteryl ester hydrolases respectively; shaded arrows indicate lipid export from the cell. CHOL, cholesterol; CEST, cholesteryl ester; PL, phospholipid; TAG, triacylglycerol; OxCHOL, oxysterols derived from the oxidation reactions over cholesterol molecules; CHOL-FA, cholesteryl esters of fatty acids.

tive incorporation into foamy-macrophage cholesteryl ester pool. This was noteworthy in the case of PGA_2 at 1 μM (60% decrease, $P = 0.0260$). [2,3-^{3}H]radioactivity found in the free cholesterol pool after the 24-h period in culture cues to the hydrolytic capacity of such cells. Accordingly, PGA_2 (1 μM) was able to decrease cholesteryl ester hydrolysis by 48% ($P = 0.0259$). Contrarily to that observed for the cholesteryl esters incorporated into foamy macrophages, the results of Fig. 2J clearly showed that foam cells exported 44% less [2,3-^{3}H]cholesterol moieties (total amounts) than did control untransformed cells ($P = 0.0001$). In this case, all the PGs tested diminished the rates of [2,3-^{3}H]cholesterol exported in 24 h, which suggests some possible interference in the mechanisms of cholesteryl ester export, since foamy macrophages took up more (Fig. 2I) and hydrolyzed less (21%-decrease, $P = 0.0398$, leftmost part of Fig. 2J) cholesteryl esters than did control cells and, except for PGA_2 (1 μM), PGs did not alter these two biochemical pathways. These data were confirmed when [2,3-^{3}H]cholesterol remaining in the cells after PG treatment was assessed: both PGA_2 (1 μM) and 15-d-PGJ_2 enhanced [2,3-^{3}H]cholesterol moieties stocked in the cells by 170% (P at least 0.0023; Fig. 2K). Taken as a whole and apart from this possible influence on cholesteryl ester transport towards the outside, the above results indicate that PGA_2 may reduce both cholesteryl ester synthesis (Fig. 2F) and hydrolysis (Fig. 2I).

Once radiotracing of biochemical pathways gives only the rates of metabolic fluxes and not necessarily implicates "mass alterations" during these periods, we investigated whether the above test PGs could influence cholesterol contents in larger periods of culture. Hence, macrophages were 18-h pre-incubated with oxLDL before PG additions and then cultivated for additional 72-h in the presence of test PGs and oxLDL (freshly re-added to the medium 15 min after PG addition to avoid PG sequestration by the lipoproteins). Although $cPGI_2$ and PGE_2 showed some diminishing effect (roughly by 10%, $P < 0.05$) on cholesterol accumulation in foamy macrophages (data not shown), PGA_2 has proven to be more effective, despite the concentration employed (Fig. 3A). After 72 h in culture, foamy macrophages showed a 42% increase in the mass of total (free + esterified) cholesterol ($P = 0.009$), which reflected a rise in both free cholesterol (by 31%, $P = 0.010$) and cholesteryl ester (by 173%, $P = 0.009$) contents. As expected, foamy macrophages doubled the proportion between cholesteryl ester to total cholesterol during this period (from 8.2% to 15.8%, $P = 0.010$). PGA_2 reduced free cholesterol mass by 27% ($P = 0.004$) at 1 μM and by 29% ($P = 0.005$) at 20 μM. Interestingly, PGA_2 decreasing effect on total cholesterol seemed to be due only to free cholesterol decrease, since cholesteryl ester contents during the same period were found to be enhanced by roughly 60% (although not significantly) owing to PGA_2 addition. On the contrary, 15-d-PGJ_2 augmented total cholesterol contents by approximately 10% ($P = 0.001$; data not shown) due to an effect of cholesteryl ester mass (increased by 54%, $P = 0.005$; data not shown).

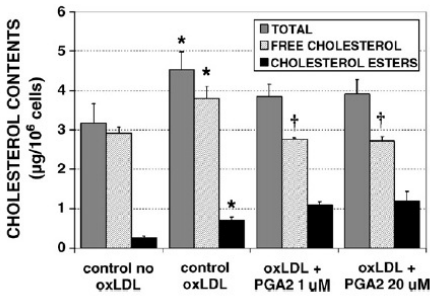

Fig. 3. Effect of PGA_2 on cholesterol accumulation in foamy macrophages. OxLDL-elicited foamy macrophages were treated with PGA_2 for 72 h and assayed for cholesterol and cholesteryl ester contents (mass). Data are the means ± S.E.M. of three different preparations, each in duplicate. Significant differences: (*) Student's t-test, between each test group and control (no oxLDL) values; (†) between each PGA_2 test group and oxLDL controls (one-way ANOVA, Bonferroni's test). Individual P values are given in the text.

3.2. Gene expression in CP-PG-treated foam cells

Since PGA_2 showed a marked inhibition on cholesterol *de novo* synthesis, we argued whether this could be related with some influence on 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the main key-enzyme of cholesterogenesis. In the following experiments we used U937 pro-monocytic cell line because of its ease and well-known behavior as foam cell after oxLDL treatment. U937 foam cells cultured for 24 h in the presence of CP-PGs and oxLDL as described in Section 2. As expected, foam cell transformation led to a 41%-rise ($P = 0.0001$) in HMG-CoA reductase mRNA expression, whereas PGA_2 (1 μM) reduced it ($P = 0.0003$; Fig. 4A). Surprisingly, however, in foamy U937 cells, PGA_2 had the opposite effect, increasing HMG-CoA reductase mRNA by 60% ($P = 0.0001$).

PPARγ mRNA expression was elevated by the 15-d-PGJ_2 treatment ($P = 0.0001$) in normal (untransformed) cells, while, in foam cells, this J-series PG reduced it by 87% ($P = 0.001$; Fig. 4B). In turn, PGA_2 reduced mRNA PPARγ expression in both normal and foamy macrophages ($P = 0.0001$). Similar results were obtained with CD36 mRNA expression in control macrophages (Fig. 4C), where PGA_2 reduced mRNA levels of the receptor ($P = 0.0001$) and 15-d-PGJ_2 rose them ($P = 0.0005$). Intriguingly, however, in foamy macrophages, while 15-d-PGJ_2 also reduced CD36 mRNA contents ($P = 0.0022$), PGA_2 induced a 110%-increase in CD36 mRNA expression ($P < 0.0001$).

In control cells, both CP-PGs had a minimal effect on caspase-3 mRNA expression. On the other hand, in foamy macrophages, in which caspase-3 mRNA was 96%-suppressed (as compared to control, $P = 0.0001$; Fig. 4D), both CP-PGs elevated mRNA levels in relation to untreated foam cells: 60-fold in PGA_2 ($P < 0.0001$) and 18-fold in 15-d-PGJ_2 group ($P < 0.0001$). However, while 15-d-PGJ_2

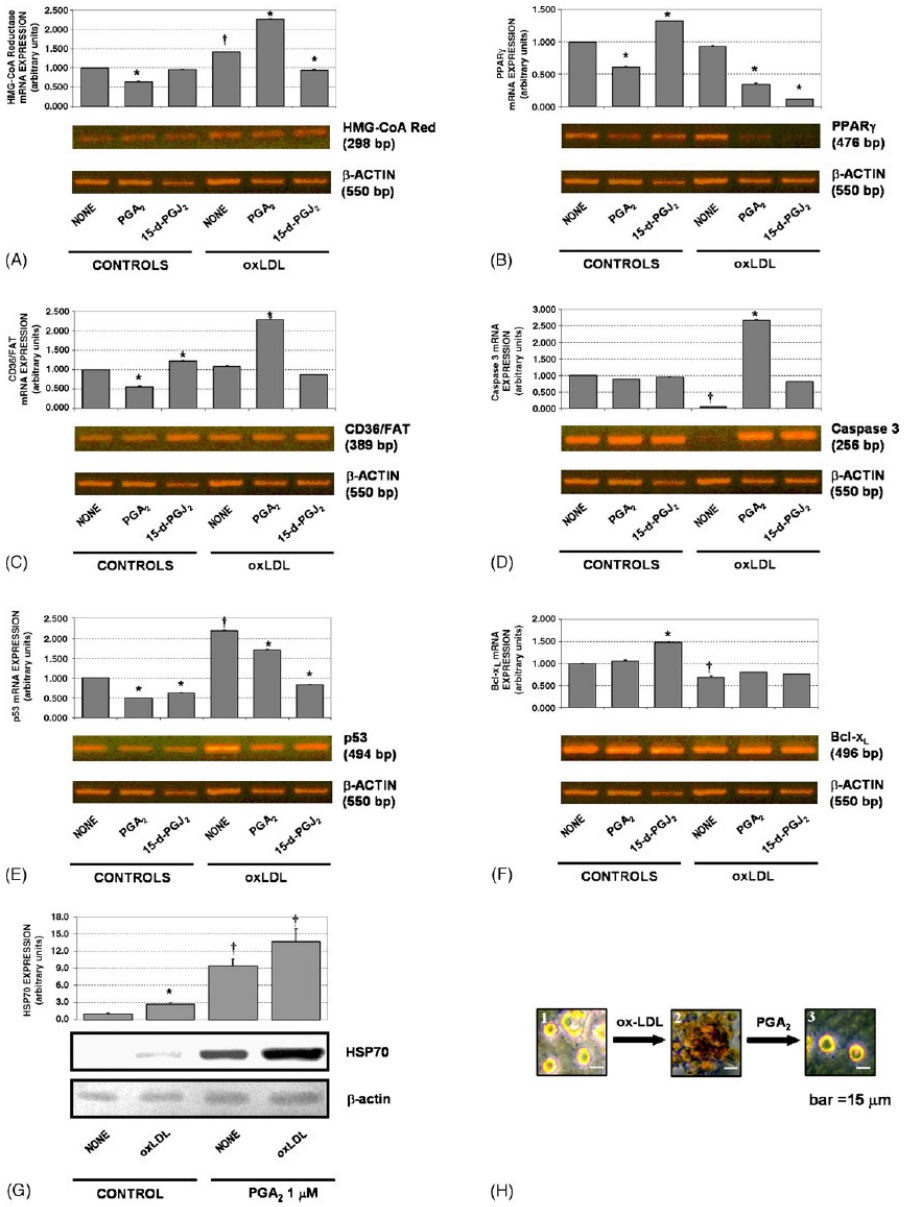

Fig. 4. Effects of PGA_2 and 15-PGJ_2 on gene expression. OxLDL-transformed human U937 foamy macrophages were treated (24 h) with PGA_2, 15-PGJ_2 or ethanol diluent and assayed for expression of mRNA encoding HMG-CoA reductase (A), PPARγ (B), CD36 (C), caspase-3 (D), p53 (E) and bcl-x_L (F). Rat-derived foamy macrophages were identically treated and assayed for hsp70 protein expression (G). Data are the means ± S.E.M. of three different experiments in duplicate. Significant differences: (*) Student *t*-test, between each test group and its control (oxLDL or no oxLDL); (†) between control (untransformed) and oxLDL controls (one-way ANOVA, Bonferroni's test); *P* values are in the text. (H) Interference photomicropgraphies of Sudan-III stained rat macrophages. Control cells (1) were oxLDL-transformed into foam cells (2) and showed the characteristic high-lipid "fatty-islet" shape. The cells were 24-h treated with 1 μM PGA_2 (3) still in the presence of oxLDL (added 15 min after PGA_2 addition).

treatment led foamy macrophage caspase-3 mRNA levels to reach approximately the same values found in control untransformed cells, PGA_2 promoted an enhancement in caspase-3 mRNA expression that was 2.7-fold higher than that found in untransformed cells, thus suggesting that PGA_2 may be much more pro-apoptotic to foam cells than 15-d-PGJ_2.

In the present work, oxLDL transformation induced a 2.2-fold rise in p53 mRNA levels relative to control untransformed cells ($P=0.001$; Fig. 4E). Also, in control macrophages both PGA_2 and 15-d-PGJ_2 reduced p53 mRNA expression ($P<0.0001$). This reducing effect persisted in CP-PG-treated oxLDL-transformed macrophages ($P<0.0001$). Moreover, oxLDL-elicited macrophage transformation into foam cells decreased the anti-apoptotic gene product Bcl-x_L mRNA levels ($P=0.0001$; Fig. 4F). PGA_2 treatment augmented Bcl-x_L mRNA content ($P=0.0079$) only in foamy macrophages, whereas 15-d-PGJ_2 significantly enhanced it in both control and foamy macrophages (at least $P=0.0161$).

Hsp70 expression was barely detectable in untransformed rat peritoneal macrophages, whereas oxLDL transformation led to a 2.7-fold higher expression ($P=0.0016$; Fig. 4G). PGA_2 treatment (24 h in the presence of oxLDL) induced a five-fold increase in hsp70 expression in foamy macrophages ($P=0.0072$) while the ability of PGA_2 to induce hsp70 expression was still higher in control-untransformed macrophages (9.4-fold, $P=0.0024$). Apparently, PGA_2 treatment was extremely beneficial for foamy macrophages, as judged by the histological manifestations in response to PG treatments. Accordingly, rat macrophages transformed into foam cells showed a striking "fatty-islet" pattern with apoptotic-like blebs, vacuoles and lipid inclusions in culture which are evident under Sudan III staining (Fig. 4H). After PGA_2 (1 μM) treatment, foamy macrophages showed a morphology that seemed to be much healthier than that of the untransformed controls themselves. Since hsp70 is cytoprotective, repairs oxidatively stressed cell proteins and was enhanced in PGA_2-treated foamy macrophages (Fig. 4G), we conjecture that hsp70 expression could be associated with PGA_2 beneficial effect in these cells. Other PGs, namely $cPGI_2$, PGE_2, PGA_2 (20 μM) and 15-d-PGJ_2, were also examined for morphological alterations under Sudan III and Sudan Black staining, but only PGA_2 at 1 μM showed this characteristic cytoprotection (data not shown). Because of this, 15-d-PGJ_2 was not tested for hsp70 induction and we started to study *in vivo* preparations containing PGA_2 at concentrations that allowed to maintain 1-μM doses within atherosclerotic lesions.

3.3. In vivo studies

Atherosclerosis was induced *in vivo* by feeding LDL receptor knockout ($ldlr^{-/-}$) adult (3 months) mice with a hypercholesterolemic diet for 4 months. After this period, animals become very sick showing characteristic diarrhea, loss of hair and of hair brightness, blindness and mutilation of extremities (e.g. fingers, ears) due to vascular complications in these fields (Fig. 5A–C). At this time, PGA_2 treatments were initiated. To overcome systemic PGA_2 toxicity, particularly the strong antiproliferative effect on immune system and rapidly proliferating cells [21], liposomes were prepared containing PGA_2 and antibodies against VCAM-1 in order to specifically direct liposome particles onto injured arterial wall lining. $Ldlr^{-/-}$ mice were therefore treated for different time periods (still under high-lipid diet) with different endothelium-directed CP-PG-based liposome (EDCPL) formulations. Other CP-PGs were also tested, but the best results were found when negatively charged liposomes containing anti-VCAM-1 antibodies and PGA_2 (at various concentrations) were employed. These preparations gave rise to LipoCardium patent deposit.

Two-week treatment (daily, i.p.) with LipoCardium was found to be sufficient to impair death by myocardium infarction or stroke that was verified in control non-treated $ldlr^{-/-}$ mice. Also, as shown below, LipoCardium treatment completely reversed vascular lesions and the sickness state of the animals (Fig. 5D) while prolonging their lifespan until the elderly age, even under high-lipid diet. By using [^{3}H]PGA_2, tissue distribution of a single dose of LipoCardium was found as follows (in percentage of total radioactivity recovered as the means ± S.E.M.): thoracic aorta, 64.7 ± 7.7; heart, 4.7 ± 0.8; lungs, 5.8 ± 1.1; kidneys, 4.0 ± 0.8; liver, 1.6 ± 0.4; spleen, 19.2 ± 0.9; other tissues, less than 0.1%. The same negatively charged EDCPL when formulated without anti-VCAM-1 antibodies have shown reduction in the distribution of [^{3}H]PGA_2 within aorta (by 47% in respect to controls with antibodies, $P=0.0343$), which was otherwise re-distributed among remaining tissues (an 89%-rise; $P=0.0234$), specially to the spleen (65%-rise; $P=0.0400$) and kidneys (up to 36% of total radioactivity recovered). Positively charged EDCPL with or without anti-VCAM-1 antibodies did not reverse atherosclerotic lesions as evaluated by histological examination. When negatively charged EDCPL formulation (LipoCardium) was injected intracardiacly, [^{3}H]PGA_2 radioactivity was recovered as follows: thoracic aorta, 40.2 ± 6.3; heart, 18.0 ± 0.7; lungs, 15.7 ± 4.5; kidneys, 4.6 ± 0.3; liver, 5.8 ± 0.4; spleen, 15.7 ± 5.2; other tissues, less than 0.1%). Neither PGA_2 nor antibodies against VCAM-1 when injected alone, at the same concentrations used in EDCPL, could reverse arterial disease as LipoCardium did. LD_{50} for i.p. administration of LipoCardium was calculated as *ca.* 25 mg/kg/day in terms of PGA_2 and body weight, while for intracardiac administration was *ca.* 1 mg/kg/day.

Histological evaluations by using Sudan Black staining (which evidences neutral lipids, such as cholesteryl esters) showed that LipoCardium treatment markedly reduced lipid accumulation in the aorta, renal and coronary arteries (Fig. 5E–L). Of note is the fact that LipoCardium reduced neointimal hyperplasia and thickness of aortas by 32% ($P=0.0020$), thus restoring the thickness to control levels (Fig. 5N) whereas the high cellular proliferative patterns

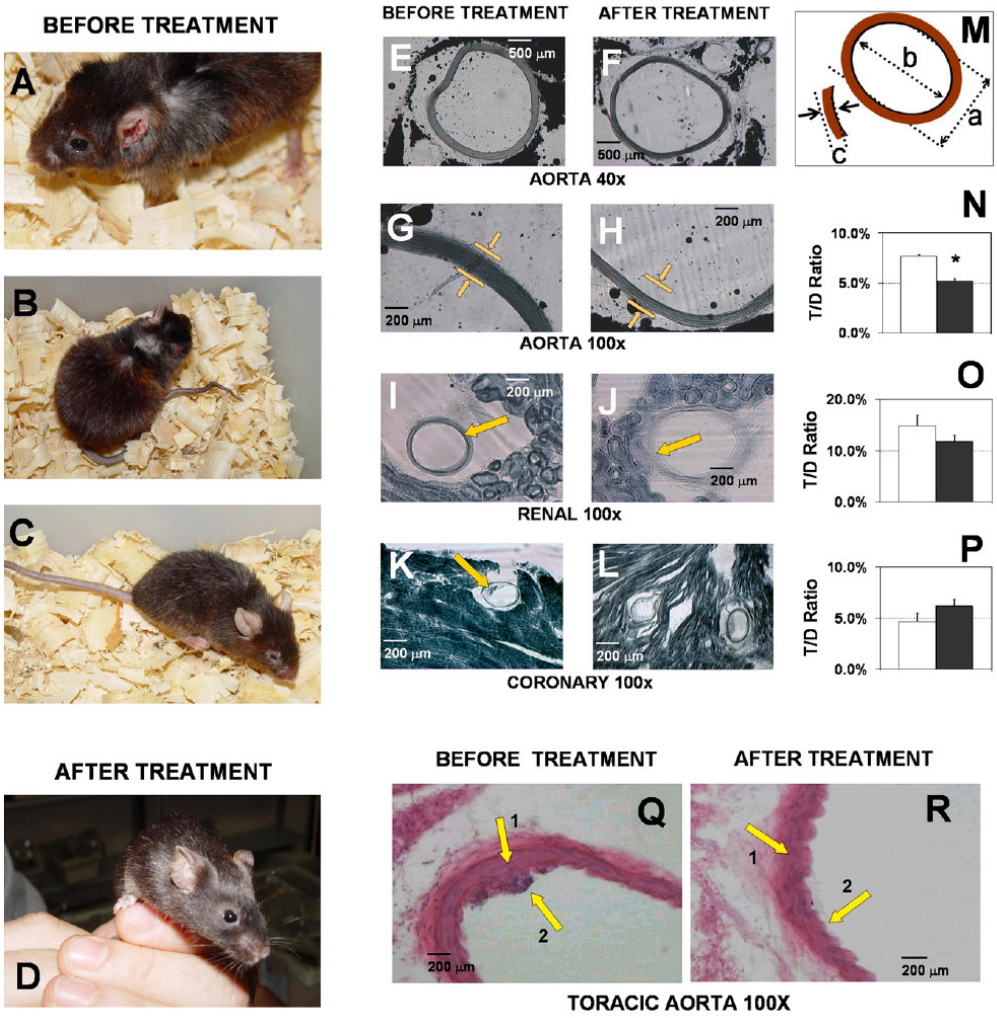

Fig. 5. LipoCardium *in vivo* effects. Male ldlr$^{-/-}$ mice under a high-fat diet during 4 months, commencing from the adulthood (3 months), showed marked evidence of cardiovascular disturbances such as lost of extremities (A) due to poor peripheral blood supply, loss of hair and of hair brightness (B), prostration, blindness and generalized sickness (C). Then, the animals were daily treated (i.p.) with LipoCardium (or EDCPL placebo injections containing no PGA_2) for 14 days when they were killed to be analyzed for anatomical-pathological alterations or maintained under high-lipid diet (with no LipoCardium treatment) until aging. A 14-day treated mouse is shown (D). The effects of LipoCardium treatment (or placebo) on the thoracic aorta (E–H), renal artery (I and J) and coronary artery (K and L) slices are shown in tissues stained with Sudan black dye (which evidences neutral lipids). Thickness-to-diameter (*T*/*D*) ratios were calculated as follows: $T/D = c/[(a+b)/2]$, where c is the average thickness (measured in three different points along the artery perimeter), and a and b are the smallest and the largest internal diameters respectively (M). Mean values ± S.E.M. (expressed as percentage values) of six different animals are shown for thoracic aorta (N), renal (O) and coronary artery (P) preparations. $^{*}P = 0.0020$ for the difference between control and LipoCardium-treated groups (Student's *t*-test). Q and R: hematoxylin–eosin stained slices of thoracic aortas. Arrows: subendothelial cell proliferation and neoitimal hyperplasia (Q1), which is absent in LipoCardium-treated animals (R1), and the typical basophilic cytoplasmic staining of infiltrating cells (characteristic of foamy macrophage subendothelial occupation, Q2), which is absent in LipoCardium-treated groups (R2).

and cell infiltration in subendothelial layer were also completely defeated (Fig. 5Q and R). Interestingly, LipoCardium seemed to suppress vascular lesions despite any alteration in cholesterolemia which persisted at high values (approximately 1600 mg/dL of total cholesterol), thus suggesting that LipoCardium effects may actually be related with local action within arterial wall.

4. Discussion

Although atherosclerosis has been demonstrated to be closely related with dysfunctions in lipid metabolism at the level of arterial wall cells [2,6,10], the possible role of combined defects in lipid synthesis, export and entry may have been neglected in favor of oxLDL uptake by foam cells, which, in fact, plays a role in foamy macrophage lipid accumulation. In this study, we demonstrated, for the first time, that CP-PGs might be of value in reducing cholesterol and cholesteryl ester accumulation in foamy macrophages (Figs. 2 and 3). Our findings indicate that all the PGs tested diminished the *de novo* cholesterol synthesis, but only CP-PGs were capable of deviate acetyl units towards phospholipid synthesis. This effect was particularly strong in the case of PGA_2 (1 μM), corroborating our previous findings with thioglycollate-elicited inflammatory macrophages [25].

Since fatty acid synthesis appeared to be not influenced by CP-PG treatment and HMG-CoA reductase mRNA expression was even enhanced, at the same time that PGA_2 deviated cholesterogenesis towards the synthesis of phospholipids, it is possible that PGA_2 inhibiting effect on cholesterogenesis should be due to a strong influence on HMG-CoA reductase activation status [36], but not with lipogenesis mediated by acetyl-CoA carboxylase or fatty acid synthase complex activities. Enzymes of phospholipid synthesis and/or export may also be targets for the PGA_2 action.

The inhibitory effect of PGA_2 on cholesterogenesis resembles in much that of statin therapy, which is known to stabilize atheroma plaques and is accompanied by antiproliferative and anti-inflammatory effects [37], the latter possibly due to statin antioxidant properties [38]. Remission of cholesterol accumulation in foamy macrophages has important consequences to atherogenesis, since intracellular cholesterol contents regulate several cellular functions [26], including expression of the scavenger receptor CD36 [39], which in turn mediates lipid accumulation.

Among CP-PGs, only the J-series ones are capable of acting as physiological ligands for the nuclear factor peroxisome proliferation-activated receptor of gamma type (PPARγ), that is responsible for the expression of the oxLDL scavenger receptor CD36, which is considered a key determinant of macrophage foam cell formation and atherogenesis [40]. Therefore, it is very interesting the finding that, in normal macrophages, PGA_2 reduced both PPARγ and CD36 mRNA levels, whereas 15-d-PGJ_2 showed opposite effects. It is much more of note that, in foamy macrophages, PGA_2 doubled CD36 mRNA levels despite its inhibitory effect over PPARγ mRNA expression. These results suggest that additional PGA_2-sensitive pathways to control the expression of the scavenger receptor CD36 may exist other than via PPARγ activation. Anyway, PGA_2 impaired cholesterol and cholesteryl ester (mass) accumulation even in the presence of augmented CD36 mRNA expression, whereas 15-d-PGJ_2 augmented intracellular cholesterol mass due to a 54%-rise in cholesteryl ester contents. Whether this dissimilitude is due to some difference in the influences that these two CP-PGs have over PPARγ remains to be elucidated. Nevertheless, this indicates that, although the inward flux of cholesterol via CD36 scavenger receptor may be of importance to dictate atherogenesis, the management of intracellular cholesterol contents through the regulation of its *de novo* synthesis, as well as the balance between import and export of cholesterol moieties, should also be of great importance. Furthermore, PGA_2 seems to block the undesirable cholesteryl ester hydrolysis/re-esterification cycle (which tends to drag and trap both cholesterol and cholesteryl esters inside the cells), but probably not via CD36 scavenger receptor uptake which is found to be enhanced in PGA_2-treated foamy macrophages. This is an important finding since ACAT inhibition has been pointed out to have anti-atherogenic effect [41,42].

Altogether, the combined results obtained with radiotracers, cholesterol mass measurements and gene expression led us to propose that CP-PGs, but specially PGA_2, should have a beneficial effect over foamy macrophage cholesterol metabolism which involves: (1) inhibition of *de novo* cholesterol synthesis with deviation of acetyl-CoA units towards the synthesis of phospholipids which are subsequently exported from cells; (2) reduced entry of cholesterol and cholesteryl esters; (3) reduced cholesterol esterification/hydrolysis recycling pathway. The summation of these individual contributions makes PGA_2 one of the most powerful agents capable of blocking intracellular cholesterol accumulation known as yet.

The striking effects of PGA_2 on foamy macrophage lipid metabolism may explain, at least partially, the benefits of LipoCardium observed *in vivo*, specially because endothelial as well as smooth muscle cells in atheromatous plaques are also transformed into foam cells and work similarly as foamy macrophages do [4–6]. Anti-inflammatory and antiproliferative PGA_2 effects should also play a role, since there was a conspicuous disappearance of inflammatory cells and neointimal hyperplasia in the aorta of LipoCardium-treated $ldlr^{-/-}$ atherosclerotic mice. This assumption is reinforced by the finding that other CP-PG-like compounds, namely 2-cyclopenten-1-one and PGJ_2, reduced restenosis after balloon angioplasty in rats, an observation that is correlated with NF-κB inhibition [43]. Since atherosclerosis is a typical proliferative inflammatory disease, we checked whether CP-PGs might influence foamy macrophage apoptotic pathways that could explain the reduction in arterial wall cellularity observed *in vivo* with LipoCardium treatment. As expected, foam cell transformation practically abolished

caspase-3 mRNA levels as compared to controls. On the other hand, treatment of foamy macrophages with either PGA_2 or 15-d-PGJ_2 promoted a dramatic rise in capase-3 expression (60- and 18-fold, respectively), which not only strengthens the usefulness of CP-PGs as anti-atherosclerotic tools, but also suggests that PGA_2 may be somehow much more pro-apoptotic to foam cells than 15-d-PGJ_2. On the other hand, CP-PG effects on the expression of the anti-apoptotic gene product Bcl-x_L, which is associated with prolonged cell survival in human atherosclerotic plaques [44], were much less evident.

In atherosclerotic lesions, there have been shown macrophage foam cell subpopulations with both proliferative and apoptotic features. In this context, p53 tumor suppressor gene expression normally colocalizes with non-proliferating or apoptotic cells [45]. In our hands, both test CP-PGs had an effect on p53 mRNA expression that was negligible as compared to that found over caspase-3. However, an undoubting striking result is that PGA_2-induced apoptosis of macrophages seems to be correlated with foam cell phenotype since, in control untransformed macrophages, PGA_2 did not affect caspase-3 mRNA expression, although some inhibition of p53 was verified in untransformed cells. This propositions are also supported by the finding that 15-d-PGJ_2 blocked proliferation in human B malignant cells by shutting down anti-apoptotic NF-κB-dependent proteins leading to apoptosis in a PPARγ-independent manner [46]. This would explain why LipoCardium had so strong effect on the lesions but not in the surrounding territories.

The results showed that hsp70 expression in foamy macrophages is extremely sensitive to PGA_2 challenge. This is especially important because many (if not all) antiproliferative as well as anti-inflammatory effects of CP-PGs have been associated with the activation of hsp70 biochemical pathways [23,24]. Hsp70 is the most prominent stress protein induced by a wide variety of cellular stressors, including oxLDL and CP-PGs. With only few exceptions, CP-PG treatment of mammalian cells results in a massive dose-dependent hsp70 induction, even at non-toxic doses. In this study, in spite of the presence of a pre-existing oxLDL-induced hsp70 expression, PGA_2 achieved a higher degree of hsp70 induction thus becoming suitable for utilization *in vivo*, inasmuch as hsp70 is also highly expressed in human atherosclerotic lesions [47]. In this specific case, enhanced hsp70 expression may be invaluable because this multifunctional PGA_2-inducible chaperone is cytoprotective and helps to repair oxidatively damaged cell proteins. This particular beneficial effect of PGA_2 may explain the disruption of the characteristic "fatty-islet" pattern observed in foamy macrophages treated with PGA_2 *in vitro* and the striking remission of atherosclerotic lesions observed *in vivo* after LipoCardium treatment. Finally, as compared to 15-d-PGJ_2 (a so powerful anti-inflammatory agent), PGA_2 (a weaker anti-inflammatory eicosanoid) is much more promising as an anti-atherosclerotic agent. We are tempted to speculate that part of this difference could be ascribed to the ability of 15-d-PGJ_2 to activate PPARγ, which does not occur in the case of PGA_2.

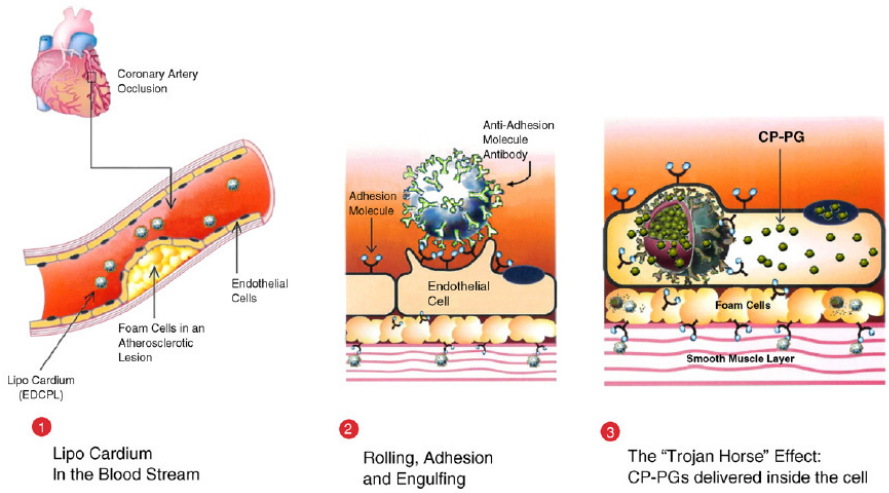

Fig. 6. Proposed mechanism for LipoCardium action. Cardiovascular risk factors determine endothelial injury which triggers inflammation via NF-κB-induced genes, including vascular cell adhesion molecule-1 (VCAM-1), which drives the firm adhesion of circulating monocytes that invade subendothelial space becoming foamy macrophages, the pivotal cell type that perpetuates atherosclerotic lesions (1). The anti-VCAM-1 antibodies of LipoCardium specifically direct PGA_2 molecules to injured cells (2). After uptake and lysosomal disassembly, PGA_2 may influence atheroma plaque progression (3) through its tetravalent effect: (a) anti-inflammatory, (b) antiproliferative, (c) anticholesterogenic and (d) cytoprotective. In view of PGA_2-mediated vanish of inflammatory signals brought about endothelial cells and foamy macrophages, no further inflammatory cells transmigrate from the blood stream towards the subendothelial space.

Although further mechanistic experiments must be done, we suggest that LipoCardium reach its targets in the arterial wall through its binding to VCAM-1, highly expressed in endothelial cells and foamy macrophages in atherosclerotic lesions (Fig. 6), leading to the downregulation of NF-κB-dependent inflammatory and pro-proliferative genes. The shunt in lipogenesis that decreases cholesterol accumulation, and HSP70-mediated cytoprotection should perform remaining tasks. Our results suggest that this PGA_2-based EDCPL has a tetravalent action: anti-inflammatory, antiproliferative, anticholesterogenic and cytoprotective. Now, this Laboratory of Cellular Physiology, in collaboration with Faculty of Pharmacy and the Center of Biotechnology of the Federal University of Rio Grande do Sul, is currently working on a pharmaceutical preparation (christened LipoCardium^{PLUS}) for the chronic slow delivery of LipoCardium constituents into the circulation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2006.08.049.

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