UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
DEPARTAMENTO DE BIOQUÍMICA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

Avaliação do potencial antioxidante e anti-inflamatório da variedade de pêssego Maciel *(Prunus persica* L. Batsh*)* e seus produtos liofilizados em modelo *in vitro*, *ex vivo* e *in vivo*

Aluno: Juciano Gasparotto
Orientador: Daniel Pens Gelain

Porto Alegre, 2014

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Dissertação apresentada ao Programa de Pós-Graduação
em Ciências Biológicas: Bioquímica,
como requisito para obtenção do grau de Mestre em Bioquímica.

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Porto Alegre, 2014

Este trabalho foi realizado no Centro de Estudos em Estresse Oxidativo, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, sendo fomentado pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Pró-Reitoria de Pesquisa desta Universidade (PROPESQ/UFRGS) e pela Embrapa Clima temperado.

Dedicatória

Esta dissertação de mestrado é dedicada a todas as pessoas que buscam melhor qualidade na alimentação e decorrente qualidade de vida. Espero que este trabalho sirva, para elucidação dos efeitos protetores do pêssego e que possa servir de inspiração para que mais pessoas pesquisem sobre frutas e que consequentemente a população possa ser beneficiada com mais informações sobre alimentos que acarretem benefícios a saúde.

Agradecimentos

Primeiramente agradeço a minha esposa, companheira para todos os momentos que me incentivou durante toda a jornada acadêmica, dando total suporte para as minhas decisões, se não fosse por ela possivelmente está dissertação não iria existir. Giu tenho muita sorte em ter tua companhia diariamente sou muito feliz ao teu lado. Te amo pequena.

Aos meus pais, que, apesar de todos os problemas financeiros, nunca deixaram de incentivar os meus estudos sempre buscando de forma incansável me auxiliar no que fosse necessário para que eu pudesse continuar com minha trajetória acadêmica. Parafraseando meu pai: “para ser alguém na vida tem que estudar”. Amo vocês dois.

Ao professor Dr. José Cláudio Fonseca Moreira, o qual me deu a oportunidade de ingressar no laboratório 32, onde prontamente fui muito bem recebido e atualmente tenho o privilégio de fazer parte deste grupo de pesquisa.

Ao competente orientador Daniel Pens Gelain, pessoa pela qual tenho imensa admiração e respeito. Um grande incentivador da pesquisa de qualidade, SEMPRE disponível para discutir resultados e novos projetos.

A todos os colegas do Centro de Estudos em Estresse Oxidativo (Laboratório 32, Departamento de Bioquímica, UFRGS), que contribuíram de alguma forma para minha evolução científica.

A Embrapa Clima temperado que é uma grande parceira, com pessoas muito competentes envolvidas, que estão empenhadas em desenvolver frutas de alta qualidade e que acarretem benefícios para a população.

A todas as pessoas que trabalham no campo diariamente fazendo da lida uma forma de sustento, cultivando e mantendo nossa tradição, tornando o Rio Grande do Sul um estado forte e próspero.

Às agências financiadoras brasileiras e riograndense de fomento à pesquisa e de bolsas de pós-graduação – CAPES, CNPq, PROPESQ/UFRGS e FAPERGS – bem como aos funcionários do Departamento de Bioquímica da UFRGS.

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Parte I

Resumo

O presente estudo investigou o potencial antioxidante e anti-inflamatório em modelos *in vitro*, *ex vivo* e *in vivo* dos extratos de pêssego in natura, casca *in natura*, pêssego em calda (compota) e a calda da compota. Nos testes *in vitro*, os extratos do pêssego e a casca *in natura* apresentaram atividade antioxidante e inibição de glicação de proteínas, assim como altas concentrações de polifenois e carotenoides. O pêssego em calda também desempenhou resultados semelhantes, porém em menor proporção. Fatias de fígado, rim e córtex cerebral de ratos foram utilizadas como modelo *ex vivo*. Os extratos do pêssego *in natura*, casca *in natura* e o pêssego em calda foram capazes de proteger o dano lipídico e proteico induzidos pela reação de Fenton, assim como inibir a produção de citocinas pró-inflamatórias. Como o pêssego é ingerido regularmente na dieta, os efeitos antioxidantes e anti-inflamatórios dos extratos foram investigados *in vivo*. Ratos machos Wistar receberam tratamento intragástrico dos diferentes extratos (200 e 400 mg/kg) durante 30 dias, no último dia de tratamento uma dose de CCl_4 (3 mL/kg, i.p.) foi administrada. Os efeitos antioxidantes e anti-inflamatórios dos extratos foram avaliados em soro, fígado e rim. Os ratos que receberam pré-tratamentos com os extratos demonstraram dano hepático e renal menor em comparação com aqueles que receberam somente CCl_4, resultado semelhante foi encontrado nos marcadores de dano e inflamação no soro. Os resultados obtidos neste estudo indicam que os extratos analisados são fontes potenciais de antioxidantes e anti-inflamatórios naturais capazes de proteger o fígado e os rins.

Abstract

The present study investigated the antioxidant and anti-inflammatory potential of fresh and canned (preserve) peaches in *in vitro*, *ex vivo* and *in vivo* models. In *in vitro* tests, extracts of fresh peach pulps and peel demonstrated antioxidant activity and inhibition of protein glycation, as well as high concentrations of polyphenols and carotenoids; preserve peach pulps also had similar results, although to a lesser extent. Slices of liver, kidney and cerebral cortex of rats were used as *ex vivo* model. The extracts of fresh peach pulps, peels and canned peaches were able to protect lipids and proteins against Fenton reaction-induced damage, as well as inhibit the production of pro-inflammatory cytokines. Antioxidant and anti-inflammatory effects of the extracts were investigated also *in vivo*. Male Wistar rats received intragastric treatment of each extract (200 and 400 mg/kg) for 30 days, and at the last day of treatment a dose of CCl_4 (3 mL/ kg, i.p) was administered. Antioxidant and anti-inflammatory effects of the extracts were evaluated in serum, liver, and kidney. Rats that received pre-treatment with the extracts showed less hepatic and renal damage compared with those receiving CCl_4, similar results were found in markers of inflammation and damage in serum. The results of this study indicate that extracts analyzed are potential sources of natural antioxidants and anti-inflammatory capable of protecting the liver and kidneys.

Lista de abreviaturas

ALT	alanina aminotransferase
ANVISA	agência nacional de vigilância sanitária
AST	aspartato aminotransferase
AVC	acidente vascular encefálico
CAT	catalase
CCl_4	tetracloreto de carbono
ERN	espécies reativas de nitrogênio
ERO	espécies reativas de oxigênio
$FeSO_4$	sulfato ferroso
GPx	glutationa peroxidase
GSH	glutationa reduzida
HPLC	cromatografia liquida de alta eficiência
H_2O_2	peróxido de hidrogênio
IL-1β	interleucina-1 beta
LDH	lactato desidrogenase
NFκB	fator nuclear kappa-B
NOS	óxido nítrico sintase
RAGE	receptor para produtos finais de glicação *avançada*
SOD	superóxido dismutase
TAR	reatividade antioxidante total
TNF-α	fator de necrose tumoral-alfa
TRAP	potencial antioxidante reativo total

I. Introdução

O desequilíbrio oxidativo e a consequente ação dos radicais livres em induzir, manter e prolongar efeitos nocivos ao organismo fomentaram o interesse de diversos pesquisadores em investigar maneiras de inibir ou amenizar a ação maligna do estresse oxidativo no organismo humano. A observação das populações italianas e francesas nos anos 80 (Richard *et al.* 1981) constatou que estes povos tinham maior longevidade e menor incidência de doenças cardiovasculares em decorrência da dieta rica em frutas, vegetais e vinho, apesar do alto consumo de gorduras saturadas (Burr 1995). Desde então, muitos estudos epidemiológicos associam o consumo de frutas e vegetais à diminuição do risco de desenvolvimento de processos patológicos crônicos que são associados com o estresse oxidativo, incluindo câncer, doenças cardiovasculares e doenças degenerativas que são relacionadas com o envelhecimento (Halliwell 2007, Stanner *et al.* 2004). A presença de compostos antioxidantes em frutas e vegetais pode ser associada a estes efeitos benéficos à saúde, protegendo biomoléculas dos danos oxidativos.

Frutas apresentam grandes quantidades de compostos fenólicos e carotenoides, que são um importante grupo de metabólitos secundários que têm sido amplamente estudados por ter grande potencial antioxidante (Rice-Evans & Miller 1995). Além destes compostos outros constituintes das frutas são potencialmente responsáveis por seus efeitos benéficos tais como, potássio, folato, fibras, acido ascórbico (Ward *et al.* 1997, Joshipura *et al.* 1999, Gaziano *et al.* 1995), flavonoides e carotenoides. Esses compostos são investigados intensamente por possuírem propriedades que auxiliam na

manutenção da saúde humana, e as evidencias científicas confirmam a potência destes compostos bioativos (Landete 2013).

Dentre os milhões de compostos bioativos existentes na natureza, muitos são primeiramente testados pela população por metodologias tradicionais ou de medicina popular, através de chás ou ingestão de alimentos que até então são desconhecidos para a maioria da população, e normalmente após a popularização do produto este é avaliado sistematicamente por pesquisadores treinados. Nos últimos anos, vários compostos bioativos têm sido submetidos a investigação em modelos pré-clínicos e também em ensaios clínicos por apresentarem potencial antioxidante, anticancerígeno, e anti-inflamatório.

As doenças crônicas são a causa mais prevalente de morte no mundo, liderada por doenças cardiovasculares, seguido por câncer, doenças pulmonares crônicas e diabetes mellitus (Patil *et al.* 2009).

No sentido de prevenir diversas doenças que são causadas principalmente por hábitos alimentares desequilibrados e estilo de vida sedentário que podem culminar em produção de radicais livres e estresse oxidativo, muitos estudos vem sendo conduzidos a fim de investigar as características de alimentos que tenham capacidade de prevenir o estresse oxidativo e consequentemente doenças comuns do cotidiano.

Radicais livres e estresse oxidativo

Um radical livre é uma espécie química de existência independente, com um ou mais elétrons desemparelhados, podendo ser átomos, como hidrogênio ou cloreto, metais de transição, ou uma molécula onde o elétron

desemparelhado esteja localizado no orbital externo. O elétron desemparelhado confere uma reatividade alta a esta molécula, devido a uma grande tendência desta perder ou adquirir um segundo elétron para este orbital (Halliwell 2007, de Bittencourt Pasquali *et al.* 2013).

Em condições normais, existe um equilíbrio delicado entre a produção de espécies reativas de oxigênio (ERO) e as defesas antioxidantes que protegem as células *in vivo* (Negi *et al.* 2013). Quando existe um desequilíbrio redox há um aumento na produção de ERO, este fenômeno é considerado uma das principais causas de doenças relacionadas ao envelhecimento (Valko *et al.* 2007). Tipicamente, ERO são geradas continuamente em condições fisiológicas e são efetivamente controladas/eliminadas por sistemas antioxidantes intracelulares e extracelulares. O estresse oxidativo tem sido definido como um desequilíbrio entre o aumento da produção de espécies reativas e defesa antioxidante inadequada que pode culminar em disfunção celular (Halliwell 2007, Schnorr *et al.* 2011).

A superprodução de ERO decorrente de diferentes fontes resulta em estresse oxidativo, processo nocivo que pode ser um importante mediador de danos nas estruturas celulares, incluindo lipídeos de membranas, proteínas e DNA (Keisari *et al.* 1983). ERO ainda podem atuar na inativação de enzimas importantes como nas enzimas de reparo ao DNA devido a sua alta reatividade e natureza oxidante (Keisari et al. 1983, Valko et al. 2007, Saugstad 2001).

A relação de dano oxidativo com doenças ligadas ao câncer, doenças cardiovasculares, lesão de isquemia/reperfusão, doenças renais e hepáticas, diabetes mellitus, doenças neurodegenerativas (doença de Alzheimer e doença de Parkinson, por exemplo), a artrite reumatoide, e o envelhecimento é bem

documentado (Ikawa *et al.* 2011, Uttara *et al.* 2009). Tratamentos com antioxidantes agem de forma profilática, inibindo ou retardando o dano oxidativo, protegendo as células, restabelecendo ou mantendo o "equilíbrio redox" denominado também como "homeostase redox" (Valko et al. 2007).

Em contraste, os efeitos benéficos de ERO e espécies reativas de nitrogênio (ERN) ocorrem em concentrações moderadas e envolvem funções fisiológicas em respostas celulares diversas, como, por exemplo, na defesa contra agentes infecciosos, na modulação de vias de sinalização celular, e na indução de uma resposta mitogênica (Valko et al. 2007).

Apesar da excelente capacidade do sistema antioxidante endógeno em sustentar a homeostase redox, a demanda requer outras fontes de antioxidantes que estão presentes em larga escala nos alimentos, principalmente em frutas (Pietta 2000).

Pêssegos apresentam em sua composição diversos agentes com capacidade antioxidante, tais como, vitaminas do complexo A, B, C e E, compostos fenólicos e carotenoides além de ser uma fonte importante de minerais como cálcio, magnésio e fibras (Rickman *et al.* 2007, Durst & Weaver 2013).

A variedade de pêssego Maciel desenvolvido pela Embrapa clima temperado além de apresentar grandes quantidades destes compostos antioxidantes comumente encontrados em pêssegos, também possui significantes efeitos anti-inflamatórios. Portanto o pêssego Maciel pode ser considerado uma importante fonte de nutrientes que se enquadra como alimento funcional, prevenindo diversas doenças ocasionadas por estresse oxidativo.

Alimento funcional

Atualmente diversos grupos de pesquisa voltam seu interesse cientifico na busca por alimentos que exerçam efeitos protetores em órgãos que são alvo do estresse oxidativo (por exemplo, fígado e rim). Algumas frutas têm sido extensamente investigadas por serem considerados alimentos funcionais (BRASIL 1999), pois possuem características altamente benéficas e que praticamente não possuem efeitos colaterais.

Embora o termo ''alimento funcional'' já tenha sido definido várias vezes (Roberfroid 2002), até agora não há uma definição global para este grupo de alimentos (Alzamora *et al.* 2005). Na maioria dos países não existe uma definição legislativa para o termo que diferencie os alimentos funcionais para alimentos convencionais (Siro *et al.* 2008). As definições para estes alimentos vão de uso simplificado ao mais complexo, como "alimentos que podem fornecer benefícios de saúde" ou "alimento semelhante em aparência à alimentação convencional que faz parte da dieta normal, mas que foi modificado para ser útil para os papeis fisiológicos além do fornecimento de requisitos simples de nutrientes" (Bech-Larsen & Grunert 2003, Siro et al. 2008).

O Ministério da saúde através da Agência Nacional de Vigilância Sanitária (ANVISA) regulamentou os Alimentos Funcionais através das seguintes resoluções: ANVISA/MS 16/99; ANVISA/MS 17/99; ANVISA/MS 19/99. Segundo a ANVISA, alimento funcional é definido como "aquele alimento ou ingrediente que, além das funções nutricionais básicas, quando consumido, como parte da dieta habitual, produz efeitos benéficos à saúde". A ANVISA ainda propõe que para o alimento ser considerado funcional deve ter sua

composição química caracterizada, evidências científicas de propriedade funcional do alimento, ensaios nutricionais, fisiológicos ou toxicológicos em animais de experimentação, ensaios bioquímicos, estudos epidemiológicos ou ensaios clínicos que reconheçam as propriedades e características do produto (BRASIL 1999).

O alimento funcional é aquele que faz parte da dieta regular, porém desempenha funções nutricionais específicas por conter em sua composição compostos capazes de modular parâmetros bioquímicos/fisiológicos (Alzamora et al. 2005), tais como os carotenoides e flavonoides que são encontrados nos pêssegos e que podem ser consumidos diariamente sem causar nenhum efeito colateral.

O principal problema em determinar se um alimento é funcional ou não é a investigação das quantidades necessárias que devem ser ingeridas. Neste sentido nossa pesquisa busca mensurar as quantidades ideais para que o alimento exerça a função adequadamente.

Polifenois

Os polifenois formam um grupo complexo de moléculas presente na maioria das frutas e vegetais, estando envolvidos na defesa da planta contra patógenos, animais ou radiação ultravioleta (Rudnicki *et al.* 2007).

Dentre as classes de polifenois, os flavonoides são os compostos mais investigados. Os flavonoides são constituintes funcionais de muitas frutas e vegetais, são bem conhecidos por terem propriedades farmacologicamente ativas podendo desempenhar atividade antioxidante (Kumazawa *et al.* 2006). O termo "flavonoides" genericamente abrange mais de 8000 compostos que

apresentam uma estrutura comum de difenilpropano ($C_6O_3C_6$), consistindo em dois anéis aromáticos unidos por três carbonos.

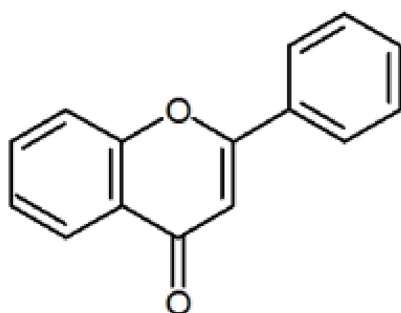

Figura 1. Estrutura Básica de diversos flavonoides

Nos últimos anos a relação entre consumo de dieta equilibrada com grandes quantidades de frutas e consequentemente polifenois está diretamente relacionada à redução de risco de doenças como acidente vascular encefálico (AVC) aterosclerose, diabetes tipo 2, artrite, câncer, entre outras (Patil et al. 2009, Joshipura et al. 1999); além de prevenir estas doenças o maior consumo de frutas pode levar a melhores resultados em testes cognitivos, e menor risco de depressão (Akbaraly *et al.* 2009).

Os polifenois possuem várias aplicações industriais, tais como na produção de tintas e cosméticos, como agentes de curtimento mais especificamente, o grupo dos taninos, e na indústria alimentícia como aditivos (como corantes naturais e conservantes). Além disso, alguns compostos fenólicos têm aplicações como anti-inflamatórios, agindo assim no tratamento de doenças tais como a hipertensão, alergia, hipercolesterolemia, entre outros (Bravo 1998).

O Interesse em compostos fenólicos de alimentos é decorrente de sua capacidade de eliminação de radicais livres e seus potenciais efeitos sobre a

saúde humana (Bravo 1998). A estimativa de consumo de polifenois é incerta e varia muito na literatura. As informações até o momento sobre as quantidades a serem consumidas variam de 23 mg/dia até 800 mg/dia (Hertog *et al.* 1993, Justesen *et al.* 1998, Pietta 2000).

Carotenoides

Carotenoides são pigmentos naturais que são sintetizados por plantas e são responsáveis pelo brilho de várias frutas e vegetais (Paiva & Russell 1999). Os carotenoides possuem uma estrutura isoprenoide, ou seja, com um número variável de duplas ligações conjugadas que permite a fácil deslocalização eletrônica nas ligações duplas. Este sistema de ligação carbono-carbono conjugado faz dos carotenoides um eficiente repressor de oxigênio singleto. Esta estrutura também cria uma lipofilicidade que faz com que os pigmentos retardem a peroxidação lipídica e estabilizem estruturas lipoproteicas tais como membranas celulares. Mamíferos não podem sintetizar carotenoides *de novo* e, por conseguinte, os carotenoides devem ser obtidos da dieta (Hammond & Renzi 2013).

Os carotenoides são classificados em dois grupos principais, os carotenos, que são hidrocarbonetos, tais como β-caroteno e licopeno ($C_{40}H_{56}$) e as xantofilas, que incluem o oxigênio, o hidrogênio e o carbono ($C_{40}H_{56}O_2$). Xantofilas, que são essencialmente produtos de oxidação dos carotenos, incluem luteína, zeaxantina, cantaxantina e β-criptoxantina (Hammond & Renzi 2013).

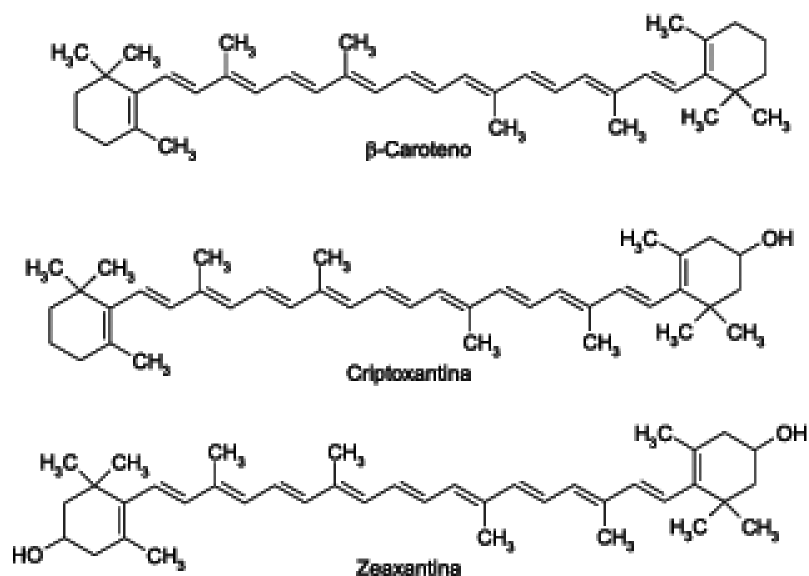

Figura 2. Principais Carotenoides (fonte: Cerqueira et al. 2007)

O β-caroteno e outros carotenoides têm propriedades antioxidantes *in vitro* e em modelos animais (Paiva & Russell 1999). Misturas de carotenoides ou associações com outros antioxidantes (vitamina E, por exemplo) podem aumentar a sua atividade contra as ERO/ERN (Paiva & Russell 1999).

Evidências científicas indicam que o caráter antioxidante dos carotenoides é decorrente da capacidade destes compostos em eliminar ERO e ERN, acarretando em efeitos benéficos sobre doenças crônicas, incluindo doenças cardiovasculares e até mesmo catarata (Rousseau *et al.* 1992, Kiokias & Gordon 2003).

O pêssego (*Prunus persica* L. Batsch)

A valorização do consumo de frutas como fontes de compostos com atividade antioxidante tem sido sugerida recentemente por diferentes grupos de pesquisa. Dentre os muitos compostos que tem perfil antioxidantes os que ganham destaque são os flavonoides, antocianos, ácido ascórbico e os carotenoides (Gil *et al.* 2002, Rossato et al. 2009). Esses compostos diferem entre as frutas, as quais algumas espécies possuem mais flavonoides enquanto que outras apresentam mais carotenoides e vice-versa, portanto o consumo de algumas frutas pode acarretar em maiores benefícios do que outras. A composição do pêssego Maciel já foi testado anteriormente demonstrando expressivas quantidades de ambos compostos (polifenois e carotenoides) agregando valor nutricional e qualidade ao produto (Rossato et al. 2009).

Pêssego (*Prunus persica* L. Batsch) é uma fruta tipicamente de clima temperado, introduzido no Brasil através da colonização portuguesa na década de 1530 (Barbosa *et al.* 2010). O pêssego foi cultivado durante séculos no Oriente, Europa e Américas em altas latitudes (30 e 50 º, do Norte e do Sul), com 500 a 2.000 horas anuais de temperatura abaixo de 7,2 º C (Barbosa et al. 2010, Chagas *et al.* 2012).

O pêssego é uma das espécies mais bem caracterizadas geneticamente na família *Rosaceae* (Shulaev *et al.* 2008, Ogundiwin *et al.* 2009), e o *Prunus* é o cultivo economicamente mais importante (Shulaev et al. 2008), gênero que inclui também nectarina, ameixa, damasco, cereja e amêndoa. O pequeno tamanho do genoma faz com que o pêssego se destaque como uma espécie para modelo para estudos de genômica em frutas (Zhebentyayeva T *et al.*

2008). Os detalhes destes recursos genéticos e genômicos são descritos no Banco de Dados do Genoma para *Rosaceae* (GDR) (Jung *et al.* 2008).

O aumento dos estudos envolvendo a capacidade antioxidante de frutas fez com que aumentasse o interesse por cultivares de pessegueiro para consumo *in natura*, que produzam frutas de baixa acidez e bom paladar. Cada região tem preferência por um determinado sabor como é o caso de São Paulo e Curitiba que preferem pêssegos de polpa branca e sabor doce (Almeida *et al.* 2006). Assim, vários programas de melhoramento de frutas estão sendo executados no sentido de obter seleções e cultivares desse tipo (Okie *et al.* 2008). A Embrapa Clima Temperado mantém uma linha de pesquisa em melhoramento genético do pessegueiro, visando este objetivo.

Melhoramento genético de novos cultivares de pêssego (Maciel)

O pessegueiro quando introduzido em baixas latitudes (22 ° S ± 2 °) exige adaptação ao clima subtropical temperado (Barbosa et al. 2010, Chagas et al. 2012), e o Rio Grande do Sul apresenta as condições ideais para o cultivo de um pêssego de boa qualidade e bom preço de mercado, tendo os municípios de Pelotas, Canguçu e Bento Gonçalves como maiores produtores do fruto, apresentando produção superior a 10.000 toneladas anualmente (AGRIANUAL, 2009).

O pêssego possui enorme importância econômica e nutricional, sendo a oitava fruta mais produzida no mundo e uma das mais consumidas *in natura*. O Rio Grande do Sul atualmente é um dos maiores produtores de pêssego do Brasil, com mais de 50% da produção nacional, seguido por São Paulo (21%),

Santa Catarina (13%), Paraná (8%) e Minas Gerais (5%). O Rio Grande do Sul produz 90% das frutas destinadas ao processamento. Em São Paulo, o pessegueiro representa a segunda principal frutífera de clima temperado, destinada principalmente ao consumo *in natura*, com cultivo em diversas regiões (Chagas et al. 2012).

A cultivar Maciel foi obtida por hibridação (cruzamento) entre duas seleções de pessegueiro (conserva 171 e conserva 334) oriundas do programa de melhoramento genético da Embrapa clima temperado. A planta desse cultivar apresenta vigor médio e forma aberta. É moderadamente suscetível à bacteriose. A densidade de gemas floríferas é de 10 a 12 pares por 25 cm de comprimento do ramo. Esta cultivar adapta-se a regiões onde o acúmulo de frio hibernal esteja entre 200 e 300 horas (Figura 1 e 2). Pode produzir até 50 kg/planta de frutos de excelente qualidade geral. Os frutos são de forma redondo-cônica e de tamanho grande, com peso médio próximo a 120 g. A película é amarelo-ouro com até 20% de vermelho. A polpa é amarela, firme, não fundente e aderente ao caroço. O sabor é doce-ácido, com leve adstringência. O teor de sólidos solúveis varia, conforme as condições do ano, de 11 a 16º Brix. No Rio Grande do Sul a plena floração ocorre ao final de julho ou início de agosto. A flor é do tipo campanulada, com pétalas de tamanho maior do que as da maioria das flores deste tipo e de cor rosa-escura. A colheita inicia-se, geralmente, na segunda ou terceira semana de dezembro. Este cultivar destaca-se pela produtividade, tamanho, aparência e resistência dos frutos ao transporte. Estes são de ótima qualidade após industrializados mas tem, também, boa aceitação para o mercado de consumo *in natura* *(Raseira 1998).*

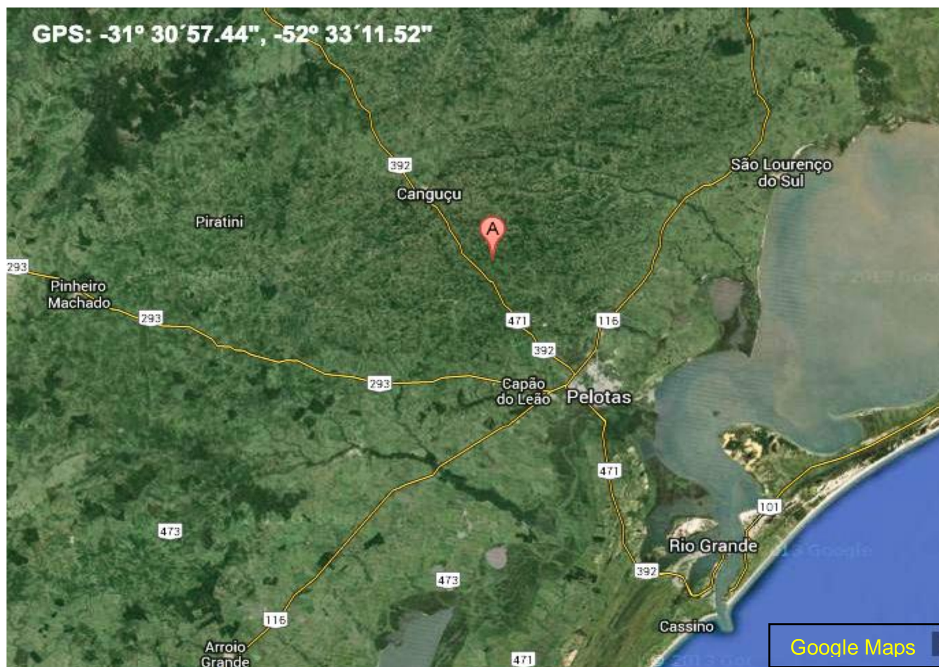

Figura 3. Coordenadas da posição global (20 km de aproximação)

Figura 4. Coordenadas da posição global (2 km de aproximação)

As propriedades das novas cultivares devem atender vários fatores: resistência a doenças, pragas e baixas temperaturas, maturidade em momento apropriado, boa qualidade das frutas (excelente sabor, alto teor de açúcares, relação equilibrada entre açúcares e ácidos). As propriedades das novas

cultivares são pesquisadas constantemente a fim de atingir as necessidades do mercado comercial.

A propriedade de pêssego, que tem um grande impacto sobre a satisfação dos produtores e consumidores é a qualidade interna dos frutos. Ela é determinada por componentes químicos, valor nutricional, firmeza, suculência, textura, frescor, doçura, acidez, aroma e sabor. A qualidade interna dos frutos pode ser avaliada de várias formas, utilizando diferentes métodos: análises químicas (avaliações cromatográficas de componentes químicos em frutos); degustação ou avaliações sensoriais, incluindo análises de cor de carne, firmeza, textura, suculência e características organolépticas da carne, medidas físicas (firmeza) (Robertson *et al.* 2013).

As análises químicas de diversas frutas mostram que os frutos de pêssego contêm uma maior quantidade de água (87-91 %), baixos teores de açúcares (4,6-9,6 %), menos ácidos orgânicos (0,5-1,3 %) e proteínas (0,8-1,7 %) (Wills *et al.* 1983). A Sacarose está presente em frutos maduros em maior quantidade, dá melhor sabor à fruta, tem efeitos antioxidantes, e é uma importante fonte de energia. Sorbitol é ao lado de sacarose, o açúcar principal, é um produto da fotossíntese nas folhas, mas não é produzido em frutas, o sorbitol é translocado a partir de outras partes da planta através do floema (Lo Bianco *et al.* 1999). Os frutos do pêssego com maiores teores de frutose são mais firmes e de melhor sabor.

Frutas de qualidade inferior contêm mais sacarose e sorbitol e a quantidade sete vezes maior de compostos fenólicos em comparação com aos frutos de maior qualidade. Tais frutos de pêssego são mais amargos com sabor azedo. O sabor depende de acidez, sólidos solúveis, açúcares individuais e

ácidos orgânicos (substâncias não voláteis), bem como sobre as substâncias polifenólicas (Robertson et al. 2013, Senter & Ann 1990).

A literatura dispõe de alguns estudos relacionando pêssegos e seu potencial antioxidante (Gil et al. 2002, Oliveira et al. 2012, Carbonaro et al. 2002). O pêssego da variedade Maciel possui esta característica preventiva, portanto a investigação do consumo desse pêssego é importante para que a população tenha conhecimento dos efeitos benéficos do pêssego Maciel e então possa agregar este alimento em suas dietas tanto na forma *in natura* quanto na forma de sucos, compotas entre outros.

II. Objetivos

Objetivo principal

Investigar os efeitos preventivos de diferentes extratos do pêssego (pêssego *in natura*, casca, calda do pêssego em conserva e a polpa do pêssego em conserva) em modelo *in vitro*, *ex vivo* e *in vivo* submetidos ao dano toxicológico experimental.

Objetivos específicos

1- Determinar as propriedades citoprotetoras, antioxidantes e anti-inflamatórias do pêssego e de seus produtos, utilizando modelo *in vitro*, *ex vivo* e *in vivo.*

2- Investigar os efeitos do consumo de diferentes produtos do pêssego em modelo de inflamação e toxicidade renal/hepática induzida por CCl_4 *in vivo.*

3- Determinar a eficácia de proteção de cada derivado de pêssego em diferentes modelos experimentais (*in vitro*, *ex vivo* e *in vivo*);

4- Investigar os efeitos da ingestão de pêssego e de seus produtos derivados na expressão de RAGE e de NFκB-p65 total.

5- Mensurar as quantidades ideais de consumo para que o alimento exerça a função de proteção no organismo humano.

Parte II

III. Resultados

Materiais, métodos e resultados

Nesta parte do trabalho, apresentamos os resultados em forma de artigos científicos. Em ambos os artigos, o efeito protetor do pêssego *in natura*, casca *in natura,* pêssego em calda (compota) e a calda foi testado em dois diferentes modelos:

Modelo *in vitro* e *ex vivo* (capítulo I): Efeitos de diferentes produtos de pêssego (*Prunus persica* L. Batsch) a partir de uma variedade desenvolvida no sul do Brasil sobre o estresse oxidativo e parâmetros inflamatórios *in vitro* e *ex vivo*.

Modelo *in vivo* (capítulo II): Suplementação preventiva com pêssego fresco e em conserva atenua o estresse oxidativo, inflamação e dano tecidual induzido pelo CCl_4, em modelo animal.

Investigamos parâmetros de estresse oxidativo, como marcadores de dano oxidativo (peroxidação lipídica, carbonilação de proteínas e estado redox de grupamentos tiois), atividade de enzimas antioxidantes superóxido dismutase e catalase (SOD, CAT) e imunoconteúdo do fator nuclear kappa-B-p65 (NFκB-p65) e do receptor para produtos finais de glicação avançada (RAGE), além de parâmetros *in vitro* como potencial antioxidante e composição química dos extratos.

Capítulo I

"Effects of different products of peach (*Prunus persica* L. Batsch) from a variety developed in southern Brazil on oxidative stress and inflammatory parameters *in vitro* and *ex vivo*"

Juciano Gasparotto, Nauana Somensi, Rafael Calixto Bortolin, Karla Suzana Moresco, Carolina Saibro Girardi, Karina Klafke, Thallita Kelly Rabelo, Maurilio Da Silva Morrone, Márcia Vizzotto, Maria do Carmo Bassols Raseira, José Claudio Fonseca Moreira, Daniel Pens Gelain

Artigo aceito para publicação no Periódico Journal of Clinical Biochemistry and Nutrition em 21/01/2014

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Date:	2014-01-21 19:09:53
To:	"Juciano Gasparotto" juciano.gasparotto@gmail.com
From:	"JCBN Editorial Secretariat" jcbn@koto.kpu-m.ac.jp
Subject:	[JCBN] Your Submission JCBN-D-13-00097R1

Ref.: Ms. No. JCBN-D-13-00097R1
Effects of different products of peach (Prunus persica L. Batsch) from a variety developed in southern Brazil on oxidative stress and inflammatory parameters in vitro and ex vivo
Journal of Clinical Biochemistry and Nutrition

Dear Mr Gasparotto,

I am pleased to tell you that your work has now been accepted for publication in Journal of Clinical Biochemistry and Nutrition.

It was accepted on 2014-01-21 19:09:50

Comments from the Editor and Reviewers can be found below.

Thank you for submitting your work to this journal.

With kind regards

Journal of Clinical Biochemistry and Nutrition

Comments from the Editors and Reviewers:

Reviewer #1: The authors carefully revised the manuscript according to the reviewers' comments.

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Journal of Clinical Biochemistry and Nutrition

Effects of different products of peach (Prunus persica L. Batsch) from a variety developed in southern Brazil on oxidative stress and inflammatory parameters in vitro and ex vivo

--Manuscript Draft--

Manuscript Number:	JCBN-D-13-00097
Article Type:	Original
Full Title:	Effects of different products of peach (Prunus persica L. Batsch) from a variety developed in southern Brazil on oxidative stress and inflammatory parameters in vitro and ex vivo
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	José Claudio Fonseca Moreira
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Abstract:	Antioxidant, anti-glycation and anti-inflammatory activities of fresh and conserved peach fruits (Prunus persica (L.) Batsch) were compared. Fresh peach pulps, peels, preserve peach pulps and the preserve syrup were prepared at equal concentrations. Fresh peach pulps and peels presented higher antioxidant and anti-glycation activities than preserve peach pulps in vitro; syrup had no effect. Rat liver, kidney and brain cortex tissue slices were pre-incubated with peach samples, subjected to oxidative stress with FeSO4 and H2O2. Fresh peach pulps and peel conferred higher protection against cytotoxicity and oxidative stress than preserve peach pulps in most tissues; no effect was observed with syrup. Release of TNF-α and IL-1β was also significantly decreased by Fresh peach pulps and peel, followed by preserve peach pulps. Total phenolic determination and HPLC analysis of carotenoids showed that the content of secondary metabolites in Fresh peach pulps and peel is significantly higher than in preserve peach pulps, while the syrup had only small or trace amounts of these compounds. Fresh peach pulps and Peel demonstrated high antioxidant and anti-inflammatory effects preventing against induced damage.
Manuscript Classifications:	Nutrition and Food Factors
Keywords:	Antioxidant, anti-inflammatory, peach, protective effect

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1 **Effects of different products of peach (*Prunus persica* L. Batsch) from a variety**
2 **developed in southern Brazil on oxidative stress and inflammatory parameters *in***
3 ***vitro* and *ex vivo***
4
5
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7 Moresco[1], Carolina Saibro Girardi[1], Karina Klafke[1], Thallita Kelly Rabelo[1], Maurilio
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23 **Summary**

24

25 **Antioxidant, anti-glycation and anti-inflammatory activities of fresh and conserved**
26 **peach fruits (*Prunus persica* (L.) *Batsch*) were compared. Fresh peach pulps, peels,**
27 **preserve peach pulps and the preserve syrup were prepared at equal**
28 **concentrations. Fresh peach pulps and peels presented higher antioxidant and**
29 **anti-glycation activities than preserve peach pulps *in vitro*; syrup had no effect. Rat**
30 **liver, kidney and brain cortex tissue slices were pre-incubated with peach samples,**
31 **subjected to oxidative stress with $FeSO_4$ and H_2O_2. Fresh peach pulps and peel**
32 **conferred higher protection against cytotoxicity and oxidative stress than preserve**
33 **peach pulps in most tissues; no effect was observed with syrup. Release of TNF-α**
34 **and IL-1β was also significantly decreased by Fresh peach pulps and peel, followed**
35 **by preserve peach pulps. Total phenolic determination and HPLC analysis of**
36 **carotenoids showed that the content of secondary metabolites in Fresh peach pulps**
37 **and peel is significantly higher than in preserve peach pulps, while the syrup had**
38 **only small or trace amounts of these compounds. Fresh peach pulps and Peel**
39 **demonstrated high antioxidant and anti-inflammatory effects preventing against**
40 **induced damage.**

41

42 **Keywords: Antioxidant, anti-inflammatory, peach, protective effect**

43

44

45

46

47

48 **Abbreviations**

49 FPP fresh poulp peach

50 PPP preserve poulp peach

51 TNF-α Tumor necrosis factor alpha

52 IL-1β Interleukin-1 beta

53 $FeSO_4$ Ferrous sulfate

54 H_2O_2 Hydrogen peroxide

55 CAT Catalase

56 SOD Superoxide dismutase

57 TBARS Thiobarbituric acid reactive substances

58 LDH Lactate dehydrogenase

59 SH Sulfhydryl

60 PBS Phosphate buffer saline

61 RAGE Receptor for advanced glycation endproducts.

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73 **Introduction**

74

75 Poor dietary intake of fruits and vegetables constitute a risk factor for several diseases
76 such as cancer, coronary heart disease, stroke and insulin resistance [1, 2]. The regular
77 consumption of fruits and vegetables is associated to prevention of esophageal,
78 stomach, pancreatic, bladder and cervical cancers; fruits and vegetables-enriched diets
79 may prevent 20% of most types of cancers [3]. A meta-analysis of cohort studies
80 observed that the risk of developing coronary heart disease and stroke decreased
81 significantly for each additional portion of fruit consumed per day, indicating a
82 protective effect [4]. It was also reported that fruit dietary intake may be associated with
83 a reduced risk of Alzheimer's disease and lower cognitive decline with age [5]. Some
84 fruit and vegetable also may play an important role in delaying the onset of Alzheimer's
85 disease, particularly among those who are at high risk for the disease [6].
86 Free radicals and related species (collectively known as reactive species) are constantly
87 produced by cells as result of aerobic metabolism. Excessive production of reactive
88 species may lead to oxidative stress, which results in oxidative damage to lipids,
89 proteins and DNA. Consequently, increased risk for developing diseases associated
90 oxidative stress, such as cancer, cardiovascular diseases and neurodegenerative
91 conditions, may arise [7]. To cope with reactive species, cells must maintain an adequate
92 pool of enzymatic and nonenzymatic antioxidants to properly clean/detoxify these
93 species. Among the nonenzymatic antioxidants, exogenous compounds obtained from
94 the diet exert an important role in the detoxification of free radicals and, in turn, in
95 disease prevention. Phenolic compounds and carotenoids obtained from dietary
96 vegetables and fruits exert prominent roles in the protection against oxidative damage

97 [8]. A reduced risk of developing diseases commonly associated to oxidative stress has
98 been associated to diets enriched in these compounds [3].
99 Different varieties of peaches (*Prunus persica* (L.) Batsch) are highly consumed
100 worldwide. Peach is the most important stone fruit crop in many western countries,
101 being grown in Europe, North and South America at a fair range of different climate
102 conditions and types of soils. Peaches are appreciated in different cultures mainly due to
103 their flavor and nutritional value; however, studies on potential benefits of peaches
104 consumption to human health are still incipient. Recently, consumers over the world
105 have been increasingly searching for foods that have a clear role in heath-promotion or
106 disease prevention, so producers have been considering such preferences when
107 developing new varieties of agricultural products. In the case of fruits, the present trend
108 is the reinforcement of the content or availability of plant endogenous compounds with
109 potential antioxidant, anti-glycemic, anti-inflammatory and anti-tumoral activities,
110 without affecting other nutritional and flavor-associated properties.
111 In Brazil, peaches of the Maciel variety have been developed at temperate climate for
112 consumption of the fresh fruit as well as its derivate products, such as juice and syrup-
113 preserved pulp. However, little is known about potential health benefits of this
114 commercial variety of peach and, especially, about the biological activity of the main
115 products commercially available from peaches, such as the fresh fruit and the syrup-
116 preserved pulp. In this regard, this study has been conducted to determine the
117 cytoprotective, antioxidant and anti-inflammatory properties of peaches of the Maciel
118 variety, developed by Embrapa (Brazilian Agricultural Research Corporation), using *in*
119 *vitro* and *ex vivo* assays. Our results indicate that fresh peach pulps (FPP) and peels
120 exhibit antioxidant, anti-glycation and anti-inflammatory properties, and that some of
121 these properties are also present in syrup-based peach pulp preserves (PPP).

122

123 **Material and Methods**

124

125 **Chemicals**

126 Catalase (CAT, EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1), thiobarbituric
127 acid (TBA), ferrous sulfate ($FeSO_4$), hydrogen peroxide (H_2O_2) were from Sigma-
128 Aldrich (St. Louis, USA). ELISA microplates were from Greiner Bio-One (Monroe,
129 USA) and ELISA TMB spectrophotometric detection kit was from BD Biosciences
130 (San Diego, USA). TNF-α rabbit polyclonal antibody, IL-1β rabbit polyclonal antibody
131 and anti-rabbit immunoglobulin linked to peroxidase were from Cell Signaling
132 (Beverly, USA). Purified recombinant TNF-α protein was from Abcam (Cambridge,
133 UK) and IL1-β was from BD. MilliQ-purified H_2O was used for preparing solutions.
134 Lactate dehydrogenase (LDH) activity kit was from Labtest (Lagoa Santa, MG, Brazil).
135 The peach samples were provided by Embrapa Clima Temperado.

136

137 **Animals**

138 Adult male Wistar rats (60 days-old; weighing 280–300 g) were obtained from our
139 breeding colony. They were caged in groups of four animals with free access to
140 standard commercial food (CR1 lab chow, Nuvilab, Curitiba, Brazil) and water and
141 were maintained in a 12-hour light–dark cycle (7:00–19:00 hours) in a temperature-
142 controlled colony room (21° C). All experimental procedures were performed in
143 accordance with the guidelines of the National Institutes of Health [9]. Our research
144 protocol was approved by the Ethical Committee for Animal Experimentation of the
145 Universidade Federal do Rio Grande do Sul. Ten healthy animals were utilized for this

146 study. A pilot test was performed with three animals to determine optimal induction of
147 hydroxyl-mediated damage by Fenton reaction ($FeSO_4$ and H_2O_2).
148
149 **Preparation of peach samples**
150 The Maciel variety was developed by Embrapa Clima Temperado by controlled
151 hybridization. The seeds were laminated in chamber at 4° ± 1° C and then seedlings
152 were cultivated in greenhouse for later being transplanted to the seedlings experimental
153 field. Fruits were obtained from this field (Pelotas, Rio Grande do Sul, Brazil, location
154 coordinates: -31º 30´57.44", -52º 33´11.52"). Immediately after harvesting the fruits, the
155 peel and pulps were separated and frozen at -20° C (the pits were removed and
156 discharged). Fruits were also used to prepare syrup-based preserves by an industrial
157 processing, which is the same procedure used for production of large-scale commercial
158 canned peach preserves. Briefly, the fruits are cut in half and the pits are separated by
159 twist. The fruits are subjected to a soda shower-based peeling procedure in a treadmill
160 and immediately washed to remove the soda. Pulps are placed into cans and hot
161 sucrose-based syrup is added. Cans are sealed, subjected to sterilization and then
162 cooled. After four months, the cans were opened and the pulps and the syrup were
163 separated and subjected to lyophilization. The samples of fresh pulp and peel were also
164 subjected to lyophilization at the same time. Lyophilization was carried out in L108
165 Liotop equipment (Liobras, São Paulo, Brazil) at the Embrapa Clima Temperado
166 experimental unit. The lyophilized samples were preserved at -20° C, dissolved in
167 ultrapure water at the moment of the experiment and then centrifuged (4000 g x 3 min)
168 to precipitate rough particles (always protected from light and temperature). Different
169 serial dilutions were obtained from this stock solution.
170

171 **Tissue slice preparation**

172 The animals were killed by decapitation. Kidney, liver and brain cortex were quickly
173 removed and were chopped in slices weighing 40 ± 5 milligrams. Tissue slices were
174 prepared with a tissue chopper device (McIlwain, Canada, USA) and immediately
175 placed on ice. Tissue was maintained in pre-warmed Kreb's Ringer Hepes (KRH)
176 oxygen-equilibrated solution (2.5 mM Hepes, 118 mM NaCl, 2.85 mM KCl, 2.5 mM
177 $CaCl_2$, 1.5 mM KH_2PO_4, 1.18 mM $MgSO_4$, 5 mM β-hydroxybutyrate, and 4.0 mM
178 glucose, pH 7.4) over a period of 1 h. The samples were washed for 30 minutes in test
179 tubes containing 2 mL KRH in a shaking water bath (60 oscillations/minute) at 37°C
180 under 95% O_2/5% CO_2. The KRH was replaced with incubation medium containing the
181 treatments and incubated for 1 h. After this pre-incubation, the $FeSO_4$ (1 mM) and H_2O_2
182 (100 mM) were added and incubated for 30 minutes to generate the Fenton reaction [(10)].
183 After incubation the medium was collected, centrifuged at 3000 x g for 5 minutes and
184 used for citotoxicity and inflammatory parameters estimation (see below). The tissue
185 slices were homogenized in phosphate buffer 50 mM (PB, KH_2PO_4 and K_2HPO_4 pH of
186 7.4) and centrifuged at 6000 x g for 5 minutes at 4°C. Protein content of the incubation
187 medium and homogenates were determined by Bradford method for data normalization
188 [(11)].

189

190 **Total reactive antioxidant potential (TRAP assay)**

191 The total reactive antioxidant potential (TRAP) was used as an index of non-enzymatic
192 antioxidant capacity. This assay is based on the quenching of peroxyl radicals generated
193 by AAPH (2,2azobis[2- amidinopropane]) by antioxidants present in a given sample [(12)].
194 Briefly, a chemical system that generates peroxyl radicals at a constant rate (an AAPH-
195 containing buffer) is coupled to a luminescent reactant (luminol) which emits photons

196 proportionally to its oxidation. The reaction was initiated by injecting luminol to the 0.1
197 M glycine buffer (pH 8.6) containing AAPH that resulted in steady luminescence
198 emission. Equal amounts of samples are then added to this reaction system, and the
199 luminescence emission at the moment following this addition (t=0) is recorded. This
200 initial emission reflects the production of free radicals by AAPH at the first moment
201 right after sample addition and is related to the endogenous oxidant state of the sample.
202 Following incubation, the thermal decomposition of AAPH produces luminescence at a
203 constant rate ("system"), and the presence of free radical scavengers in the added
204 sample will decrease this rate according to its content of non-enzymatic antioxidants.
205 Sample addition decreases the peroxyl-derived luminescence proportionately to its
206 antioxidant potential. We followed TRAP luminescence emission for 60 minutes and
207 calculated the area under the curve (AUC) relative to the system without samples
208 (which was considered as 100 % of luminescence emission at all time points), using
209 Trolox as a standard antioxidant for comparison [13]. The luminescence emission was
210 recorded in a Micro Beta luminescence counter (Perkin Elmer, USA).
211
212 **Protein glycation assay**
213 Bovine serum albumin (BSA, 10 mg/mL) in phosphate buffer (50 mM, pH 7.4)
214 containing 0.02% (w/v) sodium azide was pre-incubated with the extracts at final
215 concentrations of 1, 10 and 100 μg/mL. Glucose (25 mM) and fructose (25 mM)
216 solutions were added to the reaction mixture. All the reagents and samples were
217 sterilized by filtration through 0.25 μm membrane filters. Each solution was incubated
218 at 37°C for 21 days in the dark in a capped tube. BSA glycation during this period
219 resulted in fluorescent product formation, which was quantified in a fluorimeter (F2000,
220 Hitachi Ltd., Tokyo, Japan) with an excitation wavelength of 350 nm and an emission

221 wavelength of 450 nm [14, 15]. Glycation inhibition was calculated as follows: Inhibition

222 % = 1−(As −Ab)/ (Ac −Ab)*100, where As = fluorescence of the incubated mixture

223 with sample, Ac = the fluorescence of the incubated mixture without sample (positive

224 control for induced glycation) and Ab = the fluorescence of the sample as a blank

225 control [16].

226

227 **Determination of total phenolic content**

228 Total phenolic content of peaches and derivatives was determined using the Folin-

229 Ciocalteu method [17]. One hundred μL of Folin-Ciocalteu reagent were mixed to 100

230 μL of sample and then 200 uL of Na_2CO_3 35% were added. The volume was completed

231 to 1900 μL with ultra-pure H_2O and then homogenized. After 10 min, the absorbance

232 was measured at 725 nm and compared to a gallic acid calibration curve. Total phenols

233 in samples were determined as gallic acid equivalents [18].

234

235 **Quantification of carotenoids by High-Performance Liquid Chromatography**

236 **(HPLC)**

237 The carotenoids were separated on a polymeric reversed phase column (YMC C_{30} 250

238 micrometer x 4.6 micrometer; particle size of 3 μm) with a mobile phase gradient

239 elution starting with water/methanol/MTBE (Methyl tert-butyl ether) at 5:90:5 and

240 reaching 0:95:5 after 12 minutes, 0:89:11 after 25 minutes, 0:75:25 after 40 minutes and

241 00:50:50 after 60 minutes with a flow rate of 1 mL/minute at 33 °C [19]. The spectra

242 were conducted between 250 and 600 nm, and the chromatograms were processed at a

243 fixed wavelength of 450 nm for carotenoids. Identification was performed by

244 comparison of peak retention times obtained in each sample with the retention times of

245 standards analyzed under the same conditions.

246

247 **Cytotoxicity: measurement of lactate dehydrogenase (LDH) activity**

248 The cell viability of the tissue slices was assessed by LDH activity into the incubation
249 medium. This assay was performed by using a standard kit for LDH activity analysis,
250 according to the manufacturer's instructions. The change in absorbance at 500 nm was
251 followed in a SpectraMAX 190 plate reader (Molecular Devices, Sunnyvale, CA).

252

253 **Oxidative stress parameters**

254 Catalase (CAT, EC 1.11.1.6) activity was evaluated by following the rate of decrease in
255 hydrogen peroxide (H_2O_2) absorbance in a spectrophotometer at 240 nm [(20)]. The
256 activity of superoxide dismutase (SOD, EC 1.15.1.1) was measured by quantifying the
257 inhibition of superoxide-dependent adrenaline auto-oxidation in a sample buffer;
258 adrenochrome formation was monitored at 480 nm for 10 min (32 °C) in a
259 spectrophotometer [(21)].

260

261 **Sulfhydryl groups quantification**

262 Oxidative status of thiol groups were assessed by quantification of total reduced
263 sulfhydryl (SH) groups in samples [(22)]. Briefly, for total SH content measurement, a 60
264 μg sample aliquot was diluted in phosphate-buffered saline (PBS) and 10 mM 5,5′-
265 dithionitrobis 2-nitrobenzoic acid, and read in a spectrophotometer at 412 nm after a 60
266 minutes incubation.

267

268

269 **Lipid peroxidation**

270 The formation of thiobarbituric acid reactive species (TBARS) was quantified by an
271 acid-heating reaction with thiobarbituric acid. TBARS formation is a widely adopted
272 parameter for oxidative damage on lipids [(23)]. After precipitation with trichloroacetic
273 acid 10% (TCA), supernatant was mixed with 0.67% and heated in a boiling water bath
274 for 25 min. TBARS were determined by the absorbance in a spectrophotometer at 532
275 nm.
276

277 **Protein carbonylation**

278 The formation of carbonyl groups was used as a parameter for oxidative damage to
279 proteins, based on the reaction with dinitrophenylhidrazine (DNPH) [(24)]. Proteins were
280 precipitated by the addition of 10% TCA and re-solubilized in DNPH. Then, the
281 absorbance was read in a spectrophotometer at 370nm.
282

283 **Quantification of Tumor Necrosis Factor-α (TNF-α) and Interleukin-1β (IL-1β)**

284 To determine TNF–α and IL1-β concentration in the incubation medium, we used an
285 indirect enzyme-linked imunosorbent assay (ELISA) procedure. Samples were
286 normalized according protein content and added to ELISA plates. The antigen was
287 incubated for 24 h at room temperature, washed 3 times with Tween–Tris buffered
288 saline (TTBS: 100 mM Tris–HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween-20)
289 and then primary antibody (1:1000 dilution range) was added and incubated for 24 h at
290 4°C followed by secondary antibody incubation (rabbit anti-IgG, 1:1000 dilution range)
291 for 3 hours at room temperature. The immunoreactivity (1:1) was detected using a
292 spetrophotometric detection kit from BD Biosciences. The reaction was stopped with
293 sulfuric acid, and samples read at 450 nm. Purified recombinant TNF-α (Abcam) and
294 IL1-β (BD) were used for standard curve calculation [(25)].

295

296 **Statistical analysis**

297 The results of measurements were expressed as mean ± standard error of the mean

298 (SEM). Data were evaluated by one-way analysis of variance (ANOVA) followed by

299 Tukey's post hoc test; student's *t* test was applied to compare means between selected

300 groups. All peach samples (fresh peach pulp, peel, preserve of peach pulp and preserve

301 peach syrup), when incubated alone (i.e., in absence of the $FeSO_4$ /H_2O_2 hydroxyl

302 generating system), did not exert significant statistical effects to all parameters analyzed

303 here (data not shown). All results were calculated in GraphPad Prism 5.01 software.

304

305 **Results and Discussion**

306 We first evaluated the total antioxidant capacity of the different samples obtained from

307 peach and derivate products. We suspended the lyophilized samples of fresh peach pulp

308 (FPP), peel, preserve of peach pulp (PPP) and preserve peach syrup at the same

309 concentration each (20 µg/mL) and subjected them to the TRAP assay. This assay is

310 widely used to determine the non-enzymatic antioxidant capacity in plant extracts,

311 which is mostly dependent on the content of secondary metabolites with redox activity

312 [(13)]. The results showed that the peel has the highest antioxidant activity compared with

313 other samples; the FPP also had a significant antioxidant capacity (Fig. 1A, B). PPP and

314 syrup had no significant effects. Trolox (200 nM), hydrophilic analogue of α-

315 tocopherol, was used as a standard antioxidant.

316 The total antioxidant reactivity (TAR) index indicates the instantaneous decrease in

317 luminescence associated with the sample addition into the peroxyl-generating system.

318 While TRAP indicates the quantity of antioxidants presents in the plant extracts, the

319 TAR indicates their antioxidant effectiveness. Peel and FPP had the highest TAR

320 indexes, compared to PPP and syrup (Fig. 1C). This result indicates that the both the
321 FPP and the peel have a high content of molecules with significant antioxidant activity,
322 which is probably associated to the composition of secondary metabolites, as seen in
323 previous works [(26)] . When comparing the peel and FPP with other samples it is evident
324 that the samples from fresh fruits (i.e., peels and FPP) had a higher antioxidant activity
325 than samples from preserves (PPP and syrup). These findings suggest that some
326 properties of the peaches are lost by the preserves over time or during the processing
327 procedure, which agrees with previous observations showing that biological properties
328 of industrialized/canned fruits are lower than in fresh fruits [(27)]. It is also possible that
329 the high antioxidant potential of the fresh peaches is associated to its preservation
330 capacity over time, as it is known that antioxidants help to preserve flavor and
331 nutritional value of foods. Natural and synthetic antioxidants are widely used in the
332 food preservation industry for this reason, and it might be possible that in syrup-based
333 peach preserves they are oxidized over time, preserving other components of nutritional
334 value of oxidation and consequent degradation.
335 Glycation is a spontaneous non-enzymatic amino-carbonyl reaction between reducing
336 sugars and long-lived proteins and lipids. Glycation is one major form of chemical
337 modifications to biomolecules that compromise their function and have been recently
338 implicated in the molecular basis of several diseases, such as diabetes, cardiovascular
339 pathologies and neurodegenerative diseases [(28)]. These chemical modifications
340 frequently result in the formation of the so-called advanced glycation endproducts
341 (AGE). Glycation is a source of reactive species (RS), causing oxidative stress, which in
342 turn may trigger the production and release of inflammatory mediators [(29)]. Besides,
343 both AGE and oxidative stress enhance the expression of the receptor for AGE (RAGE)
344 in cells, which further activates pro-inflammatory pathways and NADPH oxidase-

345 derived ROS production [30]. Antioxidants are reported to prevent the oxidative reaction
346 of sugars with proteins and thus inhibit the formation of Amadori products, which is an
347 early step in AGE formation [15]. Several reports indicate that production of radicals and
348 highly reactive oxidants is increased by glycated proteins under physiological
349 conditions [15]. We subjected isolated albumin to a glycation protocol through
350 incubation with glucose and fructose during 21 days. At the end of the incubation
351 period, albumin glycation was significantly inhibited by peel and FPP by 40% at
352 different doses (Fig. 1D). PPP also inhibited albumin glycation, but at a lower extent
353 (around 10%), while the syrup alone, probably due to its high sucrose content, enhanced
354 glycation by 30%.
355 We next evaluated the effects of FPP, peel, PPP and syrup on parameters of
356 cytotoxicity, oxidative stress and inflammation by using an *ex vivo* approach. Rat
357 kidney, liver and brain cortex tissue slices were isolated and pre-incubated with the
358 different samples obtained from peaches and its products (80 μg/mL) for 60 minutes.
359 Then we subjected the tissue slices to an oxidative insult by incubation in a hydroxyl
360 radical production system with $FeSO_4$ 1 mM and H_2O_2 100 mM for 30 minutes. LDH
361 activity in the incubation medium was assessed as a parameter of cytosolic leakage
362 (cytotoxicity). The oxidative insult by the $FeSO_4/H_2O_2$ system (hydroxyl generating
363 system) increased LDH activity in the incubation medium of all tissues analyzed (Fig.
364 2A, D, G). In kidneys (Fig. 2A), FPP, peel and PPP prevented the increase in LDH
365 caused by the hydroxyl generating system, indicating a protective effect. In liver (Fig.
366 2D), FPP and peel had a significant protective effect. In brain cortex (Fig. 2G), only
367 FPP had a significant effect on LDH activity.
368 The $FeSO_4/H_2O_2$ system induces cytotoxicity by oxidative stress, as consequence of
369 Fenton reaction. Antioxidant enzymes are known to be induced in response to reactive

370 species [31]. CAT and SOD have their activities increased when H_2O_2 and superoxide
371 radicals are overproduced during cellular oxidative stress. Thus, enhanced CAT and
372 SOD activities are common parameters indicative of a increased state of reactive species
373 production [32]. As expected, incubation with the $FeSO_4/H_2O_2$ system increased CAT
374 and SOD activities in tissue slices. In kidney (Fig. 2B) and brain cortex (Fig. 2H,)
375 slices, pre-incubation with FPP and peel significantly inhibited the activation of CAT by
376 incubation with the $FeSO_4/H_2O_2$ system. In liver (Fig. 2E) only FPP was able to inhibit
377 the CAT activities. SOD activation induced by $FeSO_4/H_2O_2$ system was prevented in
378 kidney (Fig. 2C) by FPP, peel and PPP. In brain cortex (Fig. 2I), a inhibition of SOD
379 activities was observed with FPP and peel as in liver (Fig. 2F). Since SOD and CAT
380 activities are generally enhanced in conditions of increased substrate production, these
381 results altogether suggest that the pre-treatments carried out here conferred antioxidant
382 protection to kidney and brain cortex.
383 We also measured parameters of oxidative damage in biomolecules to assess the
384 antioxidant properties of peaches to tissue slices. The oxidative damage to the proteins
385 in tissue slices was measured by determining levels of the carbonyl groups based on the
386 reaction of the groups with dinitrophenylhydrazine (DNPH). Formation of protein
387 carbonyl groups is a well-known parameter of protein oxidation [24]. Protein
388 carbonylation was greatly enhanced by the $FeSO_4/H_2O_2$ system in all tissues, but pre-
389 incubation with FPP protected all tissues against this effect (Fig. 3A, D, G). PPP was
390 able to prevent carbonyl formation in kidney (Fig. 3A). We also measured the total
391 content of thiol groups, which indicates the level of protein sulfhydryl groups oxidation,
392 as sulfhydryl groups are oxidized in response to pro-oxidant stimuli [22]. Protein
393 sulfhydryl oxidation was not prevented statically by any pre-treatment (Fig 3. B, E, H),

394 however in liver (Fig. 3E) FPP group had no difference to control group indicating a
395 possible protection.
396 Lipid peroxidation is considered one of the basic mechanisms involved in reversible and
397 irreversible cell and tissue damage. Lipid peroxidation has been implicated in the
398 pathogenesis of many diseases. In liver, it is an early marker of cell membrane damage
399 associated with the subsequent leakage of hepatotoxicity markers to the bloodstream
400 [(33)]. Lipid peroxidation (expressed as TBARS) was significantly increased in samples
401 treated with the $FeSO_4/H_2O_2$. Pretreatment with peel significantly reduced increase in
402 TBARS formation in all tissue slice samples (Fig. 3C, F, I). In brain cortex slices
403 (Fig.3I), FPP also had a protective effect. The observation that peach peels presented
404 antioxidant activity mainly in the lipid fraction (Fig 3C, F, I), while FPP had a major
405 antioxidant effect to soluble protein fractions (Fig. 3A, D, G) suggest that different
406 secondary compounds present in distinct parts of the fruit (i.e. pulp and peel) are
407 responsible for these effects.
408 In response to acute or chronic infection, the production and release of TNF-α and IL-
409 1β is increased. These cytokines trigger pro-inflammatory signal cascades in tissues,
410 enhancing reactive species production and further cytokine expression and release. In
411 order to analyze the potential anti-inflammatory effects of peaches on tissues, TNF-α
412 and IL-1β levels in the incubation medium were quantified by ELISA as previously
413 described [(25)]. The incubation with the $FeSO_4/H_2O_2$ system led to a significant increase
414 in the levels of TNF-α and IL-1β in the incubation medium of all tissues, indicating an
415 acute inflammatory response (Fig. 4). In kidney tissue (Fig. 4A, B), the FPP, peel and
416 PPP prevented the release of TNF-α and IL-1β. In the liver Fig. 4C, D), only the peel
417 caused a similar effect, preventing the increase of TNF-α and IL-1β release caused by
418 the pro-oxidant insult. FPP also inhibited the release of TNF-α in brain cortex (Fig. 4E).

419 As mentioned earlier, plant secondary metabolism is responsible for the synthesis of
420 many compounds that exert important biological activities in animal cells when ingested
421 as part of animal diet. Phenolic compounds are found in many different foods,
422 especially fruits and vegetables [33]. Dietary phenolic compounds have been considered
423 essential for prevention of oxidative stress-mediated diseases [34]. Polyphenols obtained
424 from the diet are known to inhibit the free radical production derived from xenobiotic
425 toxic agents, thus reducing the risk of liver disease [35]. Carotenoids are photosynthetic
426 pigments that provide much of the different colors seen in plants and constitute an
427 important part of the diet of many animals. In humans, carotenoids-enriched diets have
428 been linked to prevention of certain cancers and eye diseases [36]. As FPP, peels, PPP
429 and syrup presented different effects in our *in vitro* and *ex vivo* assays, we evaluated the
430 differences between the content of phenolic compounds and carotenoids in these
431 products.
432 We performed a determination of the total phenolic content of the peach-dervived
433 samples by the Folin-Ciocalteau method and observed a higher content of total
434 phenolics in peels and FPP compared to PPP and syrup, we used the gallic acid as
435 standard (Fig. 5A). In a previous study with this same variety (Maciel) of peach,
436 chlorogenic acid was found to be present in high amounts in lyophilized samples from
437 the whole fruit [26]. Chlorogenic acid is one of the most abundant polyphenols in fruits
438 and it may be one of the main phenolic compounds exerting the biological activities
439 observed here.
440 We also performed a quantification of five common carotenoid compounds (all-trans-
441 lutein, zeaxanthin, β- cryptoxanthin, α-carotene and β-carotene) in these samples by
442 HPLC (Fig. 5B). Both FPP and peel presented higher concentrations of all carotenoids
443 evaluated, while the PPP samples presented lower levels of these compounds with

444 exception of α-carotene, which was not detected. On the other hand, there were no
445 detectable amounts of any of the carotenoids analyzed in syrup samples. In previous
446 studies, it was observed that peach peels exhibited a 2 to 27-fold higher antioxidant
447 activity than the fruit pulps [(37)]. In general, the main differences between these fruit parts
448 are the richest protein content of peels and the higher carbohydrate content in the pulp
449 [(38)]. However, as we have seen here, the amount of carotenoids and phenolic compounds
450 between these fruit parts may differ.
451 High concentration of phenolic compounds has been correlated with higher antioxidant
452 activity in dietary fruits such as strawberry, raspberry, blueberry, peach, apricot and
453 pear [(39)]. However, isolated phenolic compounds, carotenoids and vitamins with known
454 antioxidant properties (such as vitamin A) are not able to exert antioxidant and anti-
455 inflammatory actions at the same level as when obtained from fruit extracts such as
456 nectarine, peach and plum, which suggests an important role for the synergism among
457 the antioxidants in the mixture [(40)].
458 FPP, Peels, PPP and Syrup present different antioxidant, anti-glycation and anti-
459 inflammatory properties, as assessed by in vitro and ex vivo assays. The assessment of
460 antioxidant and anti-inflammatory effects in liver, kidney and brain cortex slices
461 showed significant differences between the peach-derived products; FPP and Peel
462 presented the highest antioxidant and anti-inflammatory properties, followed by PPP.
463 Syrup had no significant effect in all assays. We observed that the content of phenolic
464 compounds and carotenoids is significantly higher in FPP and peels, followed by PPP,
465 and a low levels of phenolic compounds plus undectable levels of carotenoids in syrup.
466

467 **Conclusions**

468 These data strongly suggest that the biological properties observed here are correlated to
469 the content of these compounds in peach products. *In natura* extracts had excellent
470 protector effects in comparison with fruits that have undergone industrial process. FPP
471 and Peel demonstrated high antioxidant and anti-inflammatory effects preventing
472 against induced damage. Further studies will address the effects of the consumption of
473 these products derived from peaches *in vivo* models, as well as the role of the
474 micronutrients and their effects.
475

476 **Authors' contributions**

477 *J.G* conducted all the animal studies and drafted the manuscript. *N.S, K.K*, performed
478 oxidative stress assays. *R.C.B,* performed total phenolic content, protein glycation and
479 total reactive antioxidant potential. *K.S.M*, conducted High-Performance Liquid
480 Chromatography (HPLC). *C.S.G* and *T.K.R*, performed oxidative damage assays. *M.S.M*
481 was responsible by ELISA assays. *M.V* and *M.C.B.R* lyophilized the fruits. *J.C.F.M* and
482 *D.P.G* are the heads of the lab. and coordinate this work. All authors have read and
483 approved the final manuscript.
484

485 **Conflict of interest**

486 The authors declare that there are no conflicts of interest
487

488 **Acknowledgements**

489 The Brazilian research funding agencies FAPERGS (PqG 12099/8, ARD
490 11/1893-7, PRONEX 1000274) CAPES (PROCAD 066/2007), CNPq PROPESQ-
491 UFRGS supported this work.
492

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588

589 **Figure Legends**

590

591 **Figure 1**. Antioxidant and anti-glycation profile. Equal concentrations (20 μg/mL) of
592 lyophilized samples of fresh peach pulp (FPP), peel, preserve peach pulp (PPP) and
593 syrup were compared. **A)** The total reactive antioxidant potential (TRAP) was
594 performed as described in "material and methods" section and the area under curve
595 values were calculated. "System" denotes the peroxyl-radical production system. Trolox
596 was used as standard antioxidant for comparison. **B)** Kinetics of chemiluminescence
597 intensity (% counts per minute [CPM]) are also depicted. **C)** The TAR index, estimated
598 by the instantaneous decrease in chemiluminescence when samples are added to the
599 system, was calculated from the same assay. ***Different from system ($p < 0.0001$), $^{\#}$
600 different from syrup and PPP groups ($p < 0.0001$). **D)** Percentage of *in vitro* albumin
601 glycation by glucose and fructose for 21 days in the presence of FPP, peel, PPP or syrup
602 at 1,10 and 100 μg/mL. *Different from glycation- induced group ($p < 0.0001$),
603 $^{\#}$different from glycation-induced group and from asterisk-marked groups ($p < 0.05$)
604 using one-way ANOVA (Tukey's post hoc). Values in graphic bars represent mean ±
605 SEM (triplicate experiments).
606
607 **Figure 2.** Effects of FPP, peel, PPP and syrup on cytotoxicity and antioxidant
608 enzyme activities in tissue slices subjected to oxidative stress. Kidney, liver and brain
609 cortex slices were pre-incubated with FPP, peel, PPP or syrup (80 μg/mL each) for 60
610 minutes and then subjected to oxidative damage by incubation with $FeSO_4$ 1 mM and
611 H_2O_2 100 mM for 30 minutes (stress-induced group). **A)** LDH activity in the incubation
612 medium was analyzed as a parameter for cytotoxicity (cell rupture). **B)** CAT and **C)**
613 SOD activities were assessed in homogenized tissue slices of Kidney. Letters **D**, **E**, **F**
614 represent respectively the same protocols to liver homogenate, and **G, H**, and **I** to brain
615 cortex. $^{\#}$ Different from control group ($p<0.0001$) **different from stress-induced group

616 (p<0.001) and * (p<0.05). One-way ANOVA (Tukey's post hoc) was applied. Values in
617 graphic bars represent mean ± SEM (triplicate experiments, n=6 per group).
618

619 **Figure 3.** Effects of FPP, peel, PPP and syrup on biomolecule oxidative damage.
620 Kidney, liver and brain cortex slices were pre-incubated with FPP, peel, PPP or syrup
621 (80 μg/mL each) for 60 minutes and then subjected to oxidative damage by incubation
622 with $FeSO_4$ 1 mM and H_2O_2 100 mM for 30 minutes (stress-induced group). Tissues
623 were homogenized and analyzed for (**A, B, C)** protein carbonylation, reduced
624 sulphydryl content and TBARS content in kidney. The same assays were conducted to
625 liver (**D, E, F**) and brain cortex (**G, H, I**). $^{\#}$Different from control group ($p<0.05$);
626 *different from stress-induced group ($p < 0.05$) using using one-way ANOVA (Tukey's
627 post hoc). Values in graphic bars represent mean ± SEM (triplicate experiments, n=6 per
628 group).
629

630 **Figure 4**. Effects of FPP, peel, PPP and syrup on interleukin release. Kidney, liver
631 and brain cortex slices were pre-incubated with FPP, peel, PPP or syrup (80 μg/mL
632 each) for 60 minutes and then subjected to oxidative damage by incubation with $FeSO_4$
633 1 mM and H_2O_2 100 mM for 30 minutes (stress-induced group). The incubation
634 medium was collected and analyzed by ELISA. (**A)** TNF-α of kidney, (**C)** liver and **(E)**
635 brain cortex was quantified. IL1-β levels in **(B)** kidney, **(D)** liver and **(F)** brain cortex
636 were evaluated too. $^{\#}$Different from control group ($p < 0.0001$); *different from stress-
637 induced group ($p < 0.05$) using one-way ANOVA (Tukey's post hoc). Values in graphic
638 bars represent mean ± SEM (triplicate experiments, n=6 per group).
639

640 **Figure 5**. Total phenol content and HPLC quantification. **A)** Suspensions of FPP,

641 peel, PPP and syrup (100 μg/mL) were analyzed for total phenolic content. Values are

642 expressed in μg of gallic acid equivalents per gram of dry weight of samples.

643 ***Different from syrup and PPP groups ($p < 0.0001$); *different from syrup group ($p <$

644 0.05). **B)** HPLC quantification of major carotenoids in FPP, peel, PPP and syrup

645 samples. Letters denote same degree of significance between groups for each carotenoid

646 ($p < 0.05$); *different from all other groups. Values in graphic bars represent mean ±

647 SEM (triplicate experiments, n=3 per group).

648

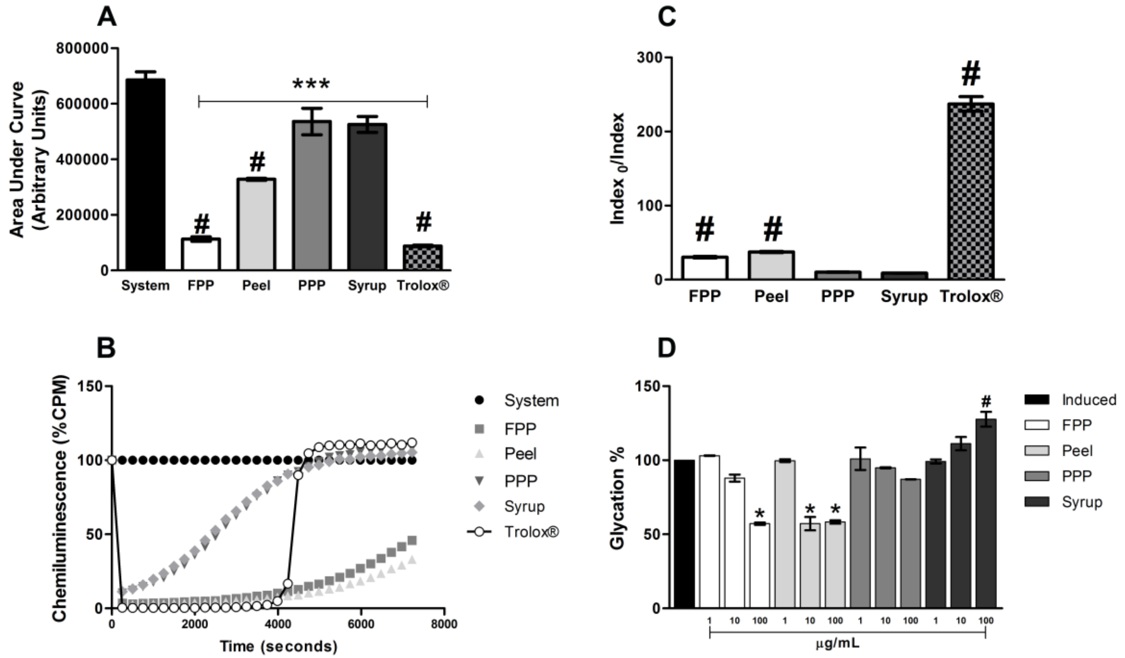


FIGURE 1. TRAP, PROFILE TRAP, TAR, GLYCATION

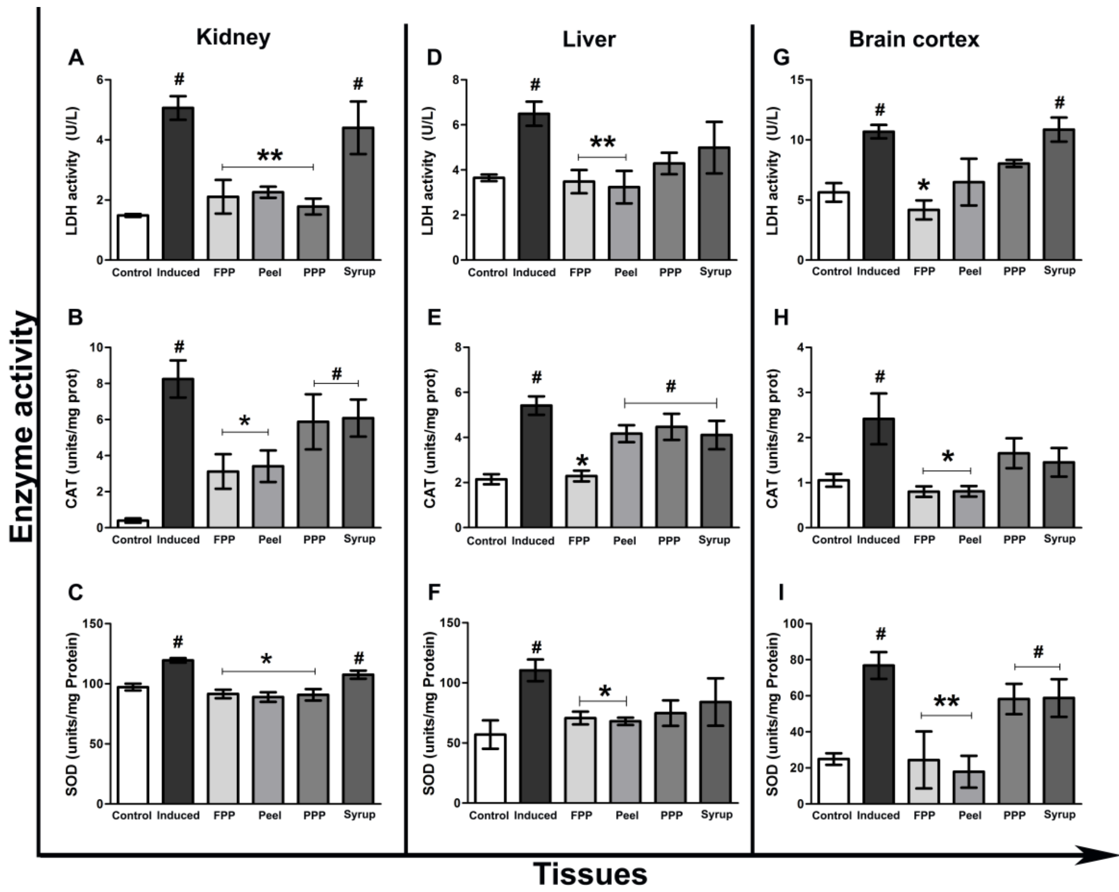


Figure 2.

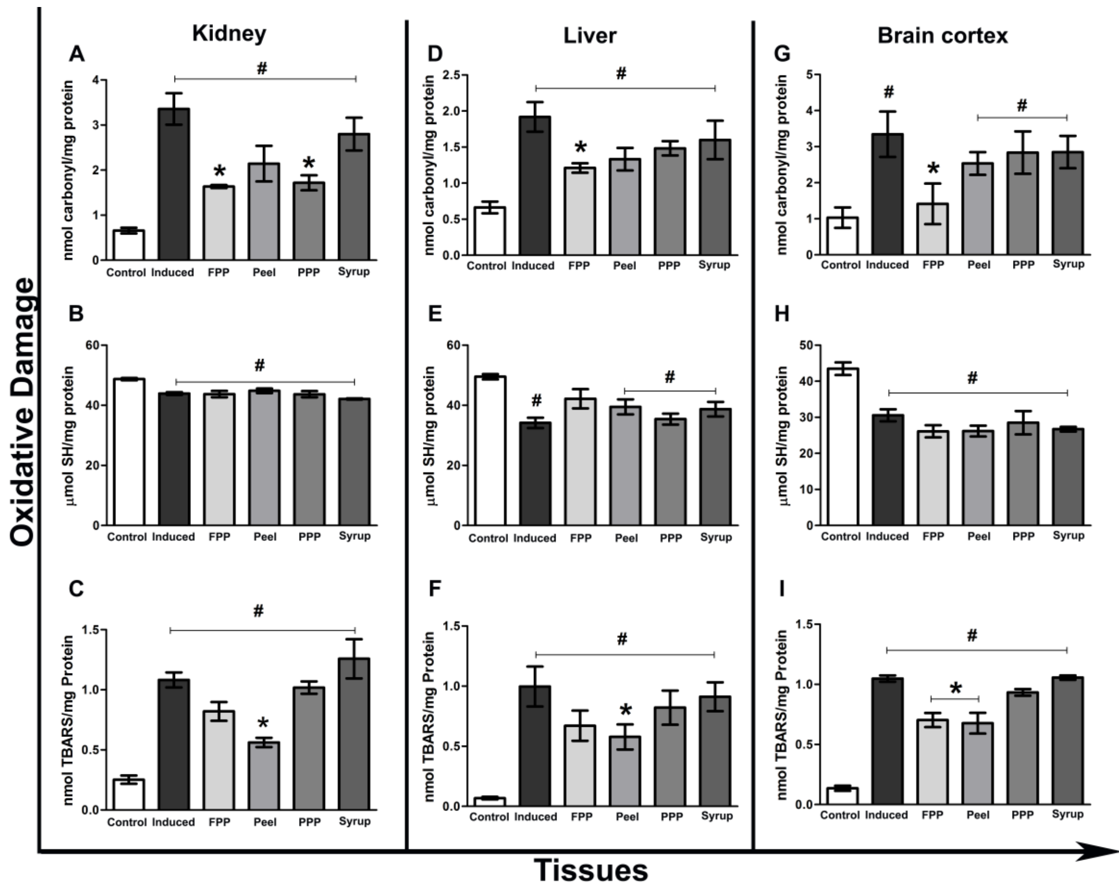


Figure 3.

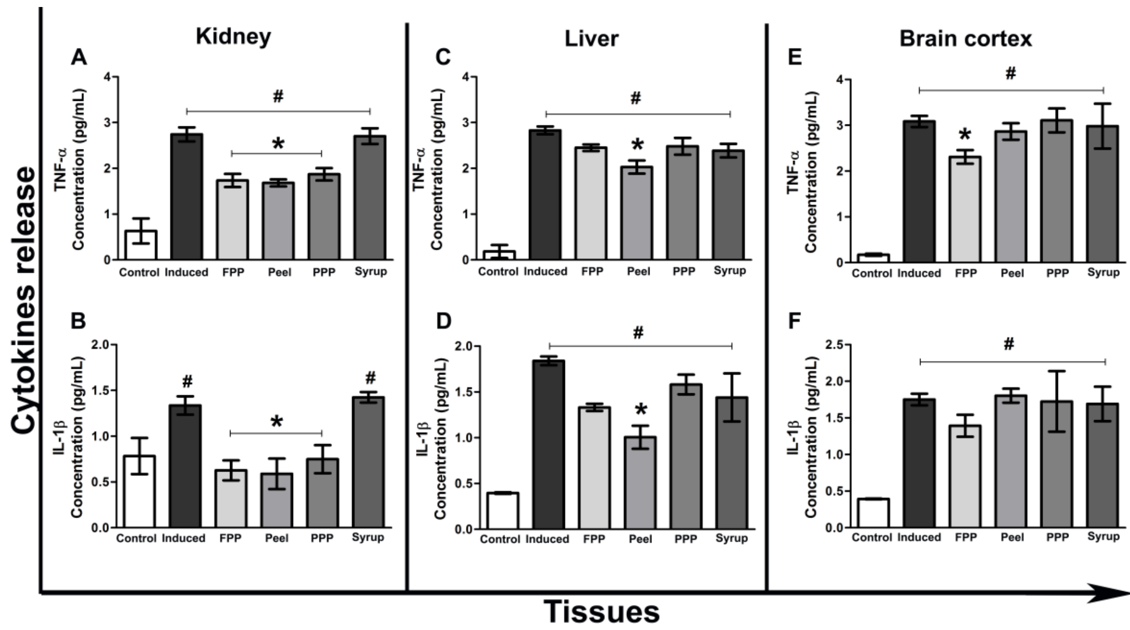


Figure 4.

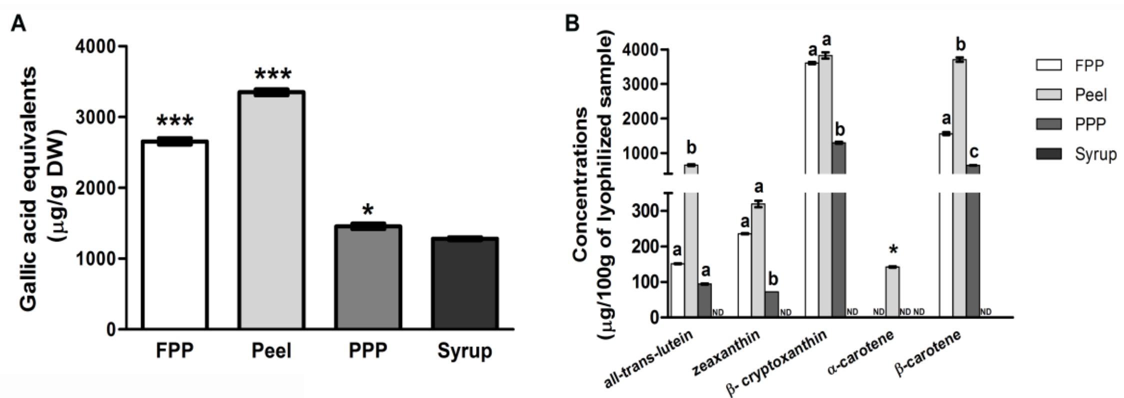
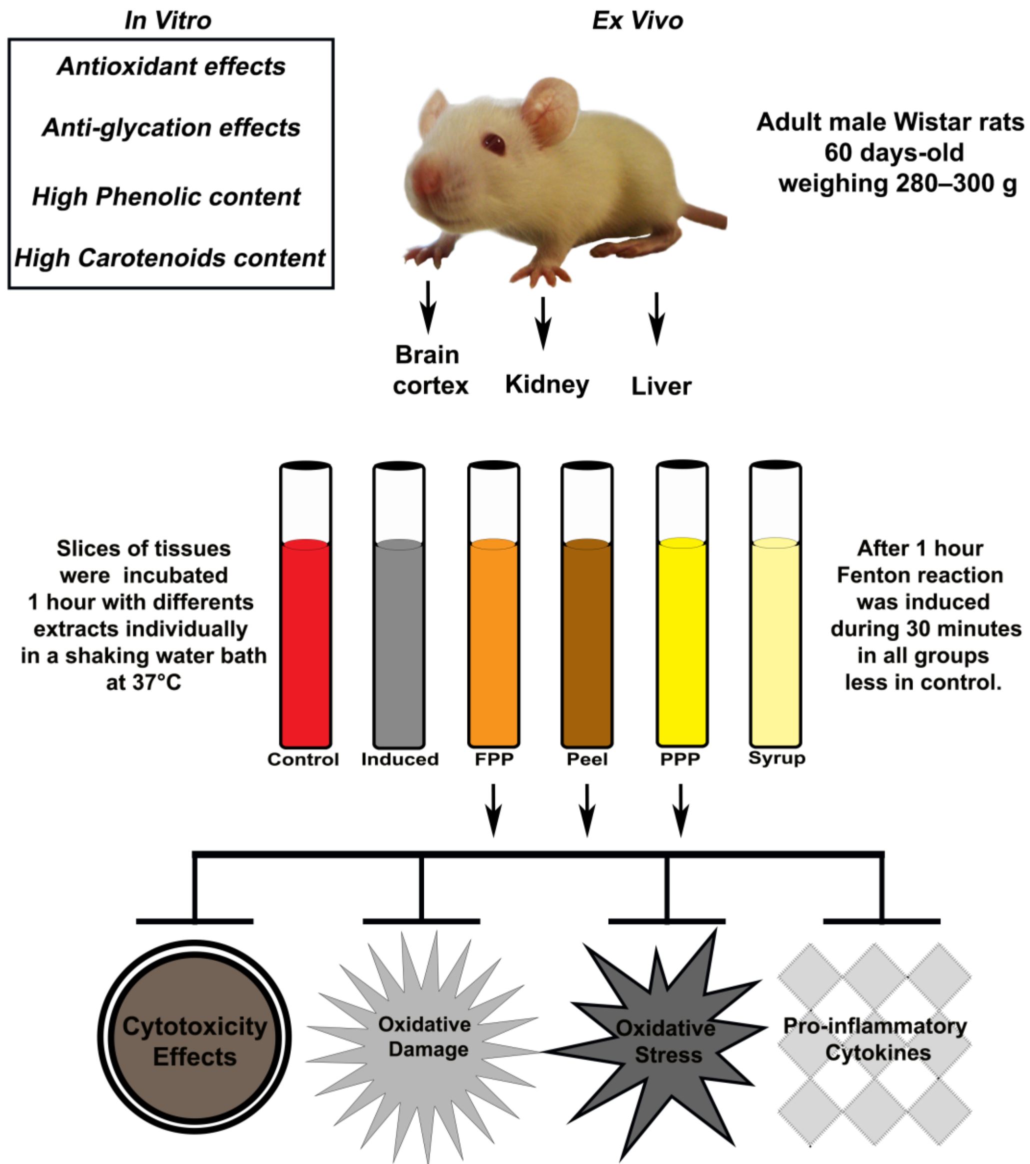


FIGURE 5. POLYPHENOLS and HPLC


Effects of different products of peach (Prunus persica L. Batsch) from
a variety developed in southern Brazil on oxidative stress and inflammatory
parameters in vitro and ex vivo
In Vitro
Ex Vivo
Antioxidant effects
Anti-glycation effects
High Phenolic content
High Carotenoids content
Adult male Wistar rats
60 days-old
weighing 280–300 g
Brain cortex
Kidney
Liver
Slices of tissues were incubated 1 hour with differents extracts individually in a shaking water bath at 37°C
After 1 hour Fenton reaction was induced during 30 minutes in all groups less in control.
Control
Induced
FPP
Peel
PPP
Syrup
Cytotoxicity Effects
Oxidative Damage
Oxidative Stress
Pro-inflammatory Cytokines

Capítulo II

"Preventive supplementation with fresh and preserved peach attenuates CCl_4-induced oxidative stress, inflammation and tissue damage in animal model"

Juciano Gasparotto; Nauana Somensi; Rafael C Bortolin; Carolina S Girardi; Alice Kunzler; Thallita K Rabelo; João Paulo A Dos Santos; Carlos E Schnorr; Karla S Moresco; Márcia Vizzotto; Maria do Carmo B Raseira ; Alfeu Zanotto-Filho; José Claudio F Moreira; Daniel P Gelain

Artigo Submetido ao Periódico Journal of Nutritional Biochemistry em 18 de Novembro de 2013.

Elsevier Editorial System(tm) for Journal of Nutritional Biochemistry
Manuscript Draft

Manuscript Number: JNB-13-762

Title: Preventive supplementation with fresh and preserved peach attenuates CCl4-induced oxidative stress, inflammation and tissue damage in animal model.

Article Type: Research Article

Keywords: Peach; NFkB-p65; RAGE; antioxidant; anti-inflammatory; carbon tetrachloride.

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Abstract: The present study was elaborated to comparatively evaluate the preventive effect of different peach lyophilized products obtained from preserved fruits (Syrup and Preserve Pulp Peach [PPP]) and from fresh peels and pulps (Peel and Fresh Pulp Peach [FPP]) in a model of liver/renal toxicity and inflammation induced by carbon tetrachloride (CCl4) in rats. Blood glucose and weight were monitored. Tissue damage (carbonyl, TBARS and sulfhydril), antioxidant enzymes activity (CAT and SOD) and inflammatory parameters (TNF-[illegible] and IL-1[illegible] levels, and RAGE and NF[illegible]B-p65 immunocontent) were investigated. HPLC analysis was carried out to evaluate the levels of carotenoids and phenols content was measured by Folin-Ciocalteu method in different peach extracts. Our findings demonstrated that Peel, PPP and FPP oral supplementation (30 days, 200-400 mg/kg/day) exerted significant potential to prevent CCl4-induced antioxidant enzymes activation and, most important, oxidative damage to lipids and proteins as well as blocked induction of inflammatory mediators such as TNF-[illegible], IL-1[illegible], RAGE and NF[illegible]B in CCl4-treated animals. This antioxidant/anti-inflammatory effect seems to be associated with the abundance of carotenoids and polyphenols present in peach fresh/preserves, since Syrup - which was the least enriched in antioxidants - displayed none protective effect in our experiments. These effects cumulated in decreased levels of transaminases and LDH leakage into serum, and maintenance of organ architecture. Therefore, the herein presented results show evidence that supplementation with peach products may be protective against organ damage caused by oxidative stress, being interesting candidates for production of antioxidant-enriched functional foods.

Cover Letter

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Porto Alegre, November 18th, 2013

To the editors of **Journal of Nutritional Biochemistry**

Dear Editor;

We are submitting our work entitled "**Preventive supplementation with fresh and preserved peach attenuates CCl_4-induced oxidative stress, inflammation and tissue damage in animal model**" to be considered for publication in Journal of Nutritional Biochemistry. The present study was designed to address the preventive effects of fresh and industry processed peaches extracts against CCl_4-induced liver and renal damage, and inflammation in animal model. We evaluated oxidative stress response enzymes, oxidative damage to lipids, proteins (cysteine groups oxidation, carbonylation and nitrotyrosine adducts), and inflammation parameters (IL-1β, TNF-α, NFκB and RAGE levels) in liver, kidney or/and serum of Wistar rats as experimental model. In addition, we characterized the abundance of carotenoids and polyphenol compounds in fresh and processed peach products, and associated with its protective potential. Taking into account that oxidative stress underlies the pathogenesis of a variety of human conditions as cardiovascular diseases, diabetes, neurodegeneration and even cancers, the comprehension of how prophylactic ingestion of antioxidant-rich fruits and how food

processing (herein focused in peaches products) may affect its preventive potential could be subject of interest to JNB readers.

We state this work is original and is not under consideration for publication elsewhere and no part of this work has been published before. We declare that this work followed the international recommendations for ethics in animal research, being evaluated by the local ethic research committee board as "approved". The experiments performed here were under "*in vitro and in vivo*" conditions. This work was funded by grants from governmental agencies for support of basic science in Brazil.

All authors contributed substantially to the work, all authors have read, approved and agreed to submit the manuscript to Journal of Nutritional Biochemistry. All individuals that contributed to this manuscript are listed as authors. The authors declare that have no conflict of interests.

Thank you for your time in handling our manuscript. Hopefully we will be pleased to publish our efforts in an important journal as JNB.

Best regards,

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*Manuscript
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1 **Preventive supplementation with fresh and preserved peach attenuates CCl_4-induced**
2 **oxidative stress, inflammation and tissue damage in animal model.**

3

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23 **Funding sources**
24 FAPERGS (PqG 12099/8, ARD 11/1893-7, PRONEX 1000274) CAPES (PROCAD
25 066/2007), CNPq PROPESQ-UFRGS.

26 **Abstract**

27 The present study was elaborated to comparatively evaluate the preventive effect of different
28 peach lyophilized products obtained from preserved fruits (Syrup and Preserve Pulp Peach
29 [PPP]) and from fresh peels and pulps (Peel and Fresh Pulp Peach [FPP]) in a model of
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31 glucose and weight were monitored. Tissue damage (carbonyl, TBARS and sulfhydril),
32 antioxidant enzymes activity (CAT and SOD) and inflammatory parameters (TNF-α and IL-
33 1β levels, and RAGE and NFκB-p65 immunocontent) were investigated. HPLC analysis was
34 carried out to evaluate the levels of carotenoids and phenols content was measured by Folin-
35 Ciocalteu method in different peach extracts. Our findings demonstrated that Peel, PPP and
36 FPP oral supplementation (30 days, 200-400 mg/kg/day) exerted significant potential to
37 prevent CCl_4-induced antioxidant enzymes activation and, most important, oxidative damage
38 to lipids and proteins as well as blocked induction of inflammatory mediators such as TNF-α,
39 IL-1β, RAGE and NFκB in CCl_4-treated animals. This antioxidant/anti-inflammatory effect
40 seems to be associated with the abundance of carotenoids and polyphenols present in peach
41 fresh/preserves, since Syrup – which was the least enriched in antioxidants – displayed none
42 protective effect in our experiments. These effects cumulated in decreased levels of
43 transaminases and LDH leakage into serum, and maintenance of organ architecture.
44 Therefore, the herein presented results show evidence that supplementation with peach
45 products may be protective against organ damage caused by oxidative stress, being interesting
46 candidates for production of antioxidant-enriched functional foods.

47

48 Keywords: Peach; NFκB-p65; RAGE; antioxidant; anti-inflammatory; carbon tetrachloride.

49

1. Introduction

The liver and kidney play pivotal roles in the systemic control of energetic metabolism, blood homeostasis as well as phaseI/II detoxification and excretion of a myriad of xenobiotics present in food, medicines, alcohol and other toxicants humans are exposed to daily in the contemporary life style [1]. Xenobiotics became extremely important in the last decades with the advances in food technology, which take use of several preservatives, sweeteners and dyes to improve taste, color and shelf-time of products. Nonetheless, several therapeutic drugs may exert hepato- and renal toxicity, which range from simple painkiller drugs such as acetaminophen and other NSAIDs, and others like allopurinol, IFN-beta1a, duloxetine (antidepressant), and some classical chemotherapeutic drugs as cisplatin and cyclophosphamide [2]. In addition, high blood sugar levels in uncontrolled diabetes may cause liver and renal inflammation and damage, which severely impairs organ functioning [3, 4]. Imbalance in the detoxification systems may be caused by a series of acute and chronic exposure to xenobiotics, which associated with genetic factors can lead to hepatocellular/renal apoptosis and inflammation. Chronic liver injury/disease is frequently featured by development of local or disseminated fibrosis and impairment in energetic and xenobiotic metabolism whereas renal disease affects excretion of waste products and toxins produced in liver from blood circulation and electrolytes homeostasis, regulation of blood pressure and hormone secretions [5].

Free radicals and non-radicals reactive oxygen species (ROS) are widely believed to contribute to development of several age/xenobiotic-related diseases by causing oxidative stress and oxidative damage [6]. When an imbalance between oxidants and antioxidants occurs, an excess of ROS forms, causing oxidative damage in vulnerable targets such as membrane unsaturated fatty acids, protein thiols, and DNA bases [7]. Excessive ROS may

75 contribute to the aging process as well as to chronic diseases such hepatic fibrosis, cancer,
76 atherosclerosis, neurodegenerative diseases and diabetes [6, 8]. Taking into account that it has
77 been established that oxidative stress and inflammation play a critical role in chronic liver
78 damage and renal injuries [5, 9], the search for drugable signaling mechanisms to counteract
79 liver/renal damage is an area of interest among scientists. On the other hand, the variety of
80 antioxidants present in vegetable sources – associated with the genetic improvement and food
81 technology – make the discovery of functional foods a good and safe strategy to prevent the
82 chronic development of organ injuries.

83 It has given proved that fruits and vegetables have a plenty of antioxidant compounds
84 including flavonoids and carotenoids, which have been associated with lower risk of stroke,
85 coronary heart disease, and markers of inflammation and oxidative stress in adults [10]. Of
86 relevance for this study, carotenoids are divided into two major structural groups: (1) oxygen-
87 containing molecules as xanthophylls such as lutein and β-cryptoxanthin, and (2) un-
88 oxygenated carotenes which include hydrocarbon carotenoids that are either cyclized such as
89 α-carotene and β-carotene or linear, like lycopene [5, 11]. High carotenoid intake leads to
90 significantly reduced risk of several chronic and degenerative diseases as coronary diseases,
91 some types of cancer and other ROS-related conditions [4, 5, 12, 13].

92 The present study was underwent to investigate the preventive effect of different peach
93 lyophilizes obtained from canned peach Syrup and pulp preserves (Syrup and PPP,
94 respectively) and from fresh peels and pulps (Peel and FPP groups, respectively) in a model
95 of liver/renal toxicity and inflammation induced by carbon tetrachloride (CCl_4) *in vivo*. We
96 monitored markers of: i) tissue damage [serum aspartate aminotransferase (AST), alanine
97 aminotransferase (ALT), bilirubin and LDH levels, and histochemistry]; ii) oxidative stress
98 and oxidative damage to biomolecules [superoxide dismutase (SOD) and catalase (CAT)
99 activities, and nitrotyrosine, protein carbonylation, sulfhydril and lipoperoxidation (TBARS)

100 levels]; iii) inflammatory parameters (TNF-α and IL-1β levels, and RAGE and NFκB-p65
101 immunocontent). HPLC was carried out to evaluate five carotenoids (All-trans-lutein,
102 zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene) in the different extracts. Total
103 phenolic content of peaches and derivatives was determined using the Folin-Ciocalteu
104 reaction. Blood glucose and weight were also monitored. The effect of treatments on
105 liverand kidneys protection was determined by performing assays in serum and tissues.
106
107 **2. Materials and Methods**
108 *2.1 Chemicals*
109 Catalase (CAT, EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1), thiobarbituric acid
110 (TBA), hydrogen peroxide (H_2O_2), carbon tetrachloride (CCl_4) were from Sigma-Aldrich®
111 (St. Louis, USA). ELISA microplates were from Greiner Bio-One (Monroe, USA) and ELISA
112 TMB spectrophotometric detection kit was from BD Biosciences (San Diego, USA). TNF-α,
113 IL-1β, nitrotyrosine, NF-κB p65, RAGE and β-actin primary antibodies and secondary-HRP
114 linked antibodies were from Cell Signaling technology ® (Beverly, USA). Purified
115 recombinant TNF-α protein was from Abcam® (Cambridge, UK) and IL1-β was from BD.
116 Bilirrubin, lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine
117 aminotransferase (ALT) activity kits were from Labtest (Minas Gerais, Brazil). The peach
118 samples were provided by Embrapa Clima Temperado (Rio Grande do Sul, Brazil). Blood
119 Glucose Meter OneTouch® ultra® was from Johnson & Johnson (New Jersey, USA).
120
121 *2.2 Preparation of peach samples*
122 The Maciel peach variety was developed by Brazilian Agricultural Research Corporation's
123 (Embrapa Clima Temperado) by controlled hybridization. The seeds were laminated in

124 chamber at 4 ± 1° C and then seedlings were cultivated in greenhouse and subsequently
125 transplanted to the seedlings experimental field. Fruits were obtained from this field (Pelotas,
126 Rio Grande do Sul, Brazil, location coordinates: -31º 30´57.44", -52º 33´11.52"). Immediately
127 after harvesting the fruits, the peel and pulps were separated and frozen at -20° C (the pits
128 were removed and discharged). Fruits were also used to prepare syrup-based preserves, which
129 involves the same industrial procedure for production of large-scale commercial canned peach
130 preserves. The fruits are subjected to a soda shower-based peeling procedure in a treadmill
131 and immediately washed to remove the soda. Pulps are placed into cans and hot sucrose-based
132 syrup is added. Cans are sealed, subjected to sterilization and then cooled. After four months,
133 the cans were opened and the "preserved peach pulps" (PPP experimental group) and the
134 remaining "syrup" (Syrup group) were separated and subjected to lyophilization. The samples
135 of "fresh peach pulp" (FPP group) and "fresh peel" (Peel group) were also subjected to
136 lyophilization, which was carried out in L108 Liotop equipment (Liobras, São Paulo, Brazil)
137 at the Embrapa Clima Temperado experimental unit. The lyophilized samples were preserved
138 at -20° C, dissolved in ultrapure water at the moment of the experiment and then centrifuged
139 (4000 g x 3 min) to precipitate rough particles.
140
141 *2.3 Animals and experimental design*
142 Male Wistar rats (21 days-old) were obtained from our breeding colony. They were caged in
143 groups of four animals with free access to standard commercial food Chow Nuvilab CR1
144 (Paraná, Brazil) and water. Rats were maintained in a 12-hour light–dark cycle in a
145 temperature-controlled colony room (21° C). All experimental procedures were performed in
146 accordance with the guidelines of the National Institutes of Health [14]. Our research protocol
147 (n° 23944) was approved by the Ethical Committee for Animal Experimentation of the
148 Universidade Federal do Rio Grande do Sul. Sixty five healthy animals were utilized for this

149 study. A pilot test was performed with five animals to determine optimal dose of CCl_4. A
150 process of adaptation lasting nine days was performed, and treatments with peach extracts
151 began at the 30th day of life (animals weighting 90-110 g).
152
153 *2.4 Weight gain and blood glucose test (BGT)*
154 One day before being sacrificed, animals were fasted for 8 hours [15], and glucose test was
155 performed. The blood sample was taken by a small tail puncture immediately before and 20,
156 40, 60 and 120 minutes after extracts administration. At each time, glucose was measured
157 with a glucose meter (OneTouch® ultra®).
158
159 *2.5 Cytokines levels in serum (IL-1β and TNF-α)*
160 TNF-α and IL-1β were quantified by indirect ELISA. Serum was incubated in an ELISA
161 plate. After 24 hours, plates were washed three times with Tween-Tris buffered saline (TTBS,
162 100 mM Tris – HCl, pH 7.5, containing 0.9 % NaCl, and 0.1 % Tween-20). Subsequently,
163 200 µl of anti-TNF-α or anti IL-1β (1:1.000) was added and incubation was carried out for 24
164 hours at 4 °C. The plates were washed three times with TTBS and incubated with rabbit or
165 mouse IgG peroxidase-linked secondary antibody (1:1.000) for 2 hours according with
166 datasheets product. After washing the plate three times with TTBS, 200 µl of substrate
167 solution (TMB spectrophotometric ELISA detection kit) were added to each well and
168 incubated for 15 min. The reaction was stopped with 50 µL/well of 12 M sulfuric acid and the
169 plate read at 450 nm in a microplate reader.
170
171 *2.6 Measurement of ALT, AST, Bilirrubin and LDH in serum*
172 ALT, AST, LDH activities and bilirubin concentrations in serum were determined using
173 Labtest kits (Minas Gerais, Brazil) according to manufacturer instructions.

174

175 *2.7 Antioxidant Enzymes (SOD and CAT)*

176 Catalase and superoxide dismutase activities were quantified in tissue homogenates (liver and
177 kidney). Tissues were homogenized with phosphate buffer (PB) 50 mM (KH_2PO_4 and
178 K_2HPO_4, pH-7.4). Catalase (CAT) activity was evaluated by following the rate of hydrogen
179 peroxide (H_2O_2) absorbance decrease at 240 nm [16]. Results are expressed as units of
180 CAT/mg of protein. The activity of superoxide dismutase (SOD) was measured by
181 quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation to
182 adrenochrome, which was monitored at 480 nm for 10 min (32 °C) in a spectrophotometer
183 [17]. Results are expressed as units of SOD/mg of protein.

184

185 *2.8 Oxidative damage markers* (Carbonyl, TBARS, Sulfhydril and nitrotyrosine)

186 As an index of protein oxidative damage, the carbonyl groups were determined based on its
187 reaction with 2,4-Dinitrophenylhydrazine (DNPH) as previously described [18].
188 Lipoperoxidation was determined from the quantification of thiobarbituric acid reactive
189 species (TBARS) originated from reaction of thiobarbituric acid with lipoperoxides in an
190 acid-heating medium [19]. After precipitation with trichloroacetic acid 10% (TCA),
191 supernatant was mixed with 0.67% and heated in a boiling water bath for 25 min. TBARS
192 were determined by the absorbance in a spectrophotometer at 532 nm. Protein Nitrotyrosine
193 was quantified by indirect ELISA in serum. Oxidative status of thiol groups were assessed by
194 quantification of total reduced sulfhydryl (SH) groups in samples [20]. Briefly, for total SH
195 content measurement, 60 μg sample aliquot was diluted in phosphate-buffered saline (PBS)
196 (NaCl, Na_2HPO_4, KH_2PO_4), and 5,5′-dithionitrobis 2-nitrobenzoic acid (10 mM), and read in
197 a spectrophotometer at 412 nm after 60 minutes of incubation in room temperature.

198

199 *2.9 Protein assay*

200 Total protein was quantified by Bradford assay and used to normalize all data [21].

201

202 *2.10 Western blotting to liver and kidney proteins*

203 To perform immunoblot experiments, the tissue was prepared using RIPA buffer protocol.
204 The proteins (30 μg/well) were fractionated by SDS-PAGE and electro-blotted onto
205 nitrocellulose membranes with Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell, Bio-
206 Rad (Hercules, CA, USA). Protein loading and electro-blotting efficiency were verified
207 through Ponceau S staining, and the membrane was washed with Tween–Tris buffered saline
208 (Tris 100 mM, pH 7.5, 0.9% NaCl and 0.1% Tween-20). Membranes were incubated 20
209 minutes at room temperature in SNAP i.d.® 2.0 Protein Detection System Merck Millipore
210 (Billerica, MA, USA) with each primary antibody (anti-RAGE, anti-NF-κb p65 or anti β-
211 actin; 1:500 dilution range) and subsequently washed with TTBS. Anti-rabbit or mouse IgG
212 peroxidase-linked secondary antibody was incubated for additional 20 minutes in SNAP
213 (1:5000 dilution), washed again and the immunoreactivity was detected by enhanced
214 chemiluminescence using Supersignal West Pico Chemiluminescent kit (Thermo Scientific;
215 Luminol/Enhancer and Stable Peroxide Buffer). Densitometric analysis of the films was
216 performed using Image J. software. Blots were developed to be linear in the range used for
217 densitometry. All results were expressed as a relative ratio to β-actin.

218

219 *2.11 Histological Analysis*

220 Formalin-fixed samples of liver were dehydrated, diaphonized and embedded in paraffin
221 according to protocols for routine histological procedures. Five-micrometer thick sections of
222 the paraffin-embedded tissues were obtained and stained by means of haematoxylin eosin

223 histochemical (H&E) method. The sections were examined microscopically for evaluation of
224 histological changes [22].
225
226 *2.12 Quantification of carotenoids by High-Performance Liquid Chromatography (HPLC) in*
227 *vitro*
228 The carotenoids were separated on a polymeric reversed phase column (YMC C_{30} 250
229 micrometer x 4.6 micrometer; particle size of 3 μm) with a mobile phase gradient elution
230 starting with water/methanol/MTBE (Methyl tert-butyl ether) at 5:90:5 and reaching 0:95:5
231 after 12 minutes, 0:89:11 after 25 minutes, 0:75:25 after 40 minutes and 00:50:50 after 60
232 minutes with a flow rate of 1 mL/minute at 33 °C [23]. The spectra were analyzed between
233 250 and 600 nm, and the chromatograms were processed at a fixed wavelength of 450 nm for
234 carotenoids. Identification was performed by comparison of peak retention times obtained in
235 each sample with the retention times of standards analyzed under the same conditions.
236
237 *2.13 Determination of total phenolic content*
238 Total phenolic content of peaches and derivatives was determined using the Folin-Ciocalteu
239 method [24] One hundred μL of Folin-Ciocalteu reagent was mixed with 100 μL of sample
240 and then 200 uL of Na_2CO_3 35% were added. The volume was completed to 1900 μL with
241 ultra-pure H_2O and then homogenized. After 10 min, the absorbance was measured at 725
242 nm and compared to a gallic acid calibration curve. Total polyphenols in samples were
243 expressed as gallic acid equivalents [25].
244
245 *2.14 Statistical analysis*
246 Statistical analysis was performed with GraphPad 5.0 software. The results were expressed as
247 mean ± standard error (SEM). Data were evaluated by one-way analysis of variance

248 (ANOVA) followed by Tukey's post hoc test. Differences were considered significant when p

249 < 0.05.

250

251 **3. Results**

252 *3.1 Effect of peach extracts on weight gain and blood glucose levels*

253 The groups, treatments and doses are detailed in figure 1A.Timeline of experimental design is

254 shown in figure 1B (see Materias and Methods for detailing). We observed that both doses

255 (200 and 400 mg/kg) did not affect the weight of animals once all groups maintained the

256 same proportions of weight gain along the period of treatment (Fig. 2A and C). The Syrup and

257 PPP extracts at doses of 200 mg/kg (Fig. 2B) promoted a transient increase of glycemia,

258 which peaked by 20, 40 minutes (102 ± 1.6 mg/dL) and returned to basal levels after 120

259 minutes (89 ± 1.0 mg/dL). At 400 mg/kg, Syrup and PPP glycemic curves displayed the same

260 profile observed at 200 mg/Kg but glycemia peaked at higher levels (112 ± 0.5 mg/dL)

261 compared to 200 mg/Kg. Differently, FPP and Peel extracts slightly modified glucose levels

262 but this effect was not statistically different from control. However, it is important to note that

263 at 400 mg/kg (Fig. 2D) FPP has also changed blood glucose in 20 min and values decreased

264 to control levels after 120 min. This data suggest that Peel extracts contains lower levels of

265 glucose precursor carbohydrates compared to other formulations tested.

266

267 *3.2 Effects of FPP, Peel, PPP and Syrup on tissue damage serum biomarkers*

268 Table 1 shows the effect of the different peach extracts on CCl_4-induced tissue damage.

269 Treatment with a single dose of CCl_4 caused a 3-fold increase in the levels of AST and ALT

270 transaminases confirming the induction of tissue toxicity. Bilirrubin, which is frequently more

271 affected in severe hepatotoxicity, only increased 1.5-fold. At dose of 200 mg/kg, only FPP

272 was able to reduce AST activity; all other markers did not change with this dose or other

273 extracts. On the other hand, pre-treatment with 400 mg/kg/day PPP and FPP for 30 days
274 significantly prevented leakage of AST and ALT. Bilirrubin was decreased only with FPP
275 group. LDH test evidenced that one single administration of CCl_4 to wistar rats resulted in a
276 10-fold increase of LDH activities in serum, and both FPP, PPP and Peel showed capacity to
277 protect tissues against cytotoxic damage.
278

279 *3.3 Cytokines (TNF-α and IL-1β) levels in serum*

280 Taking into account that organ inflammation is an important component of tissue damage
281 signaling leading to systemic inflammation, we assessed serum levels of the inflammatory
282 cytokines IL-1β and TNF-α after CCl_4 administration. The levels of these markers were
283 significantly increased by CCl_4 treatment compared with controls, indicating that damage
284 from CCl_4 causes inflammatory responses as expected. Again, pretreatments with 200 mg/kg
285 did not prevent CCl_4 induced cytokines production (Fig. 3A and 3B) whereas 400 mg/Kg
286 Peel blocked IL-1β (Fig. 3A) and FPP blocked TNF-α induction by CCl_4 (Fig.3B).
287

288 *3.4 Status of oxidative stress parameters (nitrotyrosine in serum and SOD and CAT in liver*
289 *and kidney)*

290 Such as observed for the aforementioned parameters, pre-treatments with 200 mg/kg did not
291 prevent CCL_4 effects on nitrotyrosine levels. In agreement with the inhibitory effect upon IL-
292 1β and TNF-α, FPP and Peel at 400 mg/kg decreased protein nitration (nitrotyrosine)
293 indicating that these extracts can effectively block the effect of reactive nitrogen species
294 (RNS) as peroxynitrite on protein damage (Fig. 4A). We also observed that CCl_4 also
295 promoted a significant increase in the activity of superoxide detoxification enzymes (SOD) in
296 renal and hepatic tissues (~2-fold increase), and 400 mg/kg of Peel and FPP extracted blocked
297 induction of this enzyme by CCl_4 in liver but not in kidney (Fig. 4B and D). The hydrogen

298 peroxide detoxification enzyme catalase (CAT) was 2-fold and 30-fold increased in liver and
299 renal tissues after CCl_4 –induced damage (Fig. 4C and 4E). At 400 mg/kg, supplementation
300 with PPP and FPP significantly inhibited CAT activity induction by CCl_4 in both tissues; Peel
301 extract blocked CAT induction in liver and a non-significant trend to decrease was also
302 observed in kidney.
303
304 *3.5 Oxidative damage assays in liver and kidney*
305 Hepatic and renal oxidative damage was determined through carbonyl (protein damage by
306 hydroxyl radical), sulfhydryl (cysteine oxidation) and TBARS assays (lipoperoxidation). In
307 both liver and kidney, exposure to CCl_4 modulated all parameters of oxidative damage toward
308 a pro-oxidative status/damage. FPP extracts seem to be the most effective in inhibiting
309 oxidative damage once it inhibited protein carbonylation, formation of lipoperoxides/TBARS
310 and cysteine oxidation in liver ($p< 0.05$) as well as kidney ($p<0.001$) (Fig. 5 A-F). Peel also
311 exerted significant antioxidant activity by preventing carbonylation (liver, Fig. 5A),
312 lipoperoxidation (liver and kidney, Fig. 5C and F) and sulfhydril oxidation in liver (Fig. 5B).
313 Syrup extracts were ineffective to counteract oxidative damage, and PPP only blocked
314 lipoperoxidation in both tissues (Fig. 5C and F), suggesting modest effects on these
315 parameters at the tested doses.
316
317 *3.6 Western blot for NFκB and RAGE in liver and kidney tissues*
318 Fig. 3A and B data showed that CCl_4 increases the level of IL-1β and TNF-α, which are
319 important pro-inflammatory gene products. Western blot analysis revealed that the amount of
320 hepatic and renal NFκB- p65 protein and the receptor for advanced glycation endproducts
321 (RAGE) – which are important upstream mediators of inflammatory genes as TNF-α and IL-
322 1β - increased markedly after CCl_4 administration. CCl_4-induced NFκB- p65 protein level

323 was significantly attenuated by Peel and FPP in liver (Fig. 6A), while in kidney Peel, PPP and
324 FPP, but not Syrup, were able to prevent NFκB- p65 induction by CCl_4 (Fig. 6C). The effects
325 of CCl_4 on RAGE protein content were broadly inhibited by Peel, PPP and FPP, but not
326 Syrup, in liver (Fig. 6B) and kidney (Fig.6D) tissues.
327
328 *3.7 Liver histology*
329 Histological analysis of control animals liver sections showed normal hepatic cells with well-
330 preserved cytoplasm, prominent nucleus and nucleolus, and visible central veins (Fig. 7A).
331 The liver sections from CCl_4 treatments revealed liver injuries, such as hydropic degeneration
332 and nuclear polymorphism, which were characterized by presence of hepatocytes cloudy
333 swelling with pale cytoplasm and poorly delineated and displaced nuclei (Fig. 7B). Analysis
334 of hepatic histopathological lesions indicated that pre-treatment with Peel (Fig. 7D), PPP (Fig.
335 7E) and FPP (Fig. 7F) markedly ameliorated the morphology of liver after CCl_4 insult. Syrup
336 group (Fig. 7C) did not display improvements compared to CCl4 group.
337
338 *3.8 Quantification of carotenoids by High-Performance Liquid Chromatography (HPLC) and*
339 *total phenolic content*
340 The chemical composition analysis by HPLC of Peel, FPP and PPP extracts showed presence
341 of (a) all-trans-lutein (rt = 10 min), (b) zeaxanthin (rt = 17.5 min), (c) β-cryptoxanthin (rt =
342 32.5 min) as the major compound, furthermore (d) α-carotene (rt = 42.5 min) and (e) β-
343 carotene were also identified (Fig. 8). Interestingly, extracts like Peel and FPP, which also
344 presented a better performance to protect oxidative damage and inflammation, were enriched
345 in antioxidant carotenoids as all-trans-lutein, zeaxanthin, α-carotene and β-carotene compared
346 to FPP. Quantification of total phenolic contents showed that FPP (2656.25 μg/g) and Peel

347 (3350.196 μg/g) presented higher polyphenols concentration, while PPP (1456.446 μg/g) and
348 Syrup (1281.836 μg/g) were less enriched in polyphenols.
349

350 **4. Discussion**

351

352 In the last decade, there is a growing interest in understanding how the intake of different
353 fruits and vegetables as well as its respective processed foods can lead to health benefits in
354 short and long-terms. The presence of a variety of carotenoids, ascorbic acid, citric acid and
355 polyphenols amongst other compounds make fruits and vegetables able to interfere with
356 antioxidant, inflammatory and coagulative systems [10, 13, 26]. Understanding how
357 industrial processing affects biological properties of vegetables it is also subject of interest in
358 the field. In this study, we focused on the study of different parts of fresh (Peel and FPP) and
359 canned peaches (Syrup and PPP) and evaluated their preventive role in liver and renal damage
360 caused by CCl_4 exposure in rats in order to address its possible application as functional food.
361 CCl_4 is widely utilized to induce liver fibrosis, cirrhosis and general toxicity in rats
362 [27-29]. In our model, a single CCl_4 was administered at last day of a 30-day supplementation
363 with peach extracts, characterizing it as preventive strategy. Along the supplementation with
364 peach extracts, all groups had similar weight gain (Fig 2A and C). At 29th day, blood glucose
365 was measured and a glucose curve after extracts administration was performed. The results
366 showed that glycaemia ranged in an expected profile, being weakly elevated at early 0.5 to 1 h
367 and decreasing to basal levels afterwards (within 2 h). This glycaemic effect was more
368 pronounced in extracts prepared from canned peaches (Syrup and PPP), which is possibly
369 attributed to the higher index of simple carbohydrates as sucrose and fructose used in this
370 method of preservation.

371 In the course of hepatic/tissue damage, plasmatic membrane dysfunction and necrotic
372 cell death release a series or transaminases, LDH amidst other enzymes into blood circulation,
373 which are considered indicators of liver/tissue injury [30]. In our model, different peach
374 fractions exerted different degrees of protection when based on detection of serum
375 transaminases, LDH and bilirubin caused by CCl_4. FPP, Peel and PPP were the most efficient
376 to prevent leakage of these markers, mostly LDH. As LDH is not totally specific for liver and
377 renal damage, and AST also can be used as marker of cardiac damage, we confirmed the
378 preventive effect of Peel, FPP, PPP peach extracts by direct histological analyses in liver
379 tissues, and the protective effect was clear. However, Syrup pre-treatment had no effect in
380 none of these parameters.

381 CCl_4 is a non-polar compound which tends to interact more efficiently with lipids
382 structures as cell membranes thus propagating free radical reactions toward intracellular
383 compartments [31]. Then, we would expect to find out a significant damage to lipids
384 following CCl_4 exposure. In biological systems, lipid peroxidation creates a series of stable
385 toxic aldehydes products, and thiobarbituric acid reactive substances (TBARS) have been
386 frequently used as an indicator of lipid peroxidation. Increased lipoperoxidation, protein
387 carbonylation levels, and decreased total thiol content make it easier for intra- and inter-
388 molecular cross-links of proteins, which in turn induce conformational changes leading to
389 increased hydrophobicity, formation of protein aggregates, oxidative damage to proteins
390 inducing generalized cellular dysfunction and favoring the maintenance of the pro-oxidative
391 state [9, 32]. Indeed, lipid peroxidation seems to be an important mechanism whereby CCl_4
392 affect cell integrity once the levels of TBARS in CCl_4-treated animals were hugely increased
393 mainly in kidney tissues, in agreement with [7, 33] Peel, PPP and FPP (400 mg/kg) but not
394 Syrup pre-treatments effectively protected lipid peroxidation in liver and renal tissues likely
395 due to its antioxidant and free radical scavenging activities. Although kidney has showed

396 higher TBARS, peach extracts were able to protect this tissue even at the lowest dose,
397 suggesting a greater capacity of peaches in preventing renal injury. Protein carbonylation
398 increased in both tissues and there was a decrease of protein thiol content, indicating that CCl_4
399 injury also affects protein redox state, which may affect protein structure and function, and
400 consequently pathways and organelles functioning [34]. In our model, Peel extract protected
401 liver against sulfhydril oxidation and protein carbonylation whereas only FPP was efficient in
402 renal tissue at 400 mg/Kg for the same markers.

403 The effect of Peel and FPP against protein nitration (3-nitrotyrosine marker) suggests
404 that these fractions affect NO (nitric oxide) and its derivative-nitration-active compound
405 peroxynitrite (ONOO-) metabolism. NO is frequently increased by inflammatory signals
406 which activate NOS (nitric oxide synthase) enzymes through NFκB pathway, playing a role in
407 carcinogenesis, chronic infection, inflammation and neurodegeneration [35, 36]. High levels
408 of superoxide can interact with nitric oxide in tissues and form the highly diffusible nitrogen
409 specie peroxynitrite, a highly reactive intermediate, which can increase DNA damage, and
410 initiate lipid peroxidation [37]. Increases in peroxynitrite lead to protein tyrosine residues
411 modification to form nitrotyrosine adduct, which may affect protein structure and function.
412 For example, tyrosine nitration of mitochondrial manganese superoxide dismutase results in
413 loss of enzymatic activity [38] and in serum peroxynitrite targets mainly albumin thus
414 forming 3-nitrotyrosine groups [39]. We believe that increases in the activation of
415 inflammatory pathways as NFκB – as observed in figure 6 – as well as induction of RAGE
416 (which signals downstream by inducing NFκB) in the presence of CCl_4-induced free radicals
417 are promoting formation of peroxynitrite. Then, peach extracts might be inhibiting
418 nitrotyrosine formation by blocking either NFκB or superoxide radical formation. The
419 antioxidant effect of Peel and FPP extracts in preventing ROS accumulation is evident from
420 the inhibitory effect of it on SOD and CAT activities induction by CCl_4 in liver. SOD and

421 CAT are in the first line of antioxidant defense mechanisms by protecting the cells against
422 superoxide and hydrogen peroxide through sequential detoxification reactions thus decreasing
423 hydrogen peroxide availability to react with transition metals to form the most dangerous free
424 radical, hydroxyl [39]. CCl_4 toxicity might result in significantly increases in SOD and CAT
425 activities possibly as an attempt to counteract free radicals in liver and kidney. One could
426 conclude that if Peel and FPP peach extracts are decreasing antioxidant enzymes activity it
427 would cause even more oxidative stress. However, take into account that animals were pre-
428 treated with extracts, we interpret that Peel and FPP extracts provided an antioxidant
429 environment enough to block CCl_4-induced oxidative damage (as observed from the
430 decreased level of oxidative damage markers and liver histology, figure 5 A-C) thus not being
431 necessary the induction of SOD and CAT. PPP and FPP extracts, but not Peel, prevented
432 induction of CAT enzyme in kidney but SOD was unaltered. Syrup exerted none effect on
433 these oxidative parameters. The understanding on how a supplementation with antioxidant
434 rich compounds as fruits can prevent tissue damage involves an interesting and complicated
435 crosstalk among: i) level and type of oxidants generated by the stressor agent; ii) organ
436 intrinsic antioxidant enzymatic and non-enzymatic defenses; iii) type, bioavailability and
437 specific free-radical/oxidant quenching activity of the antioxidants present in the respective
438 fruit. For example, we did not detect preventive effects of FPP on SOD induction in kidney,
439 but FPP still blocked sulfhydril oxidation, carbonylation and lipoperoxidation as well as
440 prevented transaminases and LDH leakage in CCl_4 intoxication model, showing that the
441 understanding of the antioxidant systems as a whole is important to conclude on extracts
442 usefulness as antioxidants.
443
444 Inflammatory processes are frequently accompanied by alterations in the tissue
445 structure. Such alterations may result from tissue damage due to active proteases or toxic

446 moieties released by inflammatory cells [39]. NFκB is a transcription factor that has been
447 recognized as one of the factors involved in a series of pathological conditions, principally
448 inflammation and cancers. NFκB consists of a heterodimer of p65/p50 retained in the
449 cytoplasm as an inactive tertiary complex associated with inhibitory proteins known as IκBs
450 [40]. After stimuli, as for example tumor necrosis factor alpha (TNF-α), IκB phosphorylation
451 by IKKs leads to protesome degradation of IκB, releasing NFκB to the nucleus [40, 41].
452 RAGE ligand dependent activation was shown to downstream activate NFκB, members of the
453 MAPK family and the PI3K pathway, leading to induction of pro-inflammatory cytokines as
454 TNF and interleukins, and enhancing reactive species production and oxidative stress-related
455 cell damage [42]. RAGE is capable of inducing de novo synthesis of NFκB as well as NFκB
456 targets RAGE promoter elements, which results in cycles of increasing states of pro-
457 inflammatory cytokine production upon RAGE activation [33, 43]. In our model, CCl_4 caused
458 hepatic and renal inflammation as observed from the increased levels of NFkB and RAGE as
459 well as its downstream targets TNF-α and IL-1β; necrosis and inflammatory infiltrates were
460 also confirmed by liver histology [44], evidencing an inflammatory landscape accompanying
461 liver/renal damage. Peel, FPP and PPP blocked the increases in NFκB and RAGE in liver and
462 kidney tissues caused by CCl4, and some effect on serum TNF-alpha and IL-1β were
463 observed at 400 mg/Kg of FPP and Peel, respectively. TNF-α and IL-1β are a pro-
464 inflammatory cytokines and play a key roles in the induction and perpetuation of
465 inflammation in macrophages [45]. Prolonged excessive production of TNF-α has been
466 implicated to contribute to the pathology of liver damage and systematic toxicity [28, 37] and
467 it might lead to marked cellular death. IL-1β is rapidly expressed in response to tissue
468 damage [46]. Previous studies have hypothesized that IL-1β may directly activate hepatic
469 stellate cells (HSCs) through autocrine signaling and stimulate the matrix metalloproteinases

470 (MMPs) produced by HSCs within the space of Disse, resulting in liver fibrogenesis [45, 46].
471 The inhibition of TNF-α and IL-1β release by FPP and Peel respectively can be attributed not
472 only to its antioxidant effect but also a possible direct effect on inflammatory pathways as
473 NFκB. Although Peel, FPP and even PPP demonstrated potential to block inflammatory
474 signals, in different extents and with some trend to better effects if tested in higher than 400
475 mg/Kg, Syrup extracts again had no effect in any of these parameters.
476 Studies carried out over the past few years have shown that dietary carotenoids are associated
477 with reduced oxidative stress [47]. In addition, carotenoids are antioxidants frequently
478 present in fruits, especially in peaches, and play a role in the prevention of damage caused by
479 harmful ROS, which are continuously produced in the body during normal cellular
480 functioning or are introduced from exogenous sources [48]. Previous studies demonstrated
481 that chlorogenic acid is one of the most abundant polyphenol in fruits as peaches, and it may
482 provide health-promoting advantages to consumers [7]. Here, we add the information that
483 carotenoids also collaborate with peaches antioxidant potential. The ability of plant extract to
484 scavenge ROS seems to be related to the chemical structure of phenolic compounds [49, 50],
485 our finds suggests that the Peel, PPP and FPP may present some important antioxidant
486 properties, probably related to its carotenoids and phenolic content. In our tests, all-trans-
487 lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene were identified in three
488 extracts, Peel, PPP and FPP, but Syrup did not present any detectable amount of these
489 carotenoids. The abundance of carotenoids was higher in Peel extracts followed by FPP and
490 PPP, phenols content showed the same profile. This different carotenoid profiling is in even
491 consonance with the better antioxidant activity of Peel and FPP extracts in all the oxidative
492 stress markers herein studied when compared to PPP and Syrup. The current knowledge
493 permits some interpretations on the non-effect of Syrup compared to other extracts. Syrup did
494 not present any detectable levels of carotenoids and were shabby in phenolic compounds

495 compared to other fractions. Moreover, during the industrialization process, peaches receive
496 large amounts of sugar to be preserved. Sugars are directly related to increases in AGEs
497 levels, and it consequently increases the levels of receptor for advanced glycation endproducts
498 (RAGE). In Syrup treated animals, RAGE content was similar to those observed in CCl_4
499 group. However in preserved pulp peach group (PPP), which peaches also were exposed to
500 high amounts of sugar but kept significant levels of carotenoids and polyphenols, CCl_4-
501 induced RAGE was reduced in both tissues. PPP also was able to prevent the increase in
502 RAGE, transaminases, LDH, and some oxidative stress markers – such effect was not
503 observed in Syrup treatments. These altogether suggest that peaches processing affects some
504 of the antioxidant properties compared to fresh fractions (FPP) but still keeps some of them
505 enough to prevent oxidative damage.
506
507 **4. Conclusion**
508 In conclusion, Peel, PPP and FPP preventive treatment appears to bring a significant
509 inhibition of CCl_4-induced damage either in the level of organ morphology and serum
510 markers as well as in oxidative damage to macromolecules (TBARS, carbonyl, SH content
511 levels). Our investigation shows a direct comparison between antioxidant effects of fresh and
512 industry-processed fractions of peaches (Syrup, Peel, preserve pulp peach and fresh pulp
513 peach) in an in vivo model of liver/renal toxicity, comparing their potentials and associating
514 this to their content of carotenoid and phenolic antioxidants. Peel, PPP and FPP group also
515 were able to attenuate some inflammation markers in liver and kidney by blocking the
516 stimulatory effects of CCl_4 on NFκB, RAGE, nitrotyrosine, TNF-α and IL-1β mediators.
517 Further experiments using low-dose/long-term and high-dose/short term and other damaging
518 agents may provide additional mechanism whereby peach prevents tissue damage by pro-
519 oxidant agents.

520

Conflict of interest

522

523 The authors declare that there are no conflicts of interest

524

Acknowledgements

526

527 The Brazilian research funding agencies FAPERGS (PqG 12099/8, ARD 11/1893-7,

528 PRONEX 1000274) CAPES (PROCAD 066/2007), CNPq PROPESQ-UFRGS that supported

529 this work and Embrapa clima temperado.

530

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644

645 **Figure legends**

646

647 Fig. 1. Detailed scheme of experimental design (A); Timeline (B).

648

649 Fig. 2. Animals weight at 200 mg/kg (A) and 400 mg/kg (C); blood glucose of animals receiving peach
650 extracts at 200 mg/kg (B) and 400 mg/kg (D). Values represent mean ± SEM. ANOVA repeated measures
651 analyses was applied for comparsion between both doses in weight gain data, and one-way analysis of
652 variance and Tukey's Multiple Comparison post-hoc test were applied for comparison of both groups in
653 blood glucose test. n=5 animals per group.

654

655 Fig. 3. Effects of supplementation with peach extracts (Syrup, Peel, PPP and FPP at 200 and 400 mg/kg) on
656 serum levels of IL-1β and TNF-α in CCl_4-treated rats. IL-1β (A) and. TNF-α (B) levels were quantified by
657 ELISA as described in "materials and methods". [#]denotes difference to control group ($p < 0.05$) and
658 *represent difference to CCl_4-treated group ($p < 0.0035$). Values represent mean ± SEM. One-way analysis
659 of variance and Tukey's Multiple Comparison post-hoc test were applied for all data. n=5 animals per
660 group.

661

662 Fig. 4. Effects of supplementation with peach extracts (Syrup, Peel, PPP and FPP at 200 and 400
663 mg/kg)against CCl_4-induced oxidative stress. Nitro-tyrosine levels (A) in serum were evaluated by ELISA.
664 SOD (B) and CAT activity (C) in liver homogenates. SOD (D) and CAT activity (E) in kidney. [#]denotes
665 difference to control group ($p < 0.05$) and *represent difference to CCl_4-treated group ($p < 0.01$). Values
666 represent mean ± SEM. One-way analysis of variance and Tukey's Multiple Comparison post-hoc test were
667 applied for all data. n=5 animals per group.

668

669 Fig. 5. Effects of supplementation with peach extracts (Syrup, Peel, PPP and FPP at 200 and 400
670 mg/kg)against CCl_4-induced oxidative damage. Protein carbonyl levels (A), free sulfhydryl groups (B) and
671 TBARS levels (C) in liver homogenates. Carbonyl (D) free sulfhydryl groups (E) and TBARS levels (F) in
672 kidney. [#]denotes difference to control group ($p < 0.05$) and *represent difference to CCl_4-treated group (p
673 <0.01). Values represent mean ± SEM. One-way analysis of variance and Tukey's Multiple Comparison
674 post-hoc test were applied for all data. n=5 animals per group.

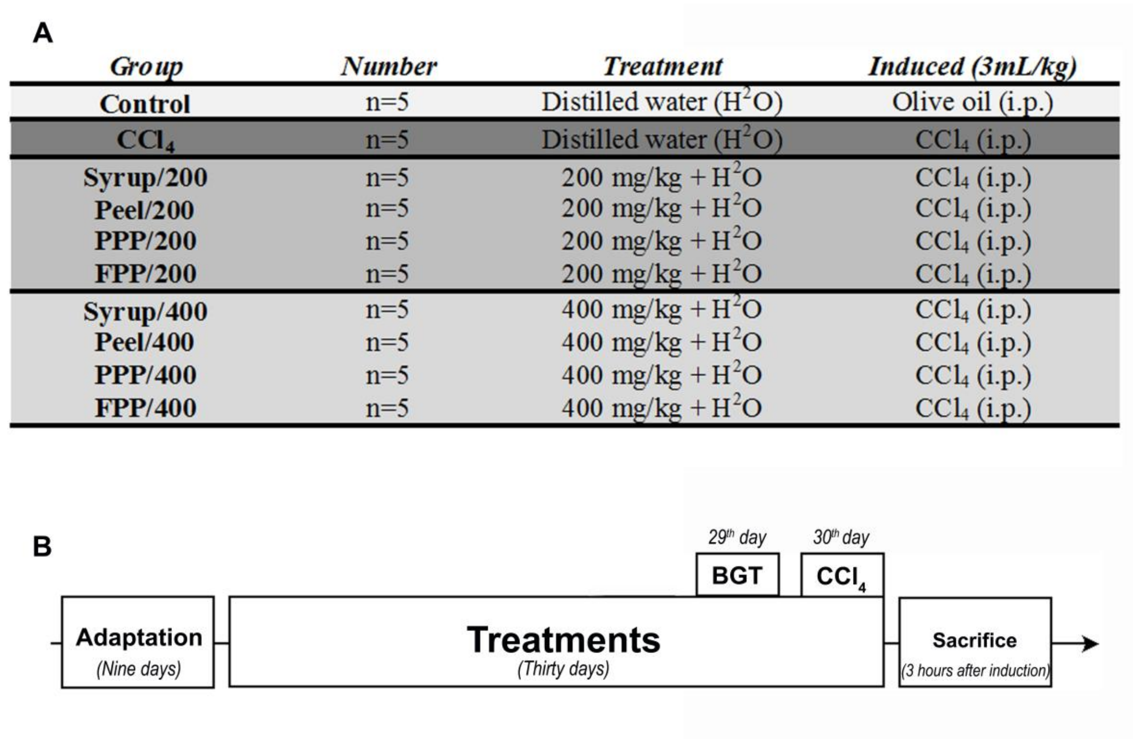
675

676 Fig. 6. Effects of supplementation with peach extracts (Syrup, Peel, PPP and FPP at 400 mg/kg) on NFκB-
677 p65 and RAGE protein content in rat liver and kidney. After supplementation, animals were subjected to
678 CCl_4 injection and western blot analysis was performed. The immunocontent of NFκB-p65 (A) and RAGE
679 in liver (B). Immunocontent of NFκB-p65 (C) and RAGE in kidney (D). $^{\#}$denotes difference to control
680 group ($p<0.001$) and * represent difference to CCl_4-treated group ($p< 0.01$). Each figure depicts
681 representative western blots gels plus mean± SEM quantification values. One-way analysis of variance and
682 Tukey's Multiple Comparison post-hoc Test were applied for all data. n=5 animals per group.
683
684 Fig. 7. Effects of supplementation with peach extracts (Syrup, Peel, PPP, and FPP 400mg/kg) on liver
685 morphological and histological characteristics (H&E staining, original magnification of 50 μm, and
686 approximation of 25 μm). Liver organ and liver tissue of normal rats (A). CCl_4 group (B). Syrup + CCl_4
687 group (C). Peel + CCl_4 (D). PPP + CCl_4 (E). FPP + CCl_4 (F).
688
689 Fig. 8. The HPLC chromatograms of the different peach extracts: Peel (A), FPP (B) and PPP (C). Each
690 Peak represents one carotenoid: a. All-trans-lutein; b. Zeaxanthin; c. β-Cryptoxanthin; d. α-carotene; e. β-
691 carotene.
692
693 Supplementary figure. Peach of Maciel Variety, (A and B)
694
695 Table 1. Effects of supplementation with peach extracts (Syrup, Peel, PPP and FPP at 200 and 400 mg/kg)
696 against CCl_4-induced oxidative stress in serum. ALT, AST, LDH activities and bilirubin concentrations in
697 serum were determined using Labtest kits. CCl_4 induced changes in all parameters. Asterisks represent
698 difference to CCl_4-treated group (* denotes $p<0.05$, ** $p<0.01$ and *** $p<0.001$).Values represent mean ±
699 SEM. One-way analysis of variance and Tukey's Multiple Comparison post-hoc test were applied for all
700 data.
701

Table 1: ALT, AST, LDH activities and bilirubin concentrations in Serum

Group	*ALT (U/L)*	*AST activity (U/L)*	*Bilirubin (mg/mL)*	*LDH activity (U/L)*
Control	31.53 ± 2.5	119.13 ± 3.4	10.50 ± 0.2	10.5 ± 1.5
CCl_4	83.80 ± 9.8	315.9 ± 11.7	15.27 ± 0.8	104.4 ± 1.1
Syrup/200	70.30 ± 10.51	229.5 ± 39.3	15. 97 ± 0.3	101.8 ± 6.4
Peel/200	63.1 ± 4.4	234.1 ± 32.3	15.31 ± 0.2	102.5 ± 8.1
PPP/200	65.9 ± 5.9	240.4 ± 40.5	15.05 ± 0.3	101.5 ± 12.1
FPP/200	62.15 ± 4.8	205.2 ± 16.5 *	14.28 ± 0.3	82.9 ± 4.2
Syrup/400	71.49 ± 9.7	289.2 ± 18.1	14.35 ± 0.6	107.3 ± 1.7
Peel/400	62.00 ± 8.7	248.0 ± 17.22	13.91 ± 0.4	78.47 ± 2.4 ***
PPP/400	42.11 ± 8.1 **	202.3 ± 12. 57 **	13.43 ± 0.8	56.99 ± 1.7 ***
FPP/400	40.94 ± 4.8 **	176.9 ± 21.47***	11.01 ± 1.1 *	59.79 ± 2.4 ***

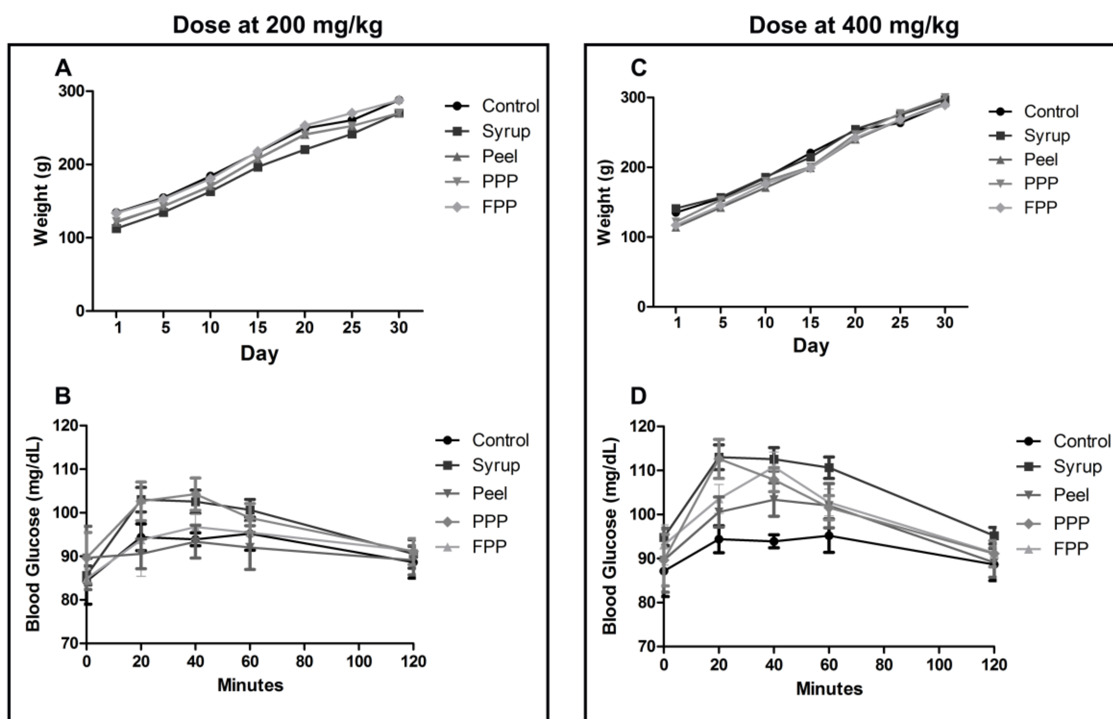
Figure 1.



A

Group	*Number*	*Treatment*	*Induced (3mL/kg)*
Control	n=5	Distilled water (H^2O)	Olive oil (i.p.)
CCl_4	n=5	Distilled water (H^2O)	CCl_4 (i.p.)
Syrup/200	n=5	200 mg/kg + H^2O	CCl_4 (i.p.)
Peel/200	n=5	200 mg/kg + H^2O	CCl_4 (i.p.)
PPP/200	n=5	200 mg/kg + H^2O	CCl_4 (i.p.)
FPP/200	n=5	200 mg/kg + H^2O	CCl_4 (i.p.)
Syrup/400	n=5	400 mg/kg + H^2O	CCl_4 (i.p.)
Peel/400	n=5	400 mg/kg + H^2O	CCl_4 (i.p.)
PPP/400	n=5	400 mg/kg + H^2O	CCl_4 (i.p.)
FPP/400	n=5	400 mg/kg + H^2O	CCl_4 (i.p.)

Figure 2.



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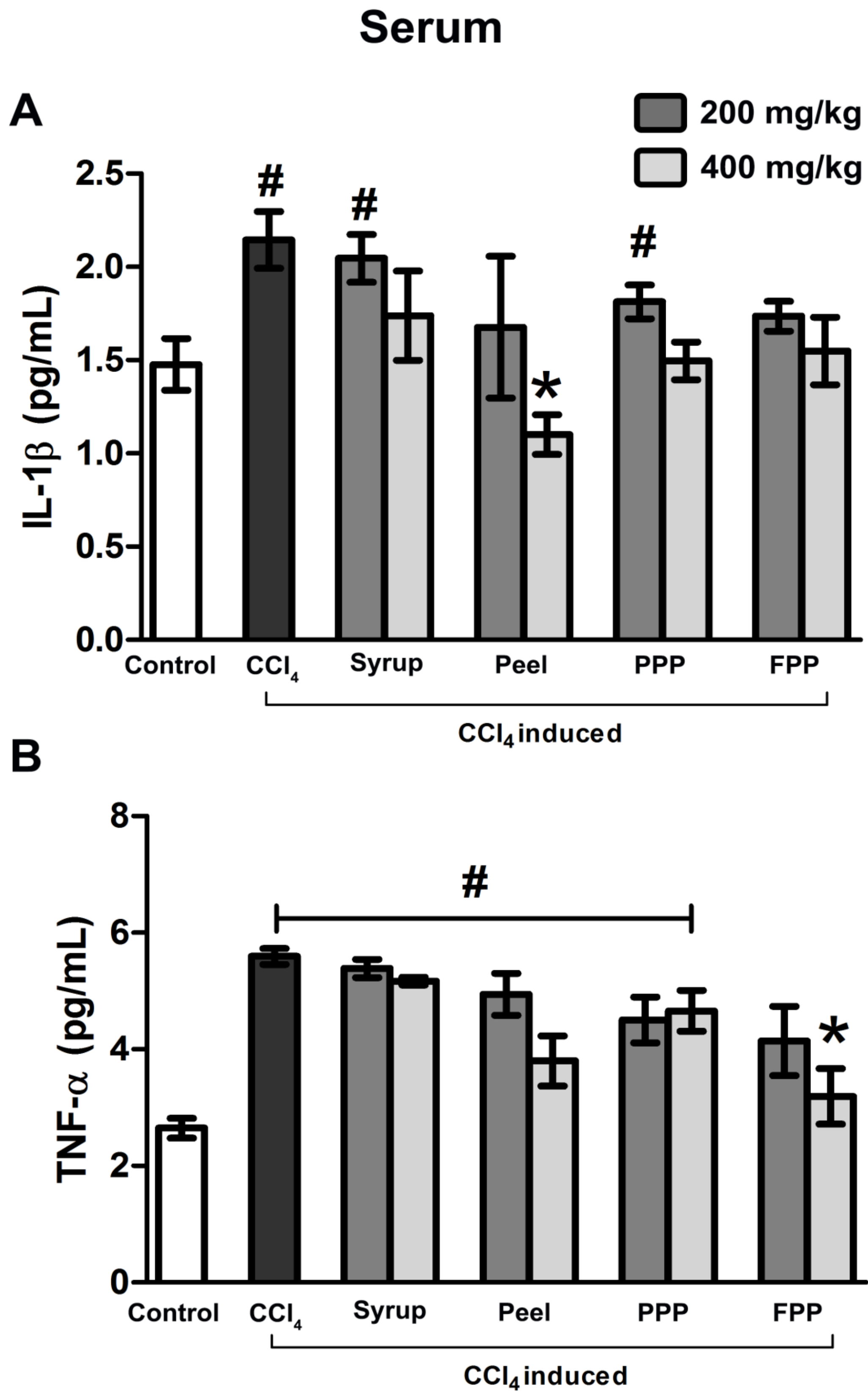


Figure 4.

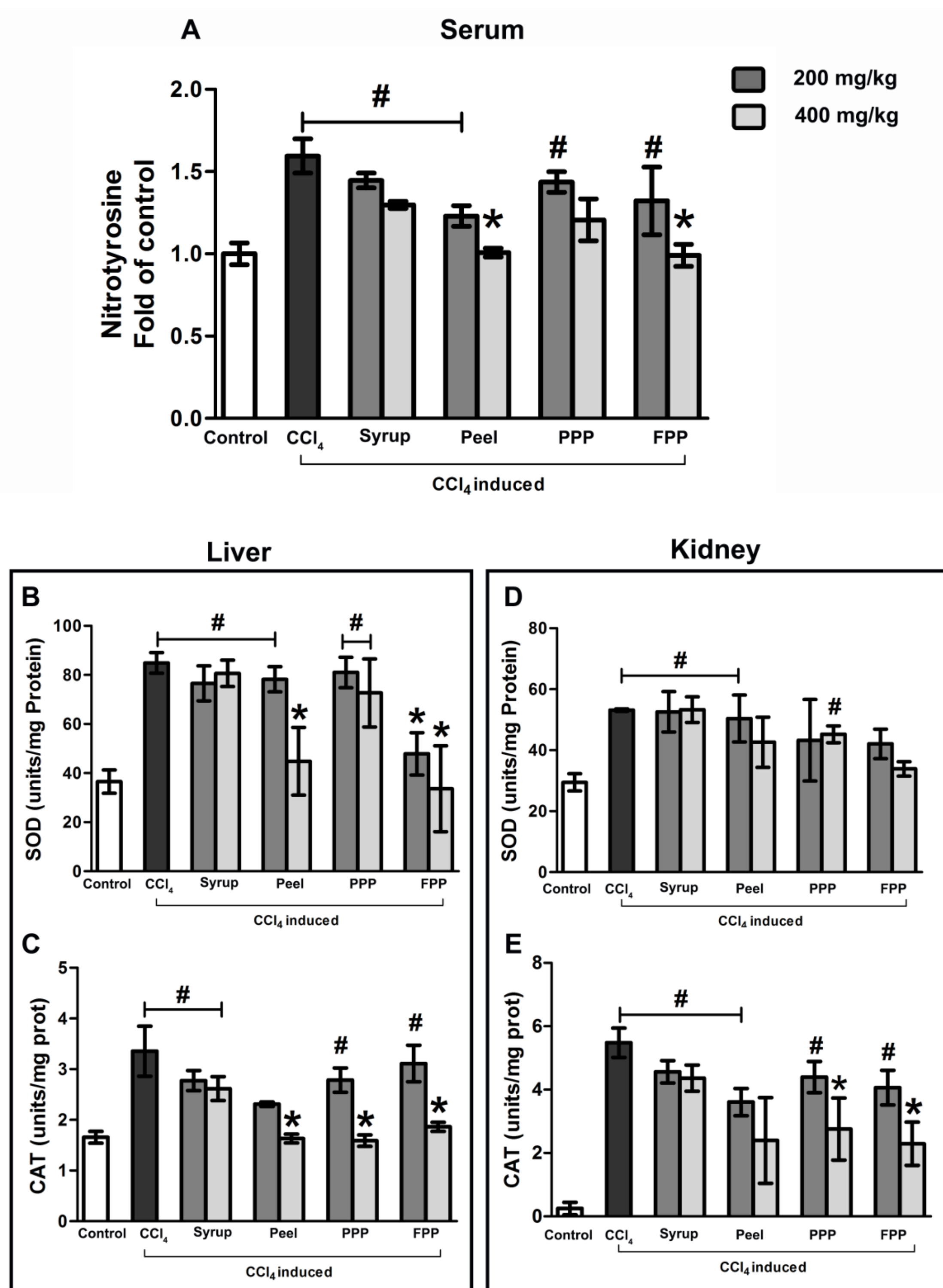
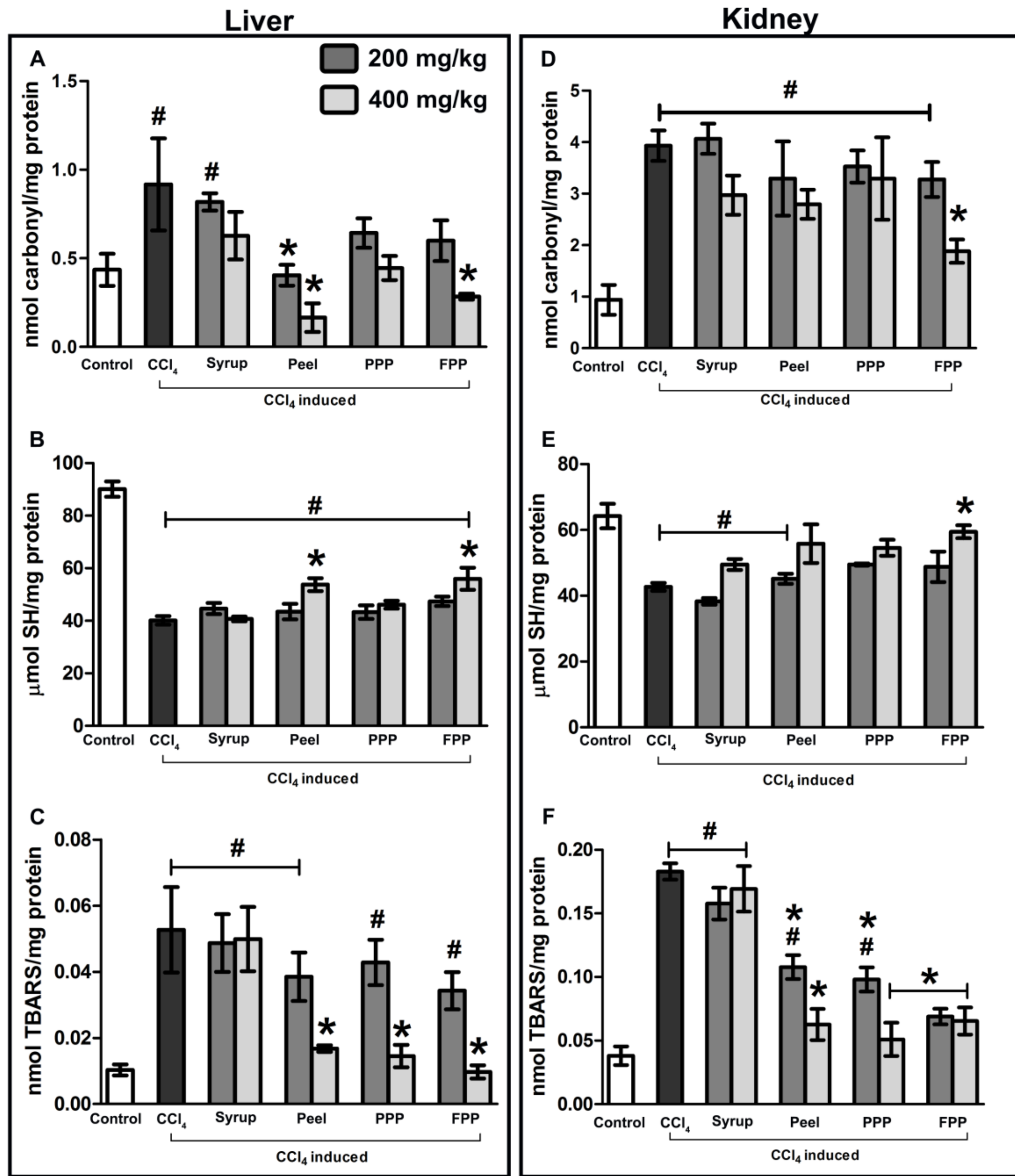


Figure 5.



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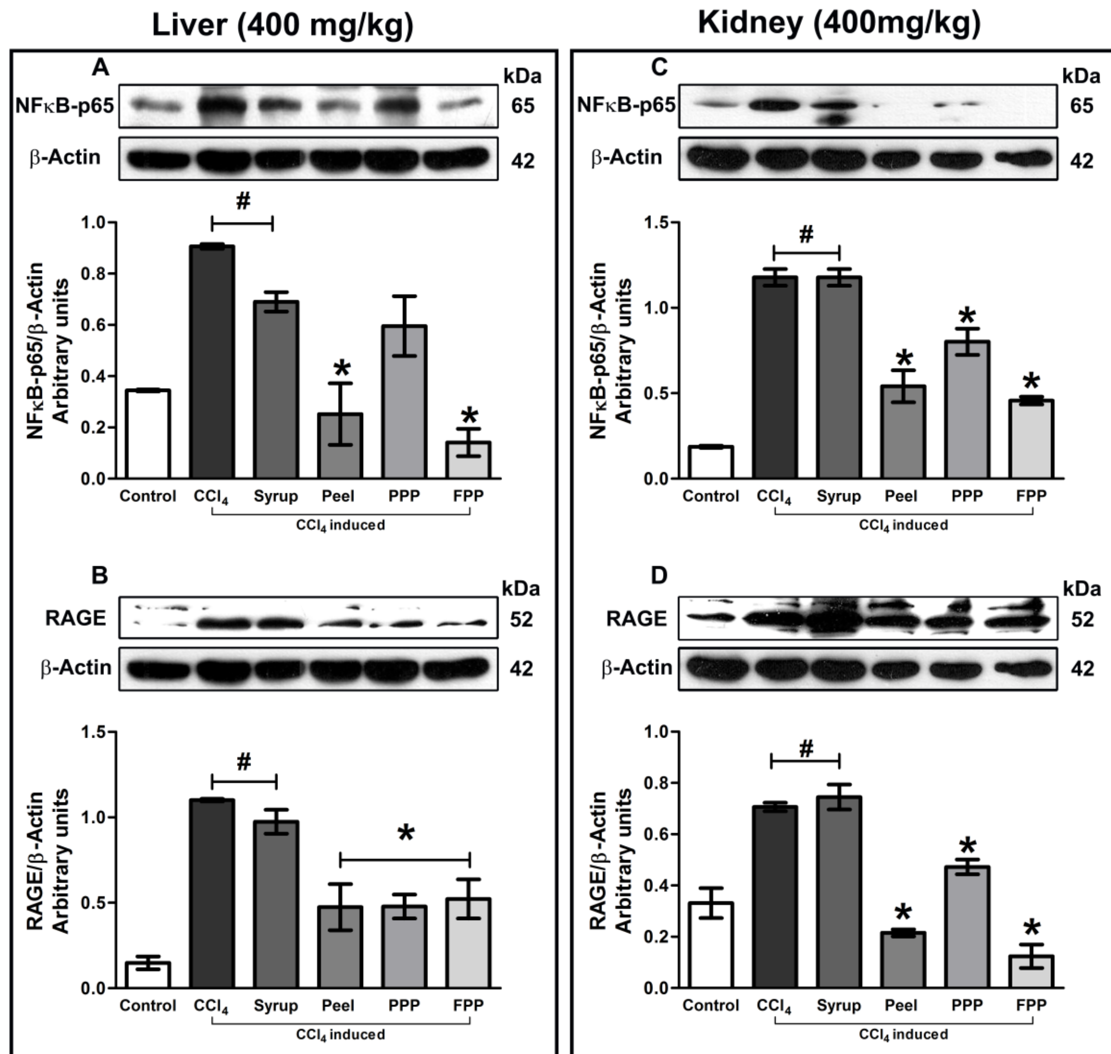

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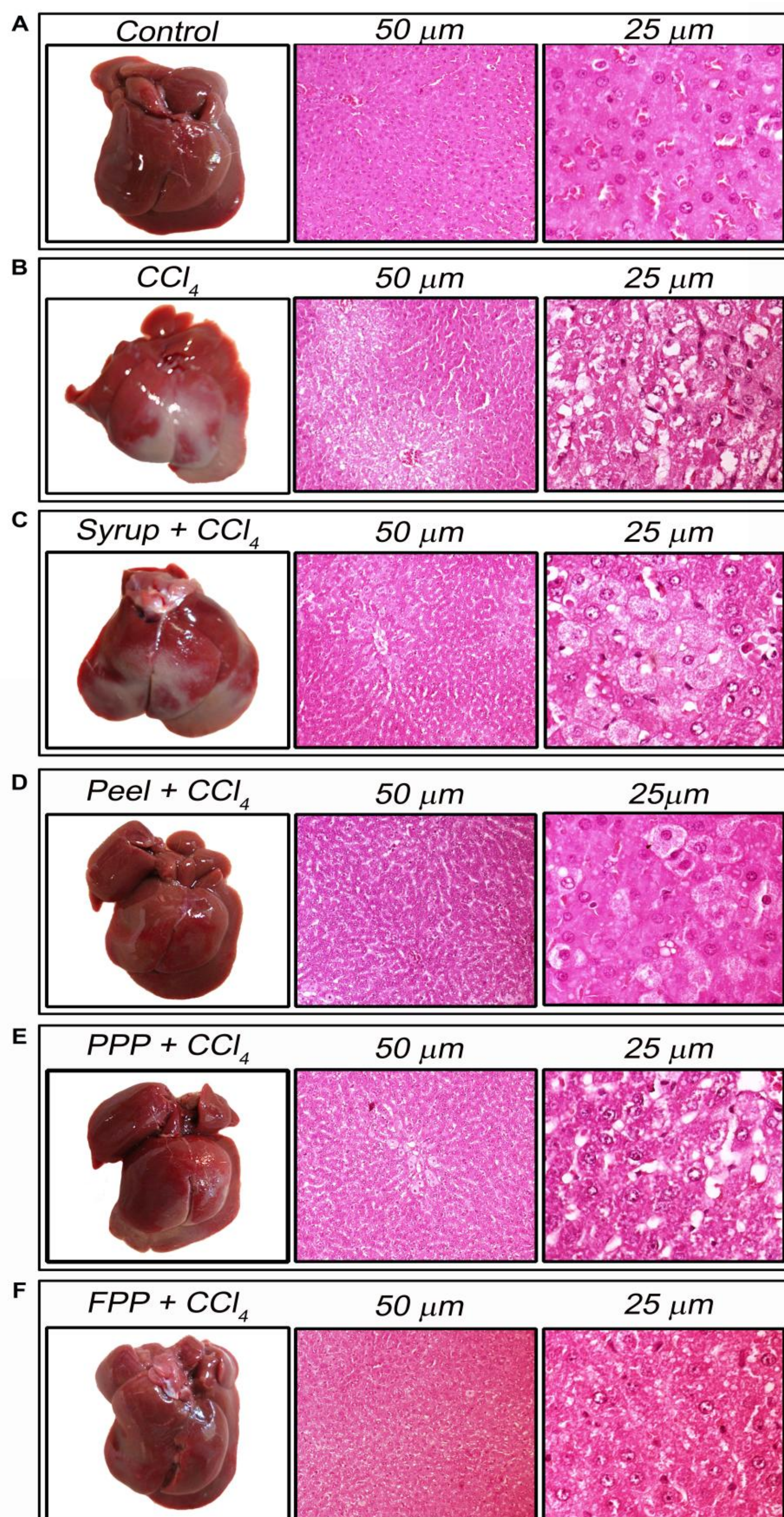
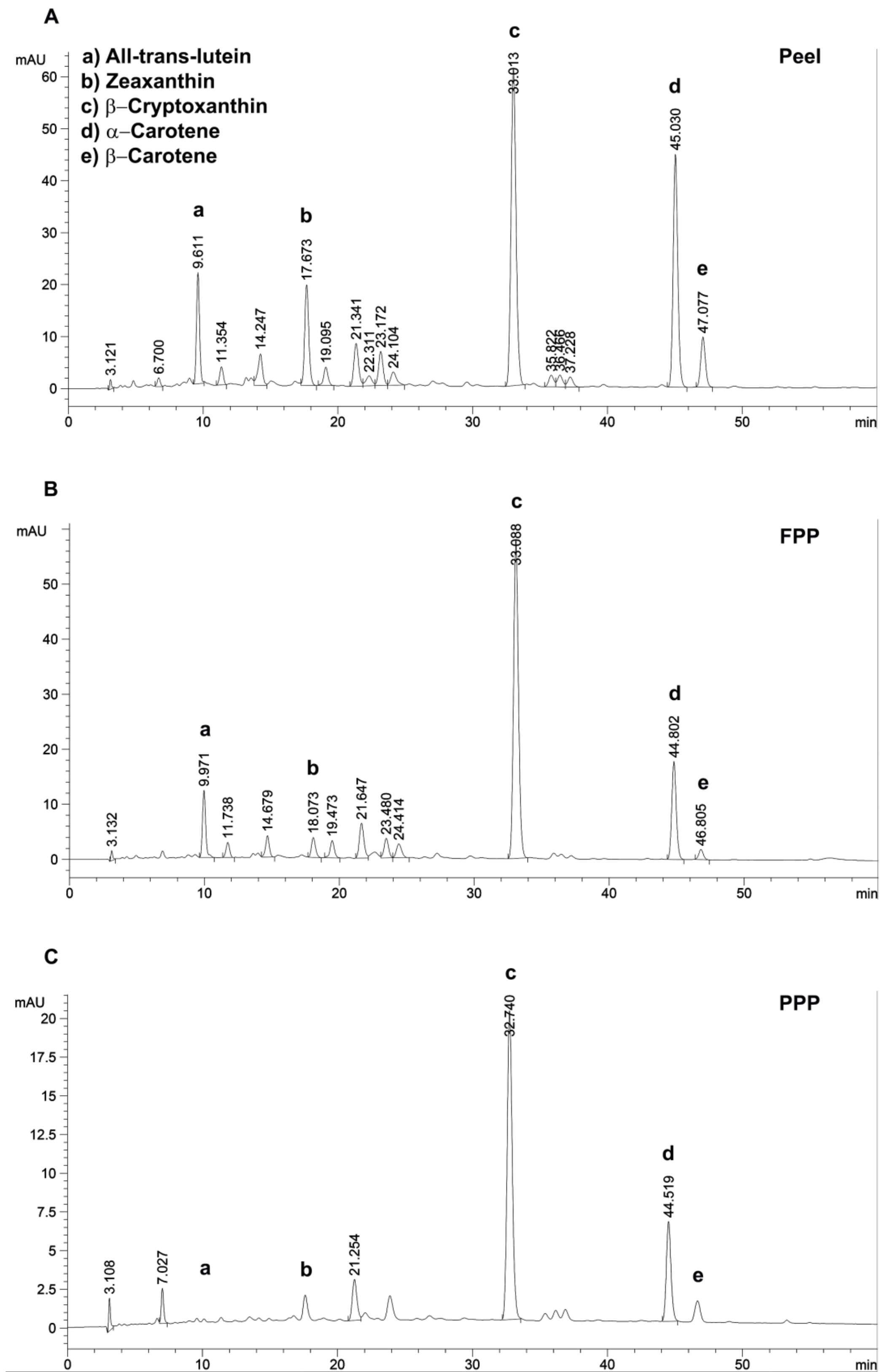


Figure 8.



Supplementary figure

A

B

Parte III

IV. Discussão geral

A literatura é bem extensa em relação a vários produtos naturais que são investigados corriqueiramente em diferentes modelos experimentais (*in vitro*, *in situ*, *in vivo* e *ex vivo*) a fim de determinar suas capacidades antioxidantes e anti-inflamatórias. Nosso trabalho teve o objetivo de avaliar a ação protetora do pêssego da variedade Maciel *in natura*, casca *in natura*, polpa do pêssego em calda e a calda do pêssego em compota em três modelos experimentais *in vitro*, *ex vivo* e *in vivo*. O efeito protetor destes extratos foi avaliado através de parâmetros bioquímicos (capacidade antioxidante e capacidade anti-inflamatória).

No capítulo I utilizamos o modelo *in vitro* e *ex vivo* a fim de avaliar e mensurar a capacidade antioxidante do extratos, e quanto os extratos seriam capazes de prevenir o estresse oxidativo em diferentes tecidos (fígado, rins e córtex cerebral).

Primeiramente foram realizados os testes *in vitro* com intuito de investigar a capacidade antioxidante dos extratos e também a qualidade antioxidante de cada um. A capacidade antioxidante total não enzimática das diferentes amostras obtidas do pêssego e de seus produtos derivados foi mensurada através do ensaio de potencial antioxidante reativo total (TRAP). A eficácia e qualidade antioxidante dos extratos foram avaliadas através do ensaio de reatividade antioxidante total (TAR). Tanto o pêssego *in natura* quanto a casca tiveram significantes capacidade antioxidantes.

Os resultados obtidos no ensaio de glicação demonstram que a casca e o pêssego *in natura* tiveram os mesmos perfis dos testes do TRAP e TAR, este

conjunto de resultados sugere que além do pêssego *in natura* e a casca são exercer atividade antioxidante, também são capazes de inibir a glicação de proteínas. O pêssego da conserva teve menor capacidade antioxidante, esta diminuição pode ser ocasionada pelo processo de industrialização. A calda foi avaliada a fim de verificar se esta seria capaz de adquirir os compostos que estão presentes no pêssego durante o processo industrial, porém a calda apresentou os menores níveis nos parâmetros avaliados nos diferentes modelos experimentais.

O teor de fenólicos totais do pêssego e casca *in natura* foi maior do que os encontrados nos extratos de pêssego em calda e na calda. Os extratos *in natura* também apresentaram maior concentração dos cinco diferentes carotenoides (all-trans-luteína, zeaxantina, e β-criptoxantina, α-caroteno β-caroteno) que foram avaliados através da cromatografia liquida de alta eficiência (HPLC).

Após fazer o *screening in vitro* determinando a composição química dos extratos conduzimos a pesquisa a fim de explorar atividades biológicas dos pêssegos e seus produtos derivados em fatias *(slices)* de tecidos no modelo *ex vivo*.

No modelo *ex vivo* os animais são sacrificados e os tecidos são dissecados e fatiados na mesma espessura, então são incubados em ambiente controlado de temperatura e oxigênio. Os tecidos foram pré-incubados com os diferentes extratos durante uma hora, após este período sulfato ferroso ($FeSO_4$) e peróxido de hidrogênio (H_2O_2) foram adicionado a fim de induzir a produção do radical hidroxil através da reação de Fenton, este radical tem alta capacidade de induzir citotoxicidade pelo estresse oxidativo.

A ação do radical hidroxil alterou parâmetros de estresse/dano oxidativo e parâmetros inflamatórios nas fatias de fígado, rim e córtex cerebral de ratos adultos machos.

A lactato desidrogenase (LDH) foi avaliada no meio de incubação dos tecidos sendo utilizada como parâmetro de citotoxicidade. Nos meios de incubação dos rins, fígado e córtex cerebral, o pêssego *in natura* e a casca foram capazes de proteger estes tecidos, e interessantemente o pêssego da compota também foi capaz de impedir o aumento da LDH causada pelo sistema de geração de hidroxilo nos rins, indicando um efeito protetor. As enzimas de defesa SOD e CAT também foram moduladas positivamente principalmente pelos dois extratos *in natura*.

O insulto oxidativo induzido (reação de Fenton) foi capaz de modular marcadores de peroxidação lipídica, de carbonilação de proteínas e de estado redox de grupamentos tiois. O pêssego *in natura* teve maior eficácia em proteger os tecidos avaliados, acompanhado pela casca, e também pelo pêssego da compota.

Parâmetros inflamatórios foram avaliados no meio de incubação (Kreb's Ringer Hepes). As citocinas pró-inflamatórias fator de necrose tumoral-alfa (TNF-α) e Interleucina-1β (IL-1β) foram quantificadas através da técnica de ELISA indireto. No meio de incubação dos rins os extratos de pêssego *in natura*, casca e pêssego da compota preveniram a liberação de TNF-α e IL-1β. No fígado apenas a casca inibiu a liberação das citocinas pró-inflamatórias e no cortex cerebral apenas a liberação de TNF-α foi inibida pelo pêssego *in natura*, portanto além do potencial antioxidante os extratos in natura apresentam ação

anti-inflamatória. O pêssego da compota também resultou inibição, porém de maneira menos expressiva, a calda não teve efeitos.

Após realizar a verificação *in vitro* dos compostos presentes nas amostras e também analisar o efeito do pêssego e de seus derivados diretamente nos tecidos *(ex vivo)*, conduzimos os experimentos na intenção de avaliar os efeitos do consumo do pêssego e seus derivados em um organismo complexo no qual os extratos foram administrados oralmente via gavagem, e passaram por todo o processo de digestão para enfim exercer ação sobre os tecidos avaliados.

O modelo *in vivo* (capitulo II) elucida quais os efeitos do consumo diário do pêssego. Ratos machos wistar (30 dias de vida) foram tratados durante 4 semanas com o pêssego *in natura* a casca *in natura*, o pêssego em calda (compota) e a calda isolada com duas doses distintas de 200 mg/kg e 400 mg/kg. Ao término dos 30 dias de tratamento cada animal recebeu um dose de CCl_4 no intuito de induzir dano hepático e renal. Após 3 horas da indução do CCl_4 os animais foram sacrificados e o soro, fígado e rins foram extraídos a fim de investigar o efeito protetor dos diferentes extratos contra o estresse e dano oxidativo além de inflamação.

A administração do pêssego e seus derivados não alterou o peso dos animais, enquanto a glicose teve aumento normal minutos depois da administração dos extratos via gavagem, e após 120 minutos a glicose sanguínea foi normalizada voltando a níveis basais.

O tratamento com uma dose única de CCl_4 causou um aumento de 3 vezes nos níveis da aspartato aminotransferase (AST) e alanina aminotransferase (ALT) no soro, confirmando a indução de toxicidade do

tecido. Bilirrubina, que é frequentemente mais afetada em casos de hepatotoxicidade grave também foi alterada.

A dose de 200 mg/kg de *pêssego in natura*, foi capaz de reduzir a atividade de AST, enquanto, a dose de 400 mg/kg de pêssego *in natura* e pêssego da compota impediu significativamente liberação de ambas as enzimas (AST e ALT). Bilirrubina foi diminuída apenas no grupo do pêssego *in natura*.

Através da avaliação da atividade da LDH em soro foi constatado que a dose de 400 mg/kg de casca, pêssego *in natura* e pêssego da compota foi capaz de proteger os tecidos contra os danos citotóxicos.

Citocinas pró-inflamatorias foram avaliadas no soro através da técnica de ELISA indireto. Com a dose de 400 mg/kg a casca foi capaz de inibir a produção da citocina IL-1β, enquanto TNF-α foi inibido pelo pêssego *in natura*. Casca e pêssego *in natura* também foram capazes de inibir a produção de nitrotirosina que é utilizada como marcador para estresse oxidativo. A dose de 200 mg/kg não apresentou alterações significativas.

A atividade de CAT e SOD foram avaliadas em homogenato de fígado e rins, mais uma vez os extratos *in natura* na dose de 400 mg/kg demonstraram maior eficácia, mantendo níveis iguais a controle destas enzimas.

Os marcadores de carbonilação de proteínas, de peroxidação lipídica e de estado redox de grupamentos tiois, foram todos alterados pelo CCl_4 e casca e pêssego *in natura* tiveram destaque na prevenção de dano incluindo ambas doses (200 e 400 mg/kg).

O imunoconteúdo de RAGE e NF-κB-p65 foi avaliado pela técnica de western blotting em homogenatos de fígado e rins dos ratos que receberam

apenas a dose de 400 mg/kg. Os resultados foram extremamente significantes onde tanto as amostras *in natura* quanto o pêssego da compota teve capacidade em inibir a expressão de RAGE e NF-κB-p65 em ambos os tecidos avaliados.

As análises histológicas confirmam os efeitos danosos do CCl_4 no fígado e também demonstram a capacidade protetora que os extratos tiveram sobre o fígado dos animais que receberam a maior dose do tratamento.

Os dados obtidos nos capítulos I e II nos permitem realizar algumas observações sobre os pêssegos e os produtos que são derivados desta fruta. A primeira constatação importante é que alimentos que passam por um processo mecanizado de manipulação visando à comercialização em escala industrial perdem ou tem diminuída a quantidade de alguns compostos que parecem ser fundamentais na sua ação protetora nos modelos biológicos aqui estudados, esse fato é consequência de que tanto as vitaminas quantos alguns compostos que estão presentes nas frutas são extremamente instáveis e perdem suas propriedades na presença de ar, calor, água ou luz, o que dificulta o armazenamento de todos nutrientes que estão disponíveis em frutas frescas (Woodside *et al.* 2013).

Quando comparadas a polpa do pêssego e a casca *in natura*, ambos demonstram uma disponibilidade maior de fatores protetores aos encontrados no pêssego em calda. Como demonstrado em ambos os capítulos, altas concentrações de polifenois e carotenoides foram detectadas principalmente nos extratos dos produtos *in natura* (polpa e casca), os quais apresentaram maiores efeitos protetores. Provavelmente os polifenois e os carotenoides estejam envolvidos diretamente nos processos de inibição do estresse

oxidativo e inflamação em diferentes órgãos. Tal observação vai de encontro com os dados que estão disponíveis na literatura (Lima & Vianello 2010) e indicam um importante potencial antioxidante e anti-inflamatório do pêssego.

Muitos dos antioxidantes naturais exibem uma gama de efeitos biológicos, incluindo antimicrobianos e (Pellegrini *et al.* 2003) anti-inflamatórios (Chen *et al.* 2008). As evidências cientificas demonstram que a maioria dos compostos que contenham propriedades antioxidantes se devem a seus compostos fenólicos (Rice-Evans *et al.* 1996). O conteúdo de compostos fenólicos e a capacidade antioxidante de frutas variam de acordo com o genótipo específico da planta (Gil et al. 2002), além de sofrer influência do ambiente e técnicas de cultivo utilizadas (Carbonaro et al. 2002).

Pêssegos orgânicos apresentam maior conteúdo de polifenois em relação com o método original de cultivo, este aumento pode ser resultado do desenvolvimento do sistema de defesa da planta como consequência do cultivo orgânico (Carbonaro *et al.* 2002), tornando o fruto mais nutritivo e com maior concentração de moléculas antioxidantes.

Os antioxidantes naturais estão presentes em praticamente todos os produtos alimentares, proporcionando-lhes um grau importante de proteção contra o ataque oxidativo. Quando os produtos alimentares são sujeitos a processamento, tais antioxidantes naturais são muitas vezes empobrecidos, pela natureza do processo físico em si, ou por degradação química. Como consequência, os produtos alimentícios processados costumam ter bem menos antioxidantes do que os produtos que lhes deram origem (Hudson 1990), tal como observado nos extratos do pêssego em calda (compota).

Apesar de o mecanismo antioxidante endógeno do organismo humano ser extremamente eficaz, a demanda no combate ao estresse oxidativo é muito grande, portanto se faz necessária a ingestão de alimentos ricos em antioxidantes para manter os radicais livres em baixas concentrações (Pietta 2000).

A exposição a ERO/ERN vindos de diversas fontes levou os organismos a desenvolver uma série de mecanismos de defesa (Cadenas & Sies 1998). Os mecanismos de defesa contra o estresse oxidativo induzido por ERO/ERN envolvem defesas antioxidantes enzimáticas SOD, CAT e glutationa peroxidase (GPX) (Uttara et al. 2009) e as defesas não enzimáticas, tais como peptídeos de histidina, proteínas ligadas ao ferro (transferrina e ferritina), ácido diidrolipólico, coenzima Q reduzida ($CoQH_2$), ácido ascórbico (vitamina C), tocoferol (vitamina E), glutationa reduzida (GSH), carotenoides, flavonoides, e outros antioxidantes presentes em diversas frutas e vegetais (Halliwell *et al.* 1995, Valko et al. 2007).

O consumo de frutas é amplamente recomendado por especialistas da área da nutrição e saúde, pois as frutas possuem grande ação antioxidante o qual protege os sistemas biológicos contra processos de estresse oxidativo e processos inflamatórios. Por esse motivo uma maior ingestão de frutas é vastamente sugerida em dietas para prevenção de diversas doenças associadas ao estresse oxidativo e ativação pró-inflamatória, tais como patologias relacionadas ao sistema cardiovascular, câncer, diabetes (Martorana *et al.* 2013, Luna-Vazquez *et al.* 2013). O pêssego e a casca *in natura* demonstraram este perfil protetor de forma bem significativa, mas deve-se observar que o pêssego em calda também mostrou potencial antioxidante e

anti-inflamatório, apesar destes serem observados em menor proporção se comparados aos extratos *in natura*.

Nossa pesquisa, de uma maneira geral indica que o consumo de pêssegos e seus produtos derivados protegem diversos órgãos contra as ações de radicais livres e citocinas pro-inflamátorias. Portanto os resultados obtidos através dos diversos testes empregados neste trabalho classifica o pêssego da variedade Maciel como alimento funcional o qual o consumo gera relevante proteção a seus consumidores.

V. Conclusões

Conclusões e perspectivas

A investigação por alimentos funcionais é constante e minuciosa. Na ultima década alimentos naturais tem despertado interesse da comunidade cientifica e galgado espaço em laboratórios interessados em investigar a composição de frutas e os efeitos que esses alimentos exercem no organismo humano. Atualmente é de conhecimento geral que uma maneira de manter uma vida saudável é ter hábitos alimentares saudáveis, é fundamental a ingestão de frutas.

Nosso trabalho investigou de maneira meticulosa os efeitos que o pêssego poderia exercer na proteção dos tecidos contra o estresse oxidativo e inflamação. Na literatura já há estudos sobre o pêssego Maciel, e tais investigações apontam que esta variedade de pêssego possui alto teor de antioxidantes (Rossato et al. 2009).

Os extratos que foram testados (pêssego *in natura*, casca *in natura*, pêssego em calda e calda), principalmente os extratos *in natura,* demonstraram excelentes resultados, prevenindo o dano renal e o dano hepático. Os extratos que passaram por processo industrial (pêssego em calda e calda) perderam grande parte de seus nutrientes afetando diretamente a ação preventiva nos tecidos.

Em ambos os modelos investigados no capítulo I e II observamos a alta capacidade desta fruta em proteger os tecidos avaliados. As concentrações utilizadas para este trabalho podem ser facilmente enquadradas na dieta humana.

O processo de liofilização das frutas acarreta em diminuição do peso do pêssego em até 80%, portanto um pêssego que é vendido comercialmente pesa cerca de 100 gramas, e após a liofilização esse pêssego pesará 20 gramas de peso seco. Um indivíduo pesando 70 kg necessita de 28 gramas de pêssego in *natura* para que possa equivaler a dose mais alta avaliada neste trabalho (400mg/kg), portanto este indivíduo deve ingerir dois a três pêssegos diariamente para exercer proteção significativa para os rins e fígado.

Nossos resultados sugerem que uma dieta contendo esta variedade de pêssego está diretamente associada com redução do perfil inflamatório e redução de estresse oxidativo. Estudos elucidando tratamentos com menores doses, tempos de tratamentos e de diferentes variedades de pêssego são necessários para que possamos ter maior compreensão dos mecanismos de ação anti-inflamatória e antioxidante encontrados nesta variedade de pêssego desenvolvido pala Embrapa.

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