UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ENGENHARIA DE ALIMENTOS

FERNANDA GUIMARÃES DRUMMOND E SILVA

CAPACIDADE ANTIOXIDANTE DE HIDROLISADOS PROTEICOS E COMPOSTOS FENÓLICOS DA LINHAÇA (*LINUM USITATISSIMUM* L.) E MODULADORA DA ATIVIDADE INFLAMATÓRIA EM MODELO EXPERIMENTAL DE COLITE

ANTIOXIDANT CAPACITY OF PROTEIN HYDROLYSATES AND PHENOLIC COMPOUNDS OF FLAXSEED (*LINUM USITATISSIMUM* L.) AND ITS MODULATORY EFFECTS ON EXPERIMENTAL COLITIS

CAMPINAS
2016

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Tese apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Alimentos e Nutrição, na área de Nutrição Experimental e Aplicada à Tecnologia de Alimentos

Thesis presented to the Faculty of Food Engineering of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor, in the area of Food and Nutrition

Orientador: Flavia Maria Netto

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA FERNANDA GUIMARÃES DRUMMOND E SILVA E ORIENTADA PELA PROFESSORA DRA. FLAVIA MARIA NETTO.

Campinas
2016

Agência(s) de fomento e nº(s) de processo(s): FAPESP, 2010/52680-7

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Engenharia de Alimentos
Claudia Aparecida Romano - CRB 8/5816

Si38c

Silva, Fernanda Guimarães Drummond e, 1983-
Capacidade antioxidante de hidrolisados proteicos e compostos fenólicos da linhaça (*Linum usitatissimum* L.) e moduladora da atividade inflamatória em modelo experimental de colite / Fernanda Guimarães Drummond e Silva. – Campinas, SP : [s.n.], 2016.

Orientador: Flávia Maria Netto.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Engenharia de Alimentos.

1. Linhaça. 2. Hidrolisados proteicos. 3. Compostos fenólicos. 4. Atividade antioxidante. 5. Atividade anti-inflamatória. I. Netto, Flávia Maria. II. Universidade Estadual de Campinas. Faculdade de Engenharia de Alimentos. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Antioxidant capacity of protein hydrolysates and phenolic compounds of flaxseed (*Linum usitatissimum* L.) and its modulatory effects on experimental colitis
Palavras-chave em inglês:
Flaxseed
Protein hydrolysates
Phenolic compounds
Antioxidant activity
Anti-inflammatory activity
Área de concentração: Nutrição Experimental e Aplicada à Tecnologia de Alimentos
Titulação: Doutora em Alimentos e Nutrição
Banca examinadora:
Flávia Maria Netto [Orientador]
Lourdes Amigo Garrido
Inar Alves de Castro
Wirla Maria da Silva Cunha Tamashiro
Caroline Dario Capitani
Data de defesa: 18-11-2016
Programa de Pós-Graduação: Alimentos e Nutrição

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A ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

DEDICATÓRIA

Dedico esta tese ao meu pai,

Luiz Fernando (*in memorian*),

exemplo de amor, força, persistência e dignidade.

AGRADECIMENTOS

Para essa tese ficar pronta foram necessários incontáveis abraços, beijos, sorrisos, olhares e palavras de incentivo, conversas, muitos chocolates e, naturalmente, alguns puxões de orelha. O caminho, apesar de árduo, proporcionou um aprendizado imensurável. Para todos os familiares, amigos, colegas, professores e pesquisadores que estiveram comigo em alguns (ou vários) momentos desta caminhada, meu muito obrigada. Essa tese leva um pouquinho de cada um de vocês! ☺

RESUMO

As proteínas da linhaça, assim como outras proteínas de origem vegetal, estão naturalmente associadas aos compostos fenólicos. Durante a hidrólise enzimática dessas proteínas, peptídeos e compostos fenólicos são liberados e podem exercer atividades antioxidante e anti-inflamatória. O presente estudo avaliou a influência do complexo proteína-fenólico e da hidrólise com Alcalase e/ou enzimas digestivas sobre a capacidade antioxidante (CA) de produtos de linhaça. Investigou-se também a capacidade anti-inflamatória *in vitro* dos hidrolisados proteicos e compostos fenólicos da linhaça, pela inibição da produção de mediadores inflamatórios em macrófagos estimuladas por IFN-γ/LPS e, *in vivo,* por meio da modulação da inflamação em animais com retocolite ulcerativa induzida por TNBS. O isolado proteico de linhaça (FPI) foi obtido a partir da farinha de linhaça desengordurada por extração alcalina (pH 9,0) e precipitação no ponto isoelétrico (pH 4,2) seguida de neutralização (pH 6,0). O hidrolisado de proteína de linhaça (FPH) foi produzido a partir da hidrólise do FPI com Alcalase. Para obter o isolado e hidrolisado proteico de linhaça com reduzido teor fenólico (phr-FPI e phr-FPH, respectivamente) a farinha de linhaça desengordurada foi lavada com etanol antes do processo de isolamento, para remoção da fração fenólica (Phi). A fração fenólica hidrolisada (Phh) foi obtida submetendo-se a Phi às mesmas condições de pH e temperatura utilizadas na hidrólise dos isolados proteicos. A análise de fluorescência intrínseca foi utilizada para avaliar a formação do complexo proteína: fenólico e a CA foi determinada pelos métodos ORAC e FRAP. As amostras Phi e Phh apresentaram a maior CA seguida dos hidrolisados e isolados proteicos. A presença de compostos fenólicos nas amostras proteicas foi responsável pelo aumento de até 80% na CA do isolado proteico, medida pelos métodos ORAC e FRAP, enquanto que, para o hidrolisado proteico, foi responsável por um aumento de 15% da CA medida por ORAC, e 80%, quando medida por FRAP. Após a simulação da digestão, a CA dos isolados e hidrolisados proteicos aumentou e a influência dos compostos fenólicos na capacidade antioxidante persistiu, especialmente quando determinada por FRAP. Nossos resultados sugerem que CA de isolados deve-se principalmente à formação do complexo proteína:fenólico, enquanto que nos hidrolisados a CA deve-se possivelmente ao sinergismo entre fenólicos e peptídeos liberados a partir da hidrólise com Alcalase ou com as enzimas digestivas. Além disso, a transferência de H^+ parece ser o principal mecanismo pelo qual as proteínas de linhaça atuam como antioxidantes. Em relação à capacidade antioxidante do phr-FPH, foram identificados quatro sequências de peptídeos usando LC-MS/MS e uma sequência foi identificada por degradação de Edman (GFPGRLDHWCASE) a qual apresentou CA maior do que a do antioxidante sintético BHA. Em relação ao efeito anti-inflamatório, as amostras Phi, Phh, FPH e phr-FPH foram capazes de inibir a produção de

NO e TNF-α em macrófagos estimulados com IFN-γ/LPS. No estudo *in vivo*, Phi e phr-FPH foram capazes de atenuar a perda de peso dos animais, as respostas inflamatórias das células T_H1 e T_H17, a proliferação de linfócitos $TCD4^+$ e os níveis de citocinas inflamatórias em animais com retocolite induzida por TNBS. De forma geral os resultados demonstram que os hidrolisados proteicos e os compostos fenólicos da linhaça possuem atividade antioxidante e anti-inflamatória. Mais estudos são necessários para avaliar a interferência do complexo proteína:fenólico na biodisponibilidade de peptídeos e fenólicos, e a relação destes compostos com a microbiota intestinal para conhecer a real capacidade destes produtos em fornecer benefícios ao organismo.

ABSTRACT

Flaxseed protein is a potential source of bioactive compounds. During the enzymatic hydrolysis of vegetable proteins, peptides and phenolic compounds, which are associated with proteins, are released and may act as antioxidants and anti-inflammatory. This study evaluated the influence of the hydrolysis with Alcalase and simulated gastrointestinal digestion on the antioxidant capacity of different flaxseed products. We also evaluated the inhibitory capacity of flaxseed protein hydrolysates and phenolic fractions on the production of inflammatory mediators by stimulated macrophages and the anti-inflammatory effects on TNBS-induced colitis. The Flaxseed protein hydrolysate (FPH) was produced with protein isolate obtained from defatted meal by alkaline extraction (pH 9.0) followed by precipitation (pH 4.2) and neutralization (pH 6.0). In order to obtain phenolic reduced flaxseed protein hydrolysate (phr-FPH), flaxseed was extracted from defatted meal with ethanol before isolating process. The hydrolysis was performed with Alcalase (pH 8.5; enzyme/substrate 1/90, w/w). Hydrolysed phenolics compounds (Phi) was obtained with the phenolics compounds extracted from the defatted flaxseed meal using the same conditions of protein hydrolysis. Four glycosylated phenolic compounds – secoisolariciresinol and ferulic, p-coumaric, caffeic acids – were identified using HPLC-MS/MS. Intrinsic fluorescence was used to analyze protein:polyphenol complex. A chromatographic characterization of the samples was performed and the AC was determined by FRAP and ORAC methods. A chromatographic separation of the phr-FPH by RP-HPLC was performed, and the AC of the six obtained fractions was determined. Flaxseed phenolic fractions exhibit the highest antioxidant capacity followed by flaxseed protein hydrolysates and isolates. The presence of phenolic compounds increased 80% of antioxidant capacity of the protein isolate, measured by ORAC and FRAP methods, while for the hydrolysates accounted for 15% measured by ORAC, and 80% when measured by FRAP. After simulated digestion, the antioxidant capacity of isolates and hydrolysates increased and the influence of phenolics on their antioxidant capacity persisted, especially when determined by FRAP. Our findings suggest that AC of isolates is mainly due to the protein: phenolic complex while in the hydrolysates is mainly by the synergism between phenolics and peptides released by Alcalase or simulated digestion. Also, the preferential mechanism that flaxseed protein may act as antioxidant is the H^+ atom transfer. Four peptide sequences could be identified by using LC-MS/MS and one by Edman degradation. The peptide sequence GFPGRLDHWCASE was synthesized and showed a value higher than that of butylated hydroxyanisole. The identified sequences represent an advance in the molecular characterization of the flaxseed protein fraction. Regarding to the anti-inflammatory effect of flaxseed extracts, Phi, Phh, FPH and phr-FPH inhibit NO and TNF-α secretion in stimulated RAW cells and also downregulating weight loss,

histological inflammation, T_H1 and T_H17 responses, T cell proliferation and inflammatory cytokine levels on TNBS-induced colitis in BALB/c mice. Together, the results demonstrate that flaxseed protein hydrolysates and phenolic isolated fractions had antioxidant activity and protective effects on a TNBS-induced colitis model. More studies are needed to evaluate the effect of the protein complex: phenol in the bioavailability of peptides and phenolics, and the relationship of these compounds with the intestinal microbiota to know the real ability of these products to provide health benefits.

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LISTA DE ABREVIATURAS

Em português

AAPH	2,2’ azobis (2-amidino-propano) dihidrocloreto
ArOH	Espécie antioxidante
AUC	Área abaixo da curva
COX	Ciclooxigenase
DC	Doença de Crohn
DII	Doença inflamatória intestinal
DMSO	Dimetilsulfoxido
EDTA	Ácido etileno diamino tetraacético
ELISA	Ensaio imunoenzimático
ENOS	Espécies reativas do nitrogênio
EROS	Espécies reativas do oxigênio
Fl	Fluoresceína
FRAP	Poder antioxidante de redução do íon ferro III
GH	Grau de Hidrólise
HAT	Transferência do átomo de hidrogênio
IFNγ	Interferon gama
IL	Interleucina
iNOS	Óxido nítrico sintase induzida
LPS	Lipopolissacarídeo
M	Metal oxidante
MAPK	Proteínas quinases ativadoras de mitógeno
MTT	3-[4,5-dimetil-tiazol-2-il]-2,5difeniltetrazólio
NF-κB	Fator de transcrição nuclear kappa B
NO	Óxído nítrico
ORAC	Capacidade de absorção do radical oxigênio
RC	Retocolite ulcerative
SECO	Secoisolariciresinol
SET	Transferência de elétrons
SRRFC	Substâncias redutoras do reagent de Folin-Ciocalteau
TEAC	Capacidade de absorção do radical Trolox
TMC	Quelação de metais de transição
TNF-α	Fator de necrose tumoral α
TE	Equivalente de Trolox

Em inglês

CD	Crohn´s Disease
DFM	Deffated flaxseed meal
DH	Degree of hydrolysis
DSS	Dextran sodium sulfate
ELISA	Enzyme linked immunosorbent assay
FCRRS	Folin-Ciocalteau reducing reagent substances
FM	Flaxseed meal
FPH	Flaxseed protein hydrolysate
FPI	Flaxseed protein isolate
FRAP	Ferric reduced antioxidant power
GAE	Gallic acid equivalents
lFNγ	Interferon gama
IL	Interleukine
NF-κB	Nuclear Factor Kappa B
NO	Nitric oxide
ORAC	Oxygen radical absorbance capacity
Phh	Phenolics hydrolysate
Phi	Phenolics isolate
phr-DFM	Phenolic reduced- deffated flaxseed meal
phr-FPH	Phenolic reduced flaxseed protein hydrolysate
phr-FPI	Phenolic reduced flaxseed protein isolate
ROS	Reactive oxygen species
RP-HPLC	Reverse phase - High Performance Liquid Chromatography
TE	Trolox equivalents
TNF-α	Tumor Necrosis Factor α
UC	Ulcerative colitis

SUMÁRIO

CAPÍTULO 1. INTRODUÇÃO GERAL

1.1. Introdução

A formação de espécies reativas de oxigênio (EROs) e de nitrogênio (ERNs) tem sido relacionada à oxidação deteriorativa de produtos alimentícios e também à patogênese de diversos doenças como aterosclerose, diabetes mellitus, inflamação crônica, desordens degenerativas e alguns tipos de câncer (MAGALHÃES, SEGUNDO, REIS *et al.*, 2008). O efeito protetor de compostos antioxidantes nas reações deletérias induzidas pela oxidação tem sido alvo de interesse crescente nas áreas biológicas, médica, nutrição e de alimentos.

No organismo, EROs são formados a partir do metabolismo energético e do consumo de oxigênio intracelular, por meio das reações da cadeia respiratória que ocorrem na mitocôndria. Quando ocorre um desbalanço entre a produção de radicais livres e a capacidade do organismo em neutralizá-los, devido a um excesso de radicais produzidos ou por enfraquecimento do sistema de defesa antioxidante, o organismo fica mais susceptível a oxidação o que caracteriza um estado de estresse oxidativo (MARTÍNEZ-CAYUELA, 1995; LIMÓN-PACHECO & GONSEBATT, 2009).

No corpo humano assim como em sistemas alimentícios, nutrientes como carotenoides, vitaminas C e E são os principais compostos com atividade antioxidante (ERDMANN, CHEUNG & SCHRÖDER, 2008). Para serem considerados como tal, essas substâncias devem estar em pequenas quantidades em relação ao substrato oxidável e serem capazes de prevenir ou retardar a oxidação desse substrato. Polifenóis e peptídeos também podem apresentar atividade redutora, capazes de reduzir as reações oxidativas que ocorrem numa matriz alimentar e em sistemas biológicos (ERDMANN, CHEUNG & SCHRÖDER, 2008).

A atividade antioxidante de hidrolisados proteicos está relacionada com a composição e sequência dos aminoácidos e com o tamanho e estrutura tridimensional do peptídeo (PEÑA-RAMOS, XIONG & ARTEAGA, 2004). O mecanismo de atuação antioxidante vai depender da composição aminoacídica da proteína. A hidrólise da proteína contribui para aumentar a exposição de resíduos de aminoácidos, principalmente os aromáticos, aumentando a atuação dos mesmos como quelantes, sequestradores de radical, redutores de hidroperóxidos e aldeídos (ELIAS, KELLERBY & DECKER, 2008).

A atividade antioxidante dos compostos fenólicos se deve à habilidade de doar hidrogênio ou elétrons e em acomodar elétrons desemparelhados nos anéis aromáticos de sua estrutura. Para ácidos fenólicos, quanto maior o número de hidroxilas livres no anel aromático, maior será a atividade antioxidante determinada pelos métodos TEAC, DPPH e ABTS. Para os flavonoides, não só a quantidade, mas também as posições dos grupos hidroxila influenciam nos valores de ORAC e ABTS (FERNANDEZ-PANCHON, VILLANO, TRONCOSO *et al.*, 2008).

Um dos mecanismos biológicos que pode perturbar o equilíbrio redox do organismo, favorecendo um ambiente pró-oxidante é o processo inflamatório. Ele pode ser agudo, caracterizando-se por vasodilatação, infiltração de leucócitos, destruição de patógenos e reparação tecidual. Quando o estímulo não cessa, se torna crônico podendo provocar o aparecimento ou progressão de doenças cardiovasculares, inflamatórias intestinais (DII), câncer, diabetes, e obesidade (DI LORENZO, DELL'AGLI, BADEA *et al.*, 2012; KUNDU & SURH, 2012). O fator de transcrição nuclear kappa B (NF-κB) é o principal responsável por ativar a expressão de citocinas e quimiocinas pró-inflamatórias como fator de necrose tumoral alfa (TNF-α) e as interleucinas (IL) 1, 6 e 8. Essas substâncias estimulam uma massiva infiltração de neutrófilos e macrófagos no tecido lesionado resultando numa elevada produção de EROS, principalmente peróxido de hidrogênio (ATREYA, ATREYA & NEURATH, 2008).

Compostos com propriedades antioxidantes e anti-inflamatórias como polifenóis (ALGIERI, ZORRILLA, RODRIGUEZ-NOGALES *et al.*, 2013; FRONTELA-SASETA, LÓPEZ-NICOLÁS, GONZÁLEZ-BERMÚDEZ *et al.*, 2013; NUNES, FERREIRA, FREITAS *et al.*, 2013), ácido α-linolênico (HASSAN, IBRAHIM, MBODJI *et al.*, 2010) e peptídeos (OSEGUERA-TOLEDO, DE MEJIA, DIA *et al.*, 2011) têm sido investigados não só pela capacidade de neutralizar espécies reativas como pela possibilidade de modular reações em cascata de sinalização celular relacionadas à inflamação. Estudos com células *in vitro* mostraram que esses compostos apresentam efeitos inibitórios na ativação do NF-κB e, consequentemente, da produção de interleucinas, COX-2 e iNOS (HASSAN, IBRAHIM, MBODJI *et al.*, 2010; FRONTELA-SASETA, LÓPEZ-NICOLÁS, GONZÁLEZ-BERMÚDEZ *et al.*, 2013; NUNES, FERREIRA, FREITAS *et al.*, 2013).

A linhaça, semente oleaginosa proveniente da planta linho (*Linum usitatissimum* L.), possui elevado teor de proteína cuja qualidade nutricional é comparável à da soja (KRAUSE, SCHULTZ & DUDEK, 2002) e, como outras proteínas de origem vegetal, estão associadas a compostos fenólicos, dentre eles a lignana (HAO & BETA, 2012). A maioria das pesquisas estuda os benefícios da linhaça na saúde focando principalmente na ação de

ácido α-linolênico, compostos fenólicos e fibras solúveis, enquanto que as proteínas, presentes em quantidades expressivas, são pouco estudadas (MARAMBE, SHAND & WANASUNDARA, 2008; UDENIGWE, LU, HAN *et al.*, 2009). Marambe e colaboradores (2008) verificaram que a proteína de linhaça hidrolisada por *Flavourzyme*, apresentou atividade antihipertensiva e antioxidante, capaz de sequestrar radical hidroxila. No estudo de Udenigwe e colaboradores (2009), a proteína de linhaça foi hidrolisada por sete enzimas (Alcalase, Pancreatina, Pepsina, Termolisina, Papaína, Tripsina e Ficina) e os hidrolisados formados apresentaram atividades anti-inflamatória e antioxidante capazes de sequestrar radical hidroxila e ânion superóxido. Em outro estudo, Marambe e colegas (2011), após submeter a proteína à simulação da digestão gastrintestinal, observaram a liberação de peptídeos inibidores da enzima conversora de angiotensina (ECA).

Em estudos anteriores realizados por nosso grupo de pesquisa verificou-se que a farinha, o concentrado e principalmente o hidrolisado proteico de linhaça apresentaram capacidade antioxidante *in vitro.* Esses produtos, após serem submetidos à simulação da digestão gastrintestinal, não tiveram sua capacidade antioxidante reduzida, e a atividade do concentrado proteico foi superior a do hidrolisado (SILVA, O'CALLAGAHAN, O'BRIEN *et al.*, 2013). No entanto, não está claro se a atividade antioxidante encontrada nesses produtos proteicos é devida aos peptídeos antioxidantes, aos compostos fenólicos ou ao complexo proteína-fenólico resultante da interação entre estes compostos.

Dessa forma, esse trabalho consiste no estudo da interação de proteínas e fenólicos na capacidade antioxidante do hidrolisado proteico de duas formas. A primeira como um redutor em meio biológico, capaz de atuar junto ao sistema de defesa antioxidante minimizando os possíveis efeitos deletérios que o estresse oxidativo pode provocar no organismo. E a segunda, como um agente anti-inflamatório, capaz de modular a resposta inflamatória por meio da sinalização redox.

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CAPÍTULO 2. OBJETIVOS

2.1. Objetivo Geral

Investigar a atividade antioxidante e anti-inflamatória de produtos proteicos e fenólicos da linhaça.

2.2. Objetivos Específicos

- Obter as farinhas de linhaça desengorduradas, os isolados e hidrolisados proteicos e a fração fenólica da linhaça.

- Caracterizar quimicamente as farinhas de linhaça desengorduradas, os isolados e hidrolisados proteicos.

- Estudar a formação do complexo proteína:fenólico nos isolados e hidrolisados proteicos por meio do espectro de fluorescência.

- Identificar e quantificar os fenólicos da linhaça nos hidrolisados proteicos e na fração fenólica por UPLC/MS/MS.

- Determinar a atividade antioxidante *in vitro* das farinhas de linhaça desengordurada, dos isolados e hidrolisados proteico e da fração fenólica da linhaça antes e após a digestão *in vitro.*

- Estudar o potencial anti-inflamatório dos hidrolisados proteicos e da fração fenólica da linhaça em cultura de células da linhagem RAW 264-7.

- Avaliar o potencial do hidrolisado proteico e da fração fenólica da linhaça na modulação dos sinais clínicos da colite experimental induzida por TNBS em camundongos BALB/c.

- Avaliar o efeito do hidrolisado proteico e da fração fenólica da linhaça na modulação da proliferação celular de linfócitos T $CD4^+$ em culturas de células de baço de camundongos BALB/c em modelo de colite experimental induzida por TNBS.

- Avaliar o efeito do hidrolisado proteico e da fração fenólica da linhaça nas frequências de populações de linfócitos T $CD4^+Foxp3^+$, T $CD4^+IL\text{-}10^+$, T $CD4^+IL\text{-}17^+$ e T $CD4^+IFN\text{-}\gamma^+$ e na secreção de citocinas Th1/Th2/Th17 em culturas de células de baço de camundongos BALB/c na colite experimental induzida por TNBS.

CAPÍTULO 3. REVISÃO BIBLIOGRÁFICA

3.1. Espécies reativas e antioxidantes em sistemas biológicos

Radicais livres são átomos ou moléculas que possuem um ou mais elétrons desemparelhados no orbital externo e por isso são bastante reativos e instáveis (WICKENS, 2001; PISOSCHI & POP, 2015). Esses radicais e outras espécies não radicalares originam-se do metabolismo do oxigênio ou do nitrogênio e, por apresentarem reatividade semelhante, recebem a denominação de espécies reativas do oxigênio (ERO) ou do nitrogênio (ERN), respectivamente. Em meio biológico destacam-se os radicais hidroxila, ânion superóxido, óxido nítrico e peroxinitrito e as não radicalares oxigênio singlet e peróxido de hidrogênio (WICKENS, 2001; PRIOR, 2015).

Em condições fisiológicas, as espécies reativas estão envolvidas em processos essenciais a homeostase e metabolismo das células, como proliferação e sinalização celular, mecanismos de defesa do sistema imune e regulação da expressão gênica. Os EROs são produzidas principalmente durante a fosforilação oxidativa na mitocôndria ou por enzimas como as lipooxigenases, xantina oxiredutase e as do complexo enzimático do citocromo P-450 (BERGER, LUNKENBEIN, STRÖHLE *et al.*, 2011; HALLIWELL, 2013; GOSTNER, BECKER, UEBERALL *et al.*, 2015). Na maioria dos casos, na cadeia transportadora de elétrons, pode ocorrer a redução do oxigênio molecular (O_2) ao ânion superóxido ($O_2^{\bullet-}$) que pode ser convertido em peróxido de hidrogênio (H_2O_2) por dismutação. Por sua vez, H_2O_2 pode reagir com íon ferroso (Fe^{+2}), formando radical hidroxila ($OH^{\bullet}$) e íons hidroxila (OH^-). Em relação às ERN, o óxido nítrico (NO) é formando a partir da reação entre L-arginina, NADPH e óxido nítrico sintetase e, o peroxinitrito ($ONOO^-$), pela reação entre $O_2^{\bullet-}$ e NO (MARTÍNEZ-CAYUELA, 1995; FERREIRA & MATSUBARA, 1997; VALKO, RHODES, MONCOL *et al.*, 2006; PISOSCHI & POP, 2015).

Embora a produção dessas espécies seja um processo contínuo e fisiológico, quando em excesso, podem oxidar as biomoléculas e provocar a formação de mais espécies reativas. Os lipídeos insaturados presentes na membrana celular, principalmente ácido araquidônico e ácido docosahexaenóico, são os mais susceptíveis à peroxidação pelo ataque do radical $OH^{\bullet}$, levando à produção de isoprostanos e aldeídos reativos como o malondialdeído. A oxidação das proteínas pode ocorrer tanto nas cadeias laterais como no esqueleto da molécula. Nos resíduos de Cys, Met, Lys, Pro e Thr formam grupos dicarbonil e nos resíduos de Tir, formam ditirosina ou outras espécies reativas nitrogenadas como 3-nitrotirosina. Essas oxidações provocam uma deformação na estrutura tridimensional da proteína levando à perda de função. Em relação aos ácidos nucléicos, a oxidação ocorre

principalmente nos nucleosídeos de guanina e podem resultar em quebra da fita de RNA, disfunções ribossomais e mutações na fita de DNA, resultante do acúmulo de alterações na estrutura de purinas e pirmidinas, danos aos nucleotídeos e formação de *crosslinking* com proteínas (PISOSCHI & POP, 2015).

O dano oxidativo nas biomoléculas provocado pelo desequilíbrio entre compostos oxidantes e antioxidantes com aumento dos oxidantes é conhecido como estresse oxidativo (BARBOSA, COSTA, ALFENAS *et al.*, 2010). O desbalanço ocorre devido ao aumento na produção de espécies reativas com concomitante ineficiência do sistema de defesa antioxidante em neutralizá-los. Ao oxidar as biomoléculas as espécies reativas interferem na permeabilidade seletiva da membrana, no funcionamento dos canais iônicos, na atividade enzimática, na função das proteínas, nos processos de transcrição e tradução do material genético e no fino controle de eventos celulares que ocorrem por mecanismos de sinalização via reações de óxido-redução (redox). Dessa forma, a maior consequência do estresse oxidativo é o comprometimento do funcionamento celular e a indução de respostas que desencadeiam a apoptose ou necrose da célula. Por isso, o termo estresse oxidativo tem sido redefinido como uma perturbação do estado redox e do controle de eventos celulares (JONES, 2006; PISOSCHI & POP, 2015).

A literatura tem mostrado que o excesso de espécies reativas e o desbalanço do estado redox celular estão envolvidos na patogênese de doenças cardiovasculares, neurodegenerativas, cânceres, doenças inflamatórias crônicas, diabetes e obesidade (WILLCOX, ASH & CATIGNANI, 2004; KARP & KOCH, 2006; LI, CHEN, ZHANG *et al.*, 2014; MANGGE, BECKER, FUCHS *et al.*, 2014). Para neutralizar as espécies reativas e reduzir o estresse oxidativo, o organismo dispõe de diversas substâncias antioxidantes. Para serem considerados como tal, essas substâncias, quando presentes em pequenas concentrações em relação ao substrato oxidável, devem ser capazes de impedir ou retardar a oxidação desse substrato (HALLIWELL, AESCHBACH et al., 1995).

No organismo, os antioxidantes podem ser classificados em enzimáticos, como a catalase, a superóxido dismutase, a glutationa peroxidase e a glutationa redutase; não enzimáticos, como as vitaminas A, C e E, compostos fenólicos e minerais como cobre, manganês, zinco e selênio (WICKENS, 2001; HUANG, OU & PRIOR, 2005). Eles também podem ser agrupados em primários ou secundários. Os primários são aqueles que reagem diretamente com as espécies reativas neutralizando-as ou impedindo a sua formação. Os secundários são aquelas substâncias que atuam em sinergismo com os antioxidantes primários, induzindo e potencializando sua atividade redutora (GOSTNER, BECKER, UEBERALL *et al.*, 2015).

O sistema antioxidante atua em três linhas de defesa de acordo com as fases na qual ocorre a neutralização da espécie reativa ou reparo do dano oxidativo (WILLCOX, ASH & CATIGNANI, 2004). A primeira ocorre quando o radical ainda não foi formado e caracteriza-se por reações enzimáticas que contribuem para redução ou impedimento da formação de espécies reativas. Os antioxidantes que atuam nesta etapa são chamados de preventivos. As enzimas catalase, superóxido dismutase, glutationa peroxidase, glutationa redutase e as proteínas transferrina, ceruloplasmina e lactoferrina, são exemplos desse tipo de antioxidantes. A segunda linha de defesa ocorre do início ao término da cascata de reações de oxirredução. Nesta fase, os antioxidantes atuam como sequestradores de radical, inibindo as etapas de iniciação e propagação e favorecendo a terminação das reações de oxirredução. Os antioxidantes capazes de sequestrar radicais livres, como os carotenoides, a vitamina C, a vitamina E e os polifenóis são chamados de *chain-breaker* (WICKENS, 2001; OU, HUANG, HAMPSCH-WOODILL *et al.*, 2002). A terceira linha de defesa acontece após o dano oxidativo das biomoléculas e consiste na ação das enzimas de reparo, reconstituindo membranas celulares e tecidos. Nesta fase participam as lipases, proteases, enzimas de reparo do DNA e transferases (WILLCOX, ASH & CATIGNANI, 2004).

Adicionalmente, os antioxidantes consumidos por meio da alimentação podem exercer papel essencial o reparo do dano sua bioatividade ativando vias de sinalização celular que estimulam a expressão de genes responsáveis pela citoproteção e detoxificação celular (GOSTNER, BECKER, UEBERALL *et al.*, 2015). Estudos sugerem que, para expandir a capacidade de defesa do sistema antioxidante, é necessário consumir alimentos que contenham substâncias com capacidade antioxidante. Uma alimentação rica em vegetais, frutas, grãos e cereais integrais podem fornecer nutrientes e compostos bioativos que irão atuar em conjunto com os antioxidantes enzimáticos a fim de minimizar os efeitos deletérios provocados pelo estresse oxidativo (VALTUENA, PELLEGRINI, FRANZINI *et al.*, 2008).

No entanto, é importante ressaltar que o efeito dos antioxidantes provenientes da dieta não está diretamente relacionado à sua ingestão. Sua atividade redutora depende de diversos fatores como reatividade química, localização intra ou extracelular, concentração, solubilidade, interação com outros radicais, absorção, distribuição, metabolismo e excreção (GOSTNER, BECKER, UEBERALL *et al.*, 2015).

Estudos recentes *in vitro* e *in vivo* questionam a hipótese de que os antioxidantes poderiam atuar como agentes preventivos de doenças que envolvem o estresse oxidativo. Esse questionamento é devido à ausência de correlação entre os resultados da capacidade

antioxidante *in vitro* e *in vivo* e pelo fato de que a suplementação com antioxidantes em indivíduos saudáveis provocou um desequilíbrio no sistema redox favorecendo a produção de espécies reativas (BERGER, LUNKENBEIN, STRÖHLE *et al.*, 2011; HALLIWELL, 2013). O excesso da ingestão de antioxidantes, além de favorecer o estresse oxidativo, também está relacionado com o desenvolvimento de asma, alergias e maior suscetibilidade à infecção (GOSTNER, BECKER, UEBERALL *et al.*, 2015).

Dessa forma, observa-se a existência de uma homeostase entre compostos antioxidantes e oxidantes no organismo. Portanto, o consumo excessivo de antioxidantes, ao invés de prevenir, pode perturbar o equilíbrio redox resultando em dano oxidativo e desenvolvimento de doenças (BERGER, LUNKENBEIN, STRÖHLE *et al.*, 2011; GOSTNER, BECKER, UEBERALL *et al.*, 2015). Porém, nos casos em que esse equilíbrio foi rompido em decorrência de uma patologia, favorecendo a produção muito elevada de espécies reativas, a suplementação com antioxidante pode ser benéfica (HALLIWELL, 2013).

3.2. Antioxidantes de origem dietética

Os antioxidantes naturais presentes nos alimentos abrangem um grupo heterogêneo de compostos (OROIAN & ESCRICHE, 2015). Os principais são a provitamina A (carotenoides), a vitamina C (ácido ascórbico), a vitamina E (tocoferóis e tocotrienóis) e os compostos fenólicos. Além desses, os micronutrientes como o selênio, zinco, cobre e manganês, presentes em frutas e vegetais participam como cofatores enzimáticos do sistema de defesa antioxidante (LIMÓN-PACHECO & GONSEBATT, 2009; LI, CHEN, ZHANG *et al.*, 2014).

Os carotenóides são pigmentos lipossolúveis encontrados em plantas e em microrganismos. A longa cadeia carbônica com duplas ligações conjugadas permite que esses compostos sejam capazes de neutralizar, principalmente, radical hidroxila, oxigênio singlet e peróxido de hidrogênio (VALKO, RHODES, MONCOL *et al.*, 2006).

O ácido ascórbico é considerado o mais importante antioxidante hidrofílico do organismo (OROIAN & ESCRICHE, 2015). Sua estrutura carbônica apresenta duas hidroxilas ionizáveis capazes de doar hidrogênios para regenerar a vitamina E oxidada ou para sequestrar as principais espécies reativas do meio biológico. A oxidação da vitamina C resulta na formação de um radical estável, o semideidroascorbato, que pode ser regenerado pela glutationa (VALKO, RHODES, MONCOL *et al.*, 2006; OROIAN & ESCRICHE, 2015).

Os tocoferóis e tocotrienóis são os principais antioxidantes lipossolúveis do organismo. Esses compostos atuam como chain-breaking e têm como principal função

impedir a peroxidação lipídica nas membranas celulares (VALKO, RHODES, MONCOL *et al.*, 2006; OROIAN & ESCRICHE, 2015).

Os compostos fenólicos, produtos do metabolismo secundário de vegetais, abrangem um amplo grupo de substâncias que apresentam em sua estrutura pelo menos um grupamento fenol (anel aromático com um ou mais hidrogênios substituídos por grupos hidroxila) (ROBBINS, 2003; BALASUNDRAM, SUNDRAM & SAMMAN, 2006). Eles são considerados antioxidantes multifuncionais, pois podem atuar como chain-breaking ou quelante de metais como o ferro (KHOKHAR & OWUSU APENTEN, 2003; FERNANDEZ-PANCHON, VILLANO, TRONCOSO *et al.*, 2008).

Além das vitaminas e polifenóis, diversos estudos *in vitro* e *in vivo* têm revelado que os peptídeos formados a partir da hidrólise de proteínas de origem animal e vegetal também podem apresentar atividade redutora (ERDMANN, CHEUNG & SCHRÖDER, 2008; MÖLLER, SCHOLZ-AHRENS, ROOS *et al.*, 2008; UDENIGWE & ALUKO, 2012; LIU, ZHENG, SONG *et al.*, 2015). O mecanismo de atuação antioxidante vai depender, principalmente, da composição e sequência aminoacídica da proteína de origem e da massa molecular do peptídeo formado (PEÑA-RAMOS, XIONG & ARTEAGA, 2004).

Embora as vitaminas tenham comprovada ação antioxidante, alguns estudos de intervenção e meta análises têm mostrado que somente a suplementação da dieta de indivíduos com ácido ascórbico, α tocoferol ou beta caroteno não foi eficaz em minimizar o estresse oxidativo e prevenir doenças cardiovasculares e câncer (BJELAKOVIC, NIKOLOVA, SIMONETTI *et al.*, 2004; LEE, FOLSOM, HARNACK *et al.*, 2004; HALLIWELL, 2007; CARVALHO & SPOSITO, 2015). Por outro lado, é consenso na literatura que uma alimentação rica em grãos, sementes, frutas e hortaliças e pobre em açúcares simples, gorduras saturadas e trans está associada à redução do risco de desenvolvimento de doenças crônicas não transmissíveis (VALTUEÑA, PELLEGRINI, FRANZINI *et al.*, 2008; FRANZINI, ARDIGÒ, VALTUEÑA *et al.*, 2010). Essa associação pode ser explicada pela presença de outros componentes como fibras, vitaminas do complexo B, ácidos graxos mono e poli-insaturados, polifenóis e enzimas detoxificantes que podem atuar como agentes imunomodulatórios, anti-colesterolêmicos e antioxidantes (HALLIWELL, 2007). Assim, diversos estudos têm investigado o potencial e o possível mecanismo de ação antioxidante de outros componentes de origem alimentar, como polifenóis e peptídeos bioativos, na redução do estresse oxidativo e na prevenção de doenças (RAHMAN, BISWAS & KIRKHAM, 2006; ERDMANN, CHEUNG & SCHRÖDER, 2008; LANDETE, 2011; UDENIGWE & ALUKO, 2012; BIELLI, SCIOLI, MAZZAGLIA *et al.*, 2015; BRANDELLI, DAROIT & CORRÊA, 2015; GIOXARI, KOGIANNOU, KALOGEROPOULOS *et al.*, 2016).

3.2.1. Ação antioxidante de compostos fenólicos

Polifenóis constituem um grupo diverso de compostos amplamente distribuídos no reino vegetal, inclusive em porções comestíveis como frutas, hortaliças, cereais, grãos e sementes (CROZIER, DEL RIO & CLIFFORD, 2010). A função dessas substâncias em plantas não é totalmente conhecida, mas sabe-se que elas estão envolvidas na síntese proteica, na atividade enzimática, na fotossíntese, proteção contra insetos e raios ultra-violeta e, quando presentes em partes comestíveis, são responsáveis pelas cores, características organolépticas e pelas propriedades antioxidantes (ROBBINS, 2003; BALASUNDRAM, SUNDRAM & SAMMAN, 2006).

Os compostos fenólicos são classificados em ácidos fenólicos, flavonoides, estilbenos e lignanas de acordo com a quantidade de anéis aromáticos presentes na estrutura e pelos elementos estruturais que ligam esses anéis uns aos outros (MANACH, SCALBERT, MORAND *et al.*, 2004; GROOTAERT, KAMILOGLU, CAPANOGLU *et al.*, 2015).

O grupo dos ácidos fenólicos compreende os compostos que possuem o grupamento funcional ácido carboxílico ligado ao grupamento fenol. Esta classe é subdivida em duas, os hidroxibenzóicos e os hidroxicinâmicos, de acordo com a posição dos grupos hidroxila no anel aromático. Os representantes mais comuns dessa classe são os ácidos caféico, p-cumárico, ferúlico e vanílico (ROBBINS, 2003).

Flavonóides são fenólicos de baixa massa molecular, caracterizado pela presença de dois grupamentos fenol unidos por um anel heterocíclico de três átomos de carbono. Variações na substituição do anel heterocíclico resultam nas diferentes subclasses, nomeadas como flavonóis, flavonas, flavononas, isoflavonas, antocianinas (CERQUEIRA, MEDEIROS & AUGUSTO, 2007; GROOTAERT, KAMILOGLU, CAPANOGLU *et al.*, 2015). Em cada subgrupo, os compostos diferem na posição e quantidade dos grupos hidroxila e no grau de glicosilação e/ou alquilação da molécula. Quercetina, luteolina, naringenina, catequina, genisteína e cianidina são alguns representantes dessa classe (GROOTAERT, KAMILOGLU, CAPANOGLU *et al.*, 2015).

Estilbenos são fenólicos que possuem dois grupamentos fenólicos unidos a dois carbonos. Esta classe de compostos é encontrada em pequenas quantidades nos alimentos e o resveratrol é o seu principal representante (MANACH, SCALBERT, MORAND *et al.*, 2004; GROOTAERT, KAMILOGLU, CAPANOGLU *et al.*, 2015).

As lignanas são formadas pela dimerização oxidativa de duas unidades de fenilpropano. A semente de linhaça é a principal fonte dos representantes dessa classe, o secoisolariciresinol (SECO) e matairesinol (MANACH, SCALBERT, MORAND *et al.*, 2004; GROOTAERT, KAMILOGLU, CAPANOGLU *et al.*, 2015).

A base molecular das propriedades antioxidantes dos polifenóis é descrita, principalmente, por meio de três mecanismos, todos oriundos da reação direta com as espécies reativas (LEOPOLDINI, RUSSO & TOSCANO, 2011). O primeiro consiste na inativação das espécies reativas por meio de reações de transferência de hidrogênio (HAT, do inglês *hydrogen atom transfer*). O antioxidante (ArOH) reage com o radical livre (R), transfere o átomo de hidrogênio por meio de uma ruptura homolítica da ligação O-H do grupamento fenol (Equação 1) (LEOPOLDINI, RUSSO & TOSCANO, 2011).

$$ArOH + R^{\bullet} \rightarrow ArO^{\bullet} + RH \qquad \text{(eq. 1)}$$

No segundo mecanismo um elétron é doado ao radical, conforme descrito na Equação 2 (LEOPOLDINI, RUSSO & TOSCANO, 2011).

$$ArOH + R^{\bullet} \rightarrow ArOH^{+} + R^{-} \qquad \text{(eq. 2)}$$

Em ambas as reações, as espécies químicas formadas, $ArO^{\bullet}$ e $ArOH^{+}$, são menos reativas que os EROs, pois o anel aromático permite que os elétrons sejam estabilizados por ressonância, tornando a molécula estável (LEOPOLDINI, RUSSO & TOSCANO, 2011).

A capacidade antioxidante dos fenólicos também está relacionada com configuração espacial da molécula. A presença de múltiplos grupos hidroxila ligados ao anel aromático, preferencialmente na posição orto-dihidroxi e/ou a presença de grupos carbonila aliado a uma estrutura planar são características que favorecem a atuação como antioxidante (LEOPOLDINI, RUSSO & TOSCANO, 2011). Para ácidos fenólicos, quanto maior o número de hidroxilas livres de um anel aromático, maior será a atividade antioxidante determinada pelos métodos TEAC, DPPH e ABTS. Para os flavonoides, não só a quantidade, mas também as posições dos grupos hidroxila influenciam nos valores de ORAC e ABTS (FERNANDEZ-PANCHON, VILLANO, TRONCOSO *et al.*, 2008)

O último mecanismo é a quelação de metais de transição (TMC, do inglês *transition metal chelation*) formando complexos de coordenação estáveis. Os potenciais sítios de ligação dos compostos fenólicos com os metais são as regiões da molécula onde há grupos hidroxila e carbonila ligados ao anel aromático (LEOPOLDINI, RUSSO & TOSCANO, 2011).

Além de minimizar o estresse oxidativo de forma direta, por meio da neutralização de espécies reativas, os polifenóis podem atuar de forma indireta, por meio da modulação de vias de sinalização redox que estão diretamente relacionadas com o desenvolvimento de doenças crônico-degenerativas e produção excessiva de espécies reativas (CROZIER, JAGANATH & CLIFFORD, 2009; OROIAN & ESCRICHE, 2015).

As vias de sinalização nas quais os compostos fenólicos podem inibir ou suprimir a ativação são as que envolvem o fator nuclear kappa B (NF-κB); a proteína 1 ativadora (AP-1) e as quinases proteicas ativadas por mitógenos (MAPK, do inglês *Mitogen-activated protein kinase*) (CROZIER, JAGANATH & CLIFFORD, 2009; OROIAN & ESCRICHE, 2015).

NF-κB é um fator de transcrição redox sensitivo que regula inúmeras funções biológicas e está relacionado com a patogênese de várias doenças. Este fator está envolvido na regulação da expressão de citocinas pró-inflamatórias, da enzima óxido nítrico sintase induzida (iNOS), da ciclooxigenase 2 (COX-2), de fatores de crescimento e inibidores de apoptose. Alterações na via de sinalização do NF-κB está associada às doenças inflamatórias como asma, Crohn e colite ulcerativa, câncer, doenças neurodegenerativas, além de doenças autoimunes como artrite reumatoide. A curcumina, a epigalocatequina-galato, o ácido elágico e o resveratrol são exemplos de potentes inibidores desta via (CROZIER, JAGANATH & CLIFFORD, 2009).

O fator de transcrição AP-1 regula a expressão de genes em resposta a diversos estímulos como citocinas, fatores de crescimento, estresse e infecções microbianas e virais. A ativação desta via promove proliferação, diferenciação e apoptose de células imunes e a inibição de genes supressores de tumor. Os compostos fenólicos presentes na maçã, no chá verde e nas uvas foram capazes de interferir no processo de ativação desta via (CROZIER, JAGANATH & CLIFFORD, 2009).

As MAP quinases (MAPKs) compreendem um grupo de proteínas quinases denominadas ERK, JNK e p38. Geralmente, elas são ativadas sob condições de estresse e por citocinas como o fator de necrose tumoral α (TNF-α), interleucina 1 (IL-1), IL-2, IL-17 e estão fortemente associadas com a inflamação, dor e morte celular programada. Os flavonóis do chá verde são capazes de suprimir a expressão de genes relacionados à tumorigênese por meio da ação antioxidante na via das MAPK quinases (CROZIER, JAGANATH & CLIFFORD, 2009).

A bioatividade dos compostos fenólicos sofre influência da concentração e da forma que é administrado, isolado ou em extratos de alimentos, do pH, da temperatura e da polaridade do solvente de extração. Em relação à atividade antioxidante, os polifenóis

podem ser agentes redutores ou oxidantes (ALMAJANO, DELGADO & GORDON, 2007). Geralmente o fator determinante deste comportamento é a concentração do composto administrada (ALGIERI, ZORRILLA, RODRIGUEZ-NOGALES *et al.*, 2013). Algieri e colaboradores (2013) mostraram que extratos de plantas em concentrações elevadas se comportaram como oxidantes. Sanchez de Medina e colaboradores (1996) observaram o mesmo para os flavonoides quercetina e rutina.

3.2.2. Ação antioxidante de peptídeos

Assim como os fenólicos, os aminoácidos, peptídeos e proteínas provenientes dos alimentos de origem vegetal e animal, além do seu papel nutricional, possuem potencial para atuar como imunomoduladores, anti-hipertensivos, antitrombóticos e antioxidantes (PIHLANTO, 2006; MÖLLER, SCHOLZ-AHRENS, ROOS *et al.*, 2008). Leite, iogurte, queijos, ovos, peixes (sardinha, salmão, atum), trigo, soja, e sorgo são alguns alimentos que se destacam como fontes de peptídeos com potencial redutor (CHEN, MURAMOTO, YAMAUCHI *et al.*, 1996; PEÑA-RAMOS, XIONG & ARTEAGA, 2004; MÖLLER, SCHOLZ-AHRENS, ROOS *et al.*, 2008; UDENIGWE & ALUKO, 2012; BRANDELLI, DAROIT & CORRÊA, 2015; YOSHIKAWA, 2015).

Os peptídeos são formados a partir da hidrólise da proteína e podem apresentar de 2 a 20 resíduos de aminoácidos em sua estrutura (PIHLANTO, 2006). O termo hidrolisado proteico refere-se ao conjunto de fragmentos de origem proteica, aminoácidos, peptídeos e polipeptídeos, resultantes da reação de hidrólise. Esta reação pode ocorrer, *in vivo* por ação de enzimas do trato digestivo e ou por enzimas da microbiota intestinal e *in vitro* por ação de enzimas isoladas de microrganismos, vegetais e animais ou durante as etapas de processamento do alimento (MÖLLER, SCHOLZ-AHRENS, ROOS *et al.*, 2008). Pepsina, papaína, quimiotripsina e o conjunto de enzimas pancreatina e Alcalase estão entre as proteases mais utilizadas na obtenção de hidrolisados proteicos (ELIAS, KELLERBY & DECKER, 2008; SAMARANAYAKA & LI-CHAN, 2011)

De forma geral, os 20 tipos de aminoácidos que podem compor uma proteína possuem atividade antioxidante (ELIAS, KELLERBY & DECKER, 2008), sendo que os mais reativos são a His, os sulfurados (Cis e Met), os aromáticos (Trp, Phe, Tir) (SAMARANAYAKA & LI-CHAN, 2011) e os demais aminoácidos hidrofóbicos (Lis, Ala, Val, Ile) (CHEN, MURAMOTO, YAMAUCHI *et al.*, 1996). Devido à sua natureza predominantemente hidrofóbica, estes resíduos tendem a se concentrar no interior da estrutura proteica, dificultando o acesso às espécies reativas e, consequentemente, limitando o potencial antioxidante da proteína (ELIAS, KELLERBY & DECKER, 2008).

Ao submeter a proteína à hidrólise, os resíduos de aminoácidos tornam-se mais expostos ao meio, aumentando a atuação dos mesmos como antioxidantes de tal forma que a atividade antioxidante do hidrolisado proteico formado tende a ser maior que a proteína nativa (ELIAS, KELLERBY & DECKER, 2008). No entanto, hidrólise extensa pode resultar na redução na atividade antioxidante, devido à diminuição do tamanho dos peptídeos e aumento no número de aminoácidos livres. Isso ocorre porque a atividade antioxidante do hidrolisado proteico se deve principalmente ao sinergismo de diferentes resíduos de aminoácidos na sequência peptídica, conferindo à estrutura maior estabilidade após as reações de neutralização das espécies reativas em relação aos aminoácidos livres (SAMARANAYAKA & LI-CHAN, 2011).

A atividade antioxidante do hidrolisado proteico está diretamente relacionada com a composição e sequência dos aminoácidos da proteína original e com a massa molecular dos peptídeos formados (PEÑA-RAMOS, XIONG & ARTEAGA, 2004). Para diferentes proteínas, o mesmo grau de hidrólise (GH) fornece uma composição distinta de aminoácidos e peptídeos e, consequentemente, diferente atividade antioxidante (YOU, ZHAO, CUI *et al.*, 2009). Isso permite que o potencial antioxidante do hidrolisado seja manipulado conforme o desejado, a partir da escolha da proteína de origem e da determinação prévia das condições de hidrólise, como a especificidade da protease, o pH e o tempo da reação (DONG, ZENG, WANG *et al.*, 2008).

Em relação ao mecanismo antioxidante, os peptídeos podem atuar como quelantes de metal, sequestradores de radical, redutores de hidroperóxidos e aldeídos (CHEN, MURAMOTO, YAMAUCHI *et al.*, 1996; SAMARANAYAKA & LI-CHAN, 2011). A capacidade de doar hidrogênio pode ser atribuída principalmente aos aminoácidos aromáticos e a Cys, a de doar elétron à Met, a de quelar metais à His, enquanto que a presença dos resíduos de Gln, Gly, Ala e Arg podem exacerbar a capacidade antioxidante nos peptídeos por qualquer um destes mecanismos (DÁVALOS, MIGUEL, BARTOLOMÉ *et al.*, 2004; HERNÁNDEZ-LEDESMA, AMIGO, RECIO *et al.*, 2007; ALEMÁN, GIMÉNEZ, PÉREZ-SANTIN *et al.*, 2011).

Além dos tipos de aminoácidos presentes na sequência, a conformação espacial do peptídeo também interfere na atividade antioxidante (HERNÁNDEZ-LEDESMA, AMIGO, RECIO *et al.*, 2007). Tanto a presença de Gly, que confere maior flexibilidade à estrutura, quanto a presença de Pro, que interrompe a estrutura secundária em α-hélice, podem deixar os resíduos de aminoácidos mais disponíveis para atuar como antioxidantes (ORSINI DELGADO, NARDO, PAVLOVIC *et al.*, 2015). .

3.3. Mecanismos para avaliação da capacidade antioxidante *in vitro*

Diversos tipos de ensaios podem ser utilizados para estimar a atividade antioxidante de produtos de origem vegetal ou animal (OU, HUANG, HAMPSCH-WOODILL *et al.*, 2002; THAIPONG, BOONPRAKOB, CROSBY *et al.*, 2006; MAGALHÃES, SEGUNDO, REIS *et al.*, 2008). Esses ensaios podem se basear em reação de transferência de elétrons (SET), como o teor de substâncias do reagente Folin-Ciocalteau (SRRFC), o poder de redução do íon ferro (FRAP) e a capacidade quelante; ou em reação de transferência de um átomo de hidrogênio (HAT) como a capacidade de absorção do radical oxigênio (ORAC) (OU, HUANG, HAMPSCH-WOODILL *et al.*, 2002; MAGALHÃES, SEGUNDO, REIS *et al.*, 2008).

Nos métodos baseados na reação SET, os antioxidantes (ArOH) interagem com as espécies reativas (M) sem a presença de uma terceira molécula que competiria com o antioxidante pela espécie oxidante (Equação 3).

$$M^{X} + Ar\text{-}OH \rightarrow M^{X-1} + ArOH \quad \text{(eq. 3)}$$

No método SRRFC, a reação SET ocorre entre os compostos fenólicos e os ácidos do reagente de Folin-Ciocalteau, em meio alcalino, formando complexos de cor azul detectados por leitura de absorbância a 765 nm. Embora este método também seja conhecido para determinação do Teor de Fenólicos Totais, ele não é específico para compostos fenólicos, uma vez que o reagente de Folin-Ciocalteau pode ser reduzido por aminoácidos aromáticos, ácido ascórbico e pelos cátions Fe^{+2} e Cu^{+1} (MAGALHÃES, SEGUNDO, REIS *et al.*, 2008).

No caso do método FRAP, a reação de transferência do elétron ocorre entre o complexo íon férrico- 2,4,6-tripiridilhidrazina $[Fe^{+3}(TPTZ)_2]$ e o antioxidante (ArOH) (Equação 4). Nesse ensaio, a habilidade dos antioxidantes em reduzir o complexo $[Fe^{+3}(TPTZ)_2]$ em meio ácido é calculada pela medida da absorbância a 595 nm, após trinta minutos de repouso, a temperatura de 37 °C (MAGALHÃES, SEGUNDO, REIS *et al.*, 2008).

$$Fe^{+3}(TPTZ)_2 + ArOH \rightarrow Fe^{+2}(TPTZ)_2 + ArOH^{+\bullet} \quad \text{(eq. 4)}$$

Esse ensaio é simples, barato e oferece um índice de atividade antioxidante confiável, porém alguns aspectos devem ser respeitados. O primeiro é em relação ao potencial redox da substância antioxidante, que deve ser superior ao par Fe^{+3}/Fe^{+2}, caso contrário uma substância que não exerce atividade antioxidante pode atuar como redutora gerando um resultado falso positivo. O outro é o fato de que nem todos os antioxidantes

reduzem os íons ferro em menos de trinta minutos, como é o caso dos ácidos fenólicos caféico, ferúlico, quercetina e ácido ascórbico, assim como antioxidantes com atividade sequestradora de radical, como tióis e carotenóides, não podem ser determinados. O terceiro se refere ao baixo valor de pH (pH 3,7), que pode induzir a precipitação de proteínas, como a caseína do leite e tornar mais lenta a transferência de elétrons. Esse ensaio também não favorece a análise de amostras biológicas, uma vez que seus mecanismos não se relacionam com os mecanismos fisiológicos (OU, HUANG, HAMPSCH-WOODILL *et al.*, 2002; MAGALHÃES, SEGUNDO, REIS *et al.*, 2008).

A capacidade quelante é determinada, na maioria das vezes, pela capacidade do antioxidante em quelar íons ferro, utilizando sulfato ferroso ou a ferrozina como fonte deste cátion. A formação do complexo antioxidante-metal pode ser determinada espectrofotometricamente utilizando ácido etilenodiamino tetra-acético (EDTA) como padrão. Este método é utilizado como indicador da atividade antioxidante de um composto, uma vez que ao quelar metais, o antioxidante pode prevenir ou retardar a indução da peroxidação lipídica provocada por metais como o ferro (SHAHIDI & ZHONG, 2015).

Nos métodos baseados na reação HAT, há um radical iniciador responsável por gerar os radicais peroxil ($ROO^{\bullet}$); uma sonda, que representa uma biomolécula, alvo em potencial para sofrer ataque do radical livre e a espécie antioxidante (Equações 5 e 6). Nesses métodos, a capacidade antioxidante depende da taxa de reação entre o radical e a espécie antioxidante bem como do radical com a sonda e da concentração entre o composto antioxidante e a sonda (MAGALHÃES, SEGUNDO, REIS *et al.*, 2008).

$$ROO^{\bullet} + ArOH \rightarrow ROOH + ArO^{\bullet} \quad \text{(eq. 5)}$$

$$ROO^{\bullet} + Fl\text{-}H \rightarrow ROOH + Fl^{\bullet} \quad \text{(eq. 6)}$$

No método ORAC, a fluoresceína (Fl) é utilizada como sonda, e o 2,2 azobis (2-amidinopropano) dihidrocloreto (AAPH) como gerador de radicais $ROO^{\bullet}$. A redução na fluorescência da fluoresceína é um indicativo do ataque do radical à sonda. Na presença de um antioxidante, ocorre a doação de um átomo de hidrogênio para o radical peroxil, transformando-se em hidroperóxido (ROOH) e num radical estável ($ArO^{\bullet}$). Isso diminui a taxa de redução da fluorescência uma vez que o radical é, em parte, neutralizado pelo antioxidante. O efeito protetor do antioxidante sobre a sonda pode ser mensurado por meio do cálculo da área abaixo da curva (AUC) de decaimento da fluorescência da fluoresceína da amostra comparada com a curva sem a presença de um antioxidante por um período superior a trinta minutos e os resultados são expressos em µM de equivalente de Trolox.

Nesse ensaio, o decaimento da fluorescência se deve principalmente à concentração de AAPH e não da fluoresceína. O AAPH não reage diretamente com a amostra, de forma que o resultado obtido é a medida direta da capacidade da amostra em atuar como *chain-braking* (OU, HUANG, HAMPSCH-WOODILL *et al.*, 2002).

O ORAC é um dos métodos mais comuns para determinação da atividade de sequestro do radical peroxil (MAGALHÃES, SEGUNDO, REIS *et al.*, 2008), muito utilizado para avaliar a capacidade antioxidante do plasma, proteínas e antioxidantes extraídos de plantas e alimentos (DÁVALOS, GÓMEZ-CORDOVÉS & BARTOLOMÉ, 2004).

Dentre os outros métodos disponíveis, o ORAC é o que mais se aproxima das condições de oxirredução que ocorrem *in vivo* (OU, HUANG, HAMPSCH-WOODILL et al., 2002; SHAHIDI & ZHONG, 2015). Primeiro porque este método prevê uma competição entre fluoresceína e antioxidante pelo peróxido de hidrogênio, assim como ocorre em meio biológico, onde biomolécula e antioxidante também podem competir pela mesma espécie reativa (OU, HUANG, HAMPSCH-WOODILL *et al.*, 2002). Segundo porque a geração de peróxido de hidrogênio ocorre de forma contínua e numa velocidade semelhante a que ocorre em fluidos biológicos (SCHAICH, TIAN & XIE, 2015) e, por último, devido à semelhança entre as condições de pH e temperatura da reação com as do organismo (OU, HUANG, HAMPSCH-WOODILL *et al.*, 2002). No entanto, este ensaio não reflete de forma realista o que acontece *in vivo*, de forma que os valores de ORAC determinados para os alimentos não são suficientes para afirmar que eles possam exercer efeitos benefícios ao organismo e não devem ser utilizados como critérios de escolha para uma alimentação rica em antioxidantes (USDA, 2010).

Para um mesmo antioxidante, as reações de SET e HAT podem ocorrer em paralelo. Neste caso, o mecanismo dominante vai depender da estrutura do composto avaliado, do coeficiente de partição, da solubilidade e do solvente no qual ocorre a reação (SHAHIDI & ZHONG, 2015). Apesar de o resultado final ser o mesmo, a neutralização do radical e a formação de uma espécie oxidada estável, as diferentes condições de pH, temperatura e cinética dos métodos são muito diversas. A reação de transferência de elétrons é muito rápida quando comparada a de transferência de hidrogênio e aumenta com o aumento do pH, uma vez que a ionização nessas condições é facilitada. Por outro lado, o meio aquoso facilita a reação de transferência de hidrogênio, enquanto que meios alcoólicos atuam como ligantes de hidrogênio e podem dificultar a reação de oxirredução. Essas diferenças podem dificultar a avaliação da atividade redutora de um mesmo antioxidante por diferentes métodos e mecanismos ou quando presente em sistemas complexos (SCHAICH, TIAN & XIE, 2015).

3.4. Influência do complexo proteína:fenólico na capacidade antioxidante de proteínas e compostos fenólicos

As proteínas, assim como os lipídeos e carboidratos, podem interagir com compostos fenólicos formando complexos (BANDYOPADHYAY, GHOSH & GHOSH, 2012; JAKOBEK, 2015; ZHOU, SEO, ALLI *et al.*, 2015). A interação da proteína com os compostos fenólicos pode ocorrer nas plantas, principalmente nos tecidos onde predominam proteínas de armazenamento e enzimas; durante as etapas de processamento do alimento, com as proteínas do alimento; durante o processo digestório, entre proteínas dos alimentos ou enzimas digestivas; e entre proteínas do plasma sanguíneo, dos tecidos e órgãos do organismo quando os fenólicos são absorvidos pela mucosa intestinal (ROHN, 2014).

A formação do complexo pode ocorrer por meio de interações não covalentes e reversíveis, como as de van der Waals, as ligações de hidrogênio e as interações iônicas ou por ligações irreversíveis como as covalentes. Na maioria dos complexos predominam múltiplas interações hidrofóbicas estabilizadas por poucas ligações de hidrogênio e, mais raramente, as ligações covalentes (BANDYOPADHYAY, GHOSH & GHOSH, 2012; JAKOBEK, 2015; OLIVEIRA, ALEXANDRE, COELHO *et al.*, 2015; ZHOU, SEO, ALLI *et al.*, 2015).

As interações hidrofóbicas ocorrem principalmente entre o anel pirrolidina dos resíduos de prolina e os anéis aromáticos do composto fenólico. Em menor extensão, os aminoácidos com cadeias laterais hidrofóbicas, Phe e Trp, também são potenciais sítios de interação com os fenólicos (JÖBSTL, O'CONNELL, FAIRCLOUGH *et al.*, 2004; BANDYOPADHYAY, GHOSH & GHOSH, 2012). A preferência por regiões ricas em prolina se dá pelo fato de estes resíduos restringirem a mobilidade da estrutura proteica e estarem mais expostos aos solventes que os aminoácidos aromáticos, o que favorece a interação com os polifenóis (OH, HOFF, ARMSTRONG *et al.*, 1980; JÖBSTL, O'CONNELL, FAIRCLOUGH *et al.*, 2004). No entanto, essas interações hidrofóbicas, podem levar a alterações na estrutura tridimensional e na carga líquida da proteína, interferindo na sua solubilidade e atividade biológica. Além disso, se as interações envolverem aminoácidos essenciais como lisina e triptofano, pode ocorrer redução da biodisponibilidade destes resíduos e consequentemente perda de parte do valor biológico da proteína (JAKOBEK, 2015).

As ligações covalentes ocorrem entre o composto fenólico oxidado, chamado de quinona, com os centros nucleofílicos das proteínas, dentre eles os resíduos de lisina, o grupamento indol dos resíduos de triptofano e o grupamento tiol do resíduo de cisteína. Na

proteína, essas interações podem ter como consequência alterações na atividade fisiológica e nas propriedades físico-químicas, tecnológicas e funcionais como solubilidade, capacidade emulsificante e atividade antioxidante, respectivamente. Nos fenólicos, a ligação covalente pode comprometer sua atividade antioxidante e sua biodisponibilidade (OZDAL, CAPANOGLU & ALTAY, 2013; ROHN, 2014).

Os principais parâmetros que podem interferir na formação do complexo são tipo da proteína e do composto fenólico, a razão entre estes compostos e a massa molecular do fenólico. O tipo de proteína influencia na afinidade de ligação com os fenólicos. Esta afinidade varia de acordo com a composição aminoacídica da proteína, caráter hidrofóbico e estrutura tridimensional. A solubilidade do complexo formado sofre forte influência da razão proteína: fenólico. Quando maior a razão proteína:fenólico, maior será a probabilidade da formação de complexos insolúveis (BANDYOPADHYAY, GHOSH & GHOSH, 2012; OZDAL, CAPANOGLU & ALTAY, 2013).

Em relação aos fenólicos, quanto maior a massa molecular, a flexibilidade da estrutura e o número de hidroxilas da molécula, maiores serão a intensidade e quantidade de interações com a proteína, resultando na formação de complexos mais estáveis (OZDAL, CAPANOGLU & ALTAY, 2013; JAKOBEK, 2015). No complexo, os fenólicos atuam como ligantes multidentados, interagindo simultaneamente em várias regiões da superfície da proteína. Dessa forma, para que um fenólico tenha alta afinidade com a proteína ele deve ser pequeno o suficiente para penetrar na estrutura proteica e grande o suficiente para interagir em diversas regiões da mesma (OLIVEIRA, ALEXANDRE, COELHO *et al.*, 2015). A flexibilidade da estrutura do fenólico também influencia na capacidade de um mesmo fenólico formar complexos de igual intensidade com diferentes tipos de proteínas (OZDAL, CAPANOGLU & ALTAY, 2013; JAKOBEK, 2015)

Força iônica, pH e temperatura também influenciam, em menor extensão, a formação dos complexos, pois podem alterar a solubilidade da proteína e a quantidade e intensidade das interações entre os compostos. Na maioria dos casos, a redução do pH provoca a dissociação da proteína aumentando a quantidade de sítios disponíveis para interação com o fenólico e também favorece a formação de complexos insolúveis devido a proximidade com o pI das proteínas. O aumento da temperatura pode desnaturar a proteína e favorecer a interação com os fenólicos na superfície, mas, por outro lado, pode enfraquecer as interações hidrofóbicas prejudicando a formação do complexo (BANDYOPADHYAY, GHOSH & GHOSH, 2012; OZDAL, CAPANOGLU & ALTAY, 2013).

Inúmeros estudos tem sido feitos para avaliar como a formação do complexo proteína:fenólico pode interferir na capacidade antioxidante de ambas as classes de compostos, no entanto ainda não há um consenso (ZHANG, YU, SUN *et al.*, 2014; HERNÁNDEZ-JABALERA, CORTÉS-GIRALDO, DÁVILA-ORTÍZ *et al.*, 2015; OLIVEIRA, ALEXANDRE, COELHO *et al.*, 2015; ZHOU, SEO, ALLI *et al.*, 2015). Em relação à proteína, as interações com os fenólicos provocam alterações nas estruturas secundária e terciária, que parecem facilitar o acesso às espécies reativas, aumentando a capacidade antioxidante das mesmas (KANAKIS, HASNI, BOURASSA *et al.*, 2011). Por outro lado, o poder redutor dos fenólicos parece ficar comprometido, uma vez que as regiões responsáveis pela neutralização das espécies reativas são as mesmas que interagem com a proteína formando o complexo (BANDYOPADHYAY, GHOSH & GHOSH, 2012; OLIVEIRA, ALEXANDRE, COELHO *et al.*, 2015). Ao estudar a atividade antioxidante do chá preto puro e do chá preto com leite, Sharma e colaboradores (2008) mostraram que a formação do complexo proteína:fenólico pode estabilizar ou reduzir a atividade antioxidante da bebida de acordo com o mecanismo e método antioxidante avaliado. Em estudo com chás inglês e verde, Dubeau e colegas (2010) mostraram que a adição de leite pode mascarar parte da atividade antioxidante dos fenólicos. No entanto, essa redução pode ser compensada pela capacidade antioxidante exercida pelas proteínas. Arts e colaboradores (2002) mostraram que o tipo de proteína, as classes dos flavonoides e a matriz do alimento influenciam na capacidade do complexo em mascarar a atividade antioxidante do fenólico.

3.5. Influência da digestão *in vitro* sobre a bioatividade de proteínas e compostos fenólicos

Por meio da simulação das condições do trato gastrintestinal, é possível predizer como o processo digestório pode interferir na bioatividade de peptídeos e compostos fenólicos (BERMÚDEZ-SOTO, TOMÁS-BARBERÁN & GARCÍA-CONESA, 2007; SANNAVEERAPPA, WESTLUND, SANDBERG *et al.*, 2007; YOU, ZHAO, REGENSTEIN *et al.*, 2010). Os métodos de digestão *in vitro* se caracterizam por serem rápidos, seguros e sem restrições éticas como acontece em métodos *in vivo* (SANNAVEERAPPA, WESTLUND, SANDBERG *et al.*, 2007), sendo, por isso, bastante utilizados para estudar os possíveis efeitos da digestão na capacidade antioxidante de compostos fenólicos (BERMÚDEZ-SOTO, TOMÁS-BARBERÁN & GARCÍA-CONESA, 2007; TAGLIAZUCCHI, VERZELLONI, BERTOLINI *et al.*, 2010; HELAL, TAGLIAZUCCHI, VERZELLONI *et al.*, 2014), de hidrolisados proteicos de origem animal (MIGUEL, ALONSO, SALAICES *et al.*, 2007; MARTOS, CONTRERAS, MOLINA *et al.*, 2010; YOU, ZHAO, REGENSTEIN *et al.*, 2010; AO & LI, 2013), vegetal (SAMARANAYAKA, KITTS & LI-CHAN, 2010; YOU, ZHAO,

REGENSTEIN *et al.*, 2010; ORSINI DELGADO, NARDO, PAVLOVIC *et al.*, 2015) e do complexo proteína:fenólico (ŚWIECA, GAWLIK-DZIKI, DZIKI *et al.*, 2013; PLUNDRICH, WHITE, DEAN *et al.*, 2015).

Para os compostos fenólicos, o processo de digestão *in vitro*, envolvendo a ação de enzimas digestivas e sais biliares, pode promover a liberação tanto dos compostos que estão armazenados na célula vegetal como aqueles que estão ligados à outras macromoléculas (SAURA-CALIXTO, SERRANO et al., 2007). Segundo Tagliazucchi e colaboradores (2010), o trato gastrintestinal atua como um extrator de compostos fenólicos, seja por trituração, liberando o conteúdo de fenólicos presentes dentro das células e dos que estão fracamente ligados à parede celular, seja pela ação de enzimas e diferentes condições de pH. No estômago, essa extração ocorre principalmente devido ao pH ácido e atividade enzimática que podem induzir a liberação de compostos fenólicos ligados a proteínas e carboidratos presentes na matriz (SAURA-CALIXTO, SERRANO & GOÑI, 2007; CHEN, CHEN, CHEN *et al.*, 2016). As condições intestinais atuam de forma semelhante às estomacais, uma vez que a liberação dos fenólicos também é favorecida pela hidrólise dos componentes da matriz provocada pela ação das enzimas pancreáticas (BOUAYED, HOFFMANN & BOHN, 2011; CHEN, CHEN, CHEN *et al.*, 2016).

Assim, para se estudar as interferências do processo digestório na bioatividade dos compostos fenólicos, tem-se utilizado preferencialmente a matriz ou extratos do alimento ao invés do fenólico isolado. Isso porque a matriz, além de liberar fenólicos que estão ligados a outras macromoléculas, exerce um forte efeito protetor contra as condições adversas do trato gastrintestinal contribuindo para maior estabilidade dos fenólicos (NUNES, FERREIRA, FREITAS *et al.*, 2013; CHEN, CHEN, CHEN *et al.*, 2016).

A atividade antioxidante dos compostos fenólicos também sofre interferências ao longo do processo da digestão devido à presença de espécies reativas e às alterações de pH. O trato gastrintestinal é constantemente exposto às espécies reativas presentes no alimento, produzidas por células do sistema imune ativadas por bactérias e toxinas provenientes do bolo alimentar e àquelas resultantes da respiração celular. A presença de compostos fenólicos na luz intestinal pode contribuir para manutenção do epitélio ao reduzir as espécies reativas por meio da transferência de elétrons ou de átomos de hidrogênio e quelação de metais (BOUAYED, HOFFMANN & BOHN, 2011).

Em relação ao pH, a literatura mostra que, apesar de facilitar a extração dos fenólicos, ele pode potencializar ou reduzir a capacidade antioxidante destes compostos. Em estudo com fenólicos da maçã, verificou-se que a atividade antioxidante é mais intensa

no intestino do que no estômago, devido à desprotonação do grupo hidroxil dos anéis aromáticos provocada pela mudança de pH de ácido para levemente básico (BOUAYED, HOFFMANN & BOHN, 2011). Por outro lado, Bermudez-Soto e colaboradores (2007) mostraram que os fenólicos da aronia, um tipo de *berry*, são degradados pelo pH alcalino do intestino e, por isso, tem sua atividade antioxidante reduzida após a digestão *in vitro*.

Em relação às proteínas e aos peptídeos, a ação das enzimas gástricas e pancreáticas pode aumentar (SANNAVEERAPPA, WESTLUND et al., 2007; SAMARANAYAKA, KITTS et al., 2010), não alterar (SAMARANAYAKA, KITTS et al., 2010) ou mesmo reduzir sua capacidade antioxidante (MÖLLER, SCHOLZ-AHRENS et al., 2008). Durante o processo digestório, os peptídeos são degradados pelas enzimas favorecendo a formação de peptídeos de menor massa molecular. Isso pode provocar aumento ou redução da atividade antioxidante, visto que a capacidade redutora do peptídeo é máxima até um determinado GH. Ultrapassado esse valor ótimo de GH, a formação de peptídeos de baixa massa molecular e aminoácidos livres resultarão na redução da atividade antioxidante (SANNAVEERAPPA, WESTLUND et al., 2007; MÖLLER, SCHOLZ-AHRENS et al., 2008). Além disso, durante esse processo, proteínas e peptídeos são expostos a diversos agentes oxidantes provenientes do próprio alimento ou do processo digestório que podem comprometer a capacidade dos hidrolisados de atuarem como redutores (YOU, ZHAO et al., 2010).

A presença de compostos fenólicos na luz intestinal pode influenciar a ação das enzimas digestivas por meio da formação de complexos. A interação do fenólico com a enzima pode afetar o microambiente do sítio ativo, resultando em perda ou redução da sua atividade biológica e, consequentemente, comprometendo a hidrólise e absorção dos macronutrientes (BANDYOPADHYAY, GHOSH & GHOSH, 2012; JAKOBEK, 2015).

A presença do complexo proteína:fenólico no alimento pode ter consequências negativas ou positivas para proteínas e fenólicos ao longo do processo digestório. O aspecto negativo se deve à redução da digestibilidade proteica, uma vez que a interação com o fenólico pode dificultar o acesso das enzimas proteolíticas às regiões de clivagem da proteína. Soma-se a isso a possível redução na qualidade nutricional devido às interações envolvendo resíduos de aminoácidos essenciais. Por outro lado, o complexo pode ser benéfico na medida em que a proteína é capaz de atuar como um carreador de fenólicos, protegendo as regiões hidrofóbicas do ataque de espécies reativas presentes no trato gastrointestinal. Dessa forma, ao final da digestão, quando ocorre a hidrólise da proteína e consequente enfraquecimento do complexo, os fenólicos são liberados somente no intestino e podem atuar como antioxidantes protegendo a própria proteína e demais nutrientes dos

agentes oxidantes presentes na luz intestinal (BANDYOPADHYAY, GHOSH & GHOSH, 2012; JAKOBEK, 2015).

3.6. Doença Inflamatória Intestinal e estresse oxidativo

A inflamação constitui um complexo mecanismo de defesa do hospedeiro em resposta a uma infecção microbiana ou estímulo nocivo (KUNDU & SURH, 2012). A inflamação pode ser aguda, caracterizando-se por vasodilatação, infiltração de leucócitos, destruição de patógenos e reparação tecidual. Quando o estímulo não cessa, ela se torna crônica, caracterizando-se pelo desencadeamento de defesas específicas que resultam em degeneração progressiva do tecido e fibrose (DELL'AGLI, DI LORENZO, BADEA *et al.*, 2012; DI LORENZO, DELL'AGLI, BADEA *et al.*, 2012). A inflamação crônica também está envolvida na patogênese de doenças neurodegenerativas, cardiovasculares, inflamatórias intestinais (DII), câncer, diabetes, e obesidade (DI LORENZO, DELL'AGLI, BADEA *et al.*, 2012; KUNDU & SURH, 2012).

As DII compreendem um grupo de desordens correlacionadas e não totalmente distintas classificadas em sua maioria como Doença de Crohn (DC) ou Retocolite Ulcerativa (RCU) (LARMONIER, SHEHAB, GHISHAN *et al.*, 2015; MALIK, 2015). Caracterizam-se por um processo inflamatório crônico e intermitente resultante de uma resposta anormal e desregulada do sistema imune frente a antígenos da microbiota comensal intestinal (KRUIDENIER, KUIPER, LAMERS *et al.*, 2003; BIASI, LEONARDUZZI, OTEIZA *et al.*, 2013). Embora a etiologia não seja totalmente conhecida, seu desenvolvimento está relacionado com a suscetibilidade genética do indivíduo, a fatores relacionados à microbiota intestinal e ambientais como dieta, tabagismo, uso de contraceptivos orais e anti-inflamatórios não esteróides (BLUMBERG, 2009; BIASI, LEONARDUZZI, OTEIZA *et al.*, 2013).

A incidência de DII, dividida entre DC e RCU, é de aproximadamente 1,5 milhão nos Estados Unidos, 2 milhões na Europa (RUTHRUFF, 2007; MALIK, 2015) e tem crescido em todo mundo, inclusive em regiões que antes era incomum como China, Coréia do Sul e Porto Rico. Possivelmente o aumento da incidência se deve à modificação nos hábitos alimentares, se assemelhando à dieta ocidental, rica em gordura e proteína e pobre em frutas e vegetais (HOU, ABRAHAM & EL-SERAG, 2011). Embora a faixa etária mais comum seja entre 15 e 30 anos, essas doenças podem acometer indivíduos de qualquer idade, sendo que a RCU é mais comum em homens e a DC em mulheres (HANAUER, 2006).

A DC é uma inflamação transmural e segmentada, podendo se manifestar em qualquer região, da boca ao ânus, mas geralmente acomete o intestino delgado.

Histologicamente, ela é caracterizada pela presença de granulomas múltiplos e agregações de macrófagos (XAVIER & PODOLSKY, 2007). A RCU caracteriza-se por alterações superficiais na mucosa intestinal do cólon e do reto, observada histologicamente pela presença de neutrófilos na lâmina própria e micro abscessos nas criptas (RUTHRUFF, 2007; XAVIER & PODOLSKY, 2007).

Embora essa inflamação crônica resulte, num primeiro momento, de defeitos na resposta do sistema imune inato, a principal hipótese do desenvolvimento e progressão das DII é baseada na contínua ativação do sistema imune adaptativo contra antígenos presentes na luz intestinal (CORRIDONI, ARSENEAU & COMINELLI, 2014; GEREMIA, BIANCHERI, ALLAN *et al.*, 2014; DE SOUZA & FIOCCHI, 2016).

A mucosa do intestino é composta por uma complexa população de células do sistema imune que são capazes de reconhecer um antígeno e elaborar uma resposta imune ou de tolerância a ele (LARMONIER, SHEHAB, GHISHAN *et al.*, 2015). A resposta imune inata constitui a primeira linha de defesa contra patógenos. Diferentemente da resposta imune adaptativa, ela é inespecífica e não é capaz de gerar memória imunológica. Durante a defesa imune inata nas DII, macrófagos, células dendríticas, epiteliais e miofibroblastos são capazes de reconhecer patógenos na lúmen intestinal e promover uma resposta rápida e efetiva contra eles. Esse reconhecimento ocorre por meio da ligação entre os receptores de reconhecimento padrão (PRRs), presentes nas células responsáveis pela imunidade inata, e os padrões moleculares associados patógenos (PAMPs), presentes nos microrganismos potencialmente patogênicos (GEREMIA, BIANCHERI, ALLAN *et al.*, 2014). Os PRRs ativam a via do fator de transcrição kappa B (NF-κB), um dos moduladores chave no processo inflamatório da DII por estar intimamente relacionado ao desencadeamento, desenvolvimento e aceleração do processo inflamatório crônico (ATREYA, ATREYA & NEURATH, 2008).

O NF-κB é um dímero formado pelas proteínas p50 e p65, existente no citoplasma das células na forma inativa devido a sua ligação à proteína inibidora Iκβ. Em resposta ao estímulo pró-inflamatório e/ou ao estresse oxidativo, Iκβ é fosforilada em dois resíduos de serina, liberando o NF-κB. Esse dímero transloca-se para o núcleo celular e ativa genes responsáveis pela síntese de citocinas, quimiocinas, moléculas de adesão, ciclooxigenases 1 e 2 (COX-1 e COX-2), óxido nítrico sintase induzível (iNOS) (BOWIE & O'NEILL, 2000; NUNES, FERREIRA, FREITAS *et al.*, 2013). A secreção de TNF-α e IL-1, IL-6, IL-12 desencadeada pela ativação do NF-κB causa danos à matriz extracelular, degradação da mucosa e diferenciação de células do sistema imune da lâmina própria,

perpetuando a inflamação. Adicionalmente, a secreção de TNF-α retroalimenta a expressão e ativação do próprio NF-κB (ATREYA, ATREYA & NEURATH, 2008).

Paralelamente à ativação da via do NF-κB, os PRRs aumentam a expressão de moléculas co-estimulatórias em células dendríticas, necessárias à completa ativação de linfócitos T após a interação com o antígeno (GEREMIA, BIANCHERI, ALLAN *et al.*, 2014).

Na resposta imune adaptativa, os linfócitos T $CD4^+$ *naive* podem se diferenciar em T helper (Th)1, Th2, Th17, entre outros. As células Th1 são essenciais na eliminação de patógenos intracelulares, as Th2 protegem o organismo contra parasitas e modulam o processo alérgico, enquanto as Th17 podem contribuir na eliminação de fungos e bactérias presentes no meio extracelular. No entanto, uma resposta imune anormal provocada por estas células resulta em inflamação da mucosa provocada pela produção descontrolada de citocinas e quimiocinas (GEREMIA, BIANCHERI, ALLAN *et al.*, 2014)

Classicamente, uma resposta excessiva de células Th1 é associada com Doença de Crohn enquanto que a Retocolite ulcerativa é relacionada com uma resposta não convencional de linfócitos Th2. A elevada produção de IL-12 ativa as células Th1, com maior liberação de IFN-γ, TNF-α, IL-1β, IL-2 e IL-6. Por outro lado, maior secreção de IL-5 e IL-13 (HANAUER, 2006; DE SOUZA & FIOCCHI, 2016) e menor de IL-4, está associada uma resposta atípica das células Th2 (DE SOUZA & FIOCCHI, 2016).

Recentemente, alguns estudos têm questionado essa divisão entre os perfis Th1 e Th2 nas respostas imunes nas DII, após a descoberta da células Th17 produtoras de IL-17 e das interações entre células Th1, Th2, Th17 e T regulatórias (T_{reg}) na mucosa intestinal. As células Th17 originam-se da diferenciação de $TCD4^+$ *naive* na presença de IL-6 e TGF-β e secretam principalmente IL-17 e IL-22. Níveis elevados dessas citocinas foram encontrados nas mucosas de pacientes com ambas as formas de DII (DE SOUZA & FIOCCHI, 2016). T_{reg} são um tipo de células T $CD4^+$ que expressam o fator de transcrição *forkhead box protein* 3 (FOXP3) e a cadeia α do receptor de IL-2 ($CD25^+$). Elas são essenciais ao desenvolvimento de processo de tolerância a antígenos próprios e não próprios e à regulação da resposta imune. A ineficiência destas células em exercer sua ação imunossupressora tem sido relatada como um dos fatores envolvidos na progressão e manutenção da DII (DE SOUZA & FIOCCHI, 2016).

O estresse oxidativo é um componente fisiopatológico importante na DII. Isso se explica pelo aumento de subprodutos da peroxidação lipídica, pela redução da expressão de enzimas antioxidantes em animais com a doença e por estudos que sugerem que a suplementação com antioxidantes pode ter um efeito preventivo ou de retardo na

progressão da doença. De fato, o excesso de espécies reativas está intimamente envolvido na expressão e liberação de citocinas bem como do aumento da infiltração de células inflamatórias por estimular diversas vias de sinalização celular, dentre elas a do NF-κB (ZHU & LI, 2012).

A infiltração de neutrófilos e macrófagos ativados no tecido resulta em grande produção de superóxido e óxido nítrico a partir da NADPH oxidase e iNOS, respectivamente. A reação entre essas duas espécies forma peroxinitrito, um potente oxidante. Estudos em pacientes mostraram que essa massiva infiltração de neutrófilos e macrófagos no tecido resulta na produção de EROS, principalmente peróxido de hidrogênio, em quantidade superior a encontrada em células de indivíduos saudáveis. Paralelamente, ação do sistema de defesa antioxidante na DII está reduzida pela queda nos níveis de glutationa e zinco-superóxido dismutase (Zn-SOD) (ZHU & LI, 2012), pela redução na capacidade antioxidante total do soro e aumento da quantidade de glutationa oxidada (ROESSNER, KUESTER, MALFERTHEINER *et al.*, 2008).

Não há tratamento específico para a DII e a maioria dos medicamentos disponíveis (anti-inflamatórios, esteroides e imunosupressores) são pouco eficazes e podem apresentar efeitos adversos (NUNES, FERREIRA, FREITAS *et al.*, 2013). Por isso, a preocupação em desenvolver novas terapias eficazes e seguras para tratar DII. Nesse contexto, compostos com propriedades antioxidantes e anti-inflamatórias como polifenóis (ALGIERI, ZORRILLA, RODRIGUEZ-NOGALES *et al.*, 2013; FRONTELA-SASETA, LÓPEZ-NICOLÁS, GONZÁLEZ-BERMÚDEZ *et al.*, 2013; NUNES, FERREIRA, FREITAS *et al.*, 2013) e ácido α-linolênico (HASSAN, IBRAHIM, MBODJI *et al.*, 2010) têm sido investigados não só pela capacidade de neutralizar espécies reativas como pela possibilidade de modular reações em cascata de sinalização celular relacionadas à inflamação. Estudos *in vitro* mostraram que esses compostos apresentam efeitos inibitórios na ativação do NF-κB e, consequentemente, da produção de interleucinas, de COX-2, iNOS e NO (HASSAN, IBRAHIM, MBODJI *et al.*, 2010; FRONTELA-SASETA, LÓPEZ-NICOLÁS, GONZÁLEZ-BERMÚDEZ *et al.*, 2013; NUNES, FERREIRA, FREITAS *et al.*, 2013).

Estudos *in vivo* também sugerem que a suplementação da dieta com antioxidantes pode ter eficácia terapêutica (LIMA DE ALBUQUERQUE, COMALADA, CAMUESCO *et al.*, 2010; BALIGA, JOSEPH, VENKATARANGANNA *et al.*, 2012; BEHERA, MOHANTY, RAMANI *et al.*, 2012; MOURA, DE ANDRADE, DOS SANTOS *et al.*, 2015). Os possíveis mecanismos incluem a neutralização de espécies reativas, a supressão da expressão de citocinas pró-inflamatórias pela redução da expressão de enzimas e fatores de transcrição (SUNG & PARK, 2013). Entretanto, mais estudos ainda são necessários para

elucidar os mecanismos de ação, a quantidade ótima desses compostos, a interação dos mesmos com outros componentes da matriz alimentícia e sua estabilidade frente às condições intestinais.

3.6.1- Colite Experimental induzida pelo ácido 2,4,6-trinitrobenzenosulfônico (TNBS)

Uma das formas de se estudar a complexidade e a patogênese das DII é por meio da indução dessa doença em modelos experimentais animais (RANDHAWA, SINGH, SINGH *et al.*, 2014). Esses modelos, embora não mimetizem exatamente as características das DII humanas, são ferramentas indispensáveis para o estudo dos mecanismos envolvidos nas alterações morfológicas e histológicas que ocorrem no trato intestinal, bem como do desenvolvimento de novas estratégias terapêuticas (WIRTZ, NEUFERT, WEIGMANN *et al.*, 2007; RANDHAWA, SINGH, SINGH *et al.*, 2014).

As DII experimentais podem ser induzidas quimicamente ou por transferência de células do sistema imune, mas também podem ocorrer de forma espontânea em linhagens de animais modificados geneticamente. Os modelos de indução química são os mais utilizados por serem de fácil execução, por induzirem rapidamente a inflamação e apresentarem boa reprodutibilidade (WIRTZ, NEUFERT, WEIGMANN *et al.*, 2007; RANDHAWA, SINGH, SINGH *et al.*, 2014).

As substâncias comumente utilizadas para indução da colite experimental são sulfato sódico de dextrana (DSS), oxazolona e ácido 2,4,6 trinitrobenzeno sulfônico (TNBS) (WIRTZ, NEUFERT, WEIGMANN *et al.*, 2007). Ácido acético, drogas anti-inflamatórias não esteróides (NSAIDs), carragenana e polissacarídeo peptídeoglicano (PGPS) também podem ser utilizados (RANDHAWA, SINGH, SINGH *et al.*, 2014).

O modelo de indução de colite experimental por TNBS consiste na administração intra-retal do TNBS dissolvido em etanol. O processo inflamatório ocorre pela ação combinada dessas duas substâncias: O hapteno TNBS liga-se às proteínas da microbiota colônica por meio da porção trinitrofenil (TNP) e ativa o sistema imune, enquanto que o etanol promove a ruptura da barreira intestinal, alterando sua permeabilidade (WIRTZ, NEUFERT, WEIGMANN *et al.*, 2007).

O grau de severidade da colite induzida experimentalmente varia de acordo com a linhagem do animal, concentração e o número de doses de TNBS administradas. No protocolo clássico, uma única dose de administração de TNBS em animais BALB/c ou SJL/J induz uma inflamação aguda cujo padrão de resposta imune predominante é do tipo Th1 (VALATAS, BAMIAS & KOLIOS, 2015). Este padrão caracteriza-se pelo espessamento da parede intestinal, infiltração de linfócitos T $CD4^+$, neutrófilos e macrófagos (KIESLER, FUSS

& STROBER, 2015) e produção exacerbada de IFN-γ, TNF-α, IL-12 e IL-2. Nos animais, os sinais clínicos são diarreia severa, prolapso retal e perda progressiva de peso (WIRTZ, NEUFERT, WEIGMANN *et al.*, 2007). Essas características, semelhantes as da doença de Crohn em humanos, fazem da colite experimental um modelo amplamente utilizado para estudos pré-clínicos que investigam novas estratégias de tratamento dessa doença (WIRTZ, NEUFERT, WEIGMANN *et al.*, 2007; RANDHAWA, SINGH, SINGH *et al.*, 2014).

3.7. Atividade anti-inflamatória de peptídeos e compostos fenólicos

Peptídeos obtidos a partir de alimentos de origem vegetal e animal também podem apresentar atividade anti-inflamatória (HERNÁNDEZ-LEDESMA, HSIEH & DE LUMEN, 2009; LEE, KOVACS-NOLAN, ARCHBOLD *et al.*, 2009; UDENIGWE, LU, HAN *et al.*, 2009; VERNAZA, DIA, GONZALEZ DE MEJIA *et al.*, 2012; MILLÁN-LINARES, BERMÚDEZ, YUST *et al.*, 2014; MILLÁN-LINARES, MILLÁN, PEDROCHE *et al.*, 2015). Esta bioatividade está relacionada com a capacidade que os peptídeos apresentam de interferir na expressão de citocinas e quimiocinas inflamatórias e na regulação de fatores de transcrição (MAJUMDER, MINE & WU, 2015).

A maioria dos estudos que investigam o potencial anti-inflamatório de peptídeos é realizada *in vitro*, por meio da indução de mediadores inflamatórios com lipopolissacarídeo (LPS) e/ou IFN-γ em linhagens celulares de macrófagos como TPH1 ou RAW 264-7 (HERNÁNDEZ-LEDESMA, HSIEH & DE LUMEN, 2009; UDENIGWE, LU, HAN *et al.*, 2009; VERNAZA, DIA, GONZALEZ DE MEJIA *et al.*, 2012; MILLÁN-LINARES, BERMÚDEZ, YUST *et al.*, 2014; MILLÁN-LINARES, MILLÁN, PEDROCHE *et al.*, 2015). A lunasina, um peptídeo de 43 aminoácidos presente na soja, trigo, cevada e triticale, tem sido um dos mais estudados por seu potencial anti-inflamatório (DINELLI, BREGOLA, BOSI *et al.*, 2014). Foi demonstrado que este peptídeo possui efeito supressor na expressão de IL-6, TNF-α (HERNÁNDEZ-LEDESMA, HSIEH & DE LUMEN, 2009), IL-1β, iNOS e da produção de NO (DE MEJIA & DIA, 2009), além de inibir a ativação do NF-κB e reduzir a expressão da COX-2 (DE MEJIA & DIA, 2009; CAM & DE MEJIA, 2012). Outras fontes alimentares de peptídeos bioativos são o salmão, capaz de inibir a expressão de IL-6, TNF-α e IL-1β e de modular as vias da COX_2 e PGE_2 (AHN, JE & CHO, 2012), a soja, capaz de reduzir a expressão de TNF-α e a produção de NO (VERNAZA, DIA, GONZALEZ DE MEJIA *et al.*, 2012) e a caseína, cujos peptídeos atuam inibindo os fatores de transcrição NF-κB e PPAR-γ (MARCONE, HAUGHTON, SIMPSON *et al.*, 2015).

Embora alguns estudos identifiquem as sequências de aminoácidos responsáveis pela atividade anti-inflamatória (DE MEJIA & DIA, 2009; AHN, CHO & JE,

2015; MILLÁN-LINARES, MILLÁN, PEDROCHE *et al.*, 2015), ainda não há um consenso sobre quais são os fatores determinantes relativos à estrutura e composição da cadeia peptídica que expliquem essa bioatividade. De acordo com os estudos de De Mejia & Dia (2009) e de Lee e colaboradores (2009), a massa molecular do peptídeo pode interferir no seu potencial anti-inflamatório, uma vez que peptídeos menores apresentam maior facilidade para acessar o meio intracelular. Hernandez-Ledesma e colaboradores (2009) sugerem que a atividade anti-inflamatória está intimamente relacionada às estruturas primária, secundária e terciária do peptídeo e não somente à presença de algum aminoácido em específico. Majumder e colegas (2013) verificaram que a atividade anti-inflamatória de tripeptídeos da clara de ovo foi maior que a de seus aminoácidos constituintes na forma livre ou combinados em dipeptídeos, mostrando que atividade inflamatória é um reflexo da combinação e da sequência aminoacídica do peptídeo.

As informações sobre o potencial anti-inflamatório de peptídeos ainda são muito limitadas. Estudos *in vitro* e *in vivo* são necessários para elucidar as bases moleculares da ação anti-inflamatória, bem como à sua farmacocinética, dosagem e frequência de administração. Além disso, são necessários estudos envolvendo possíveis efeitos adversos, como a presença de epítopos, para evitar que estes peptídeos provoquem reações alérgicas nos usuários (CHAKRABARTI, JAHANDIDEH & WU, 2014).

Os compostos fenólicos também são conhecidos por apresentar atividades antitumoral, antiaterogênica e anti-inflamatória. A literatura mostra que essas atividades não são puramente um reflexo de sua atividade antioxidante e sugere que os fenólicos também são capazes de modular vias de sinalização celular durante o processo inflamatório, ou seja, eles mesmos os próprios agentes sinalizadores (RAHMAN, BISWAS & KIRKHAM, 2006).

A atividade anti-inflamatória dos fenólicos geralmente envolve a modulação de vias intracelulares, principalmente NF-κB e MAPK quinases, que regulam a expressão de citocinas pró-inflamatórias. A modulação do NF-κB pelos fenólicos pode ocorrer antes da ativação do fator, por meio da redução dos níveis de oxidante no meio e na regulação da atividade da enzima quinase IKK e, após a ativação, ao impedir que ele se ligue ao DNA. As MAPK quinases são uma família de serina/treonina quinases envolvidas na transdução de sinais de diversos processos celulares como proliferação, diferenciação e inflamação. Os compostos fenólicos, principalmente os flavonoides, possuem capacidade de modular diferentes etapas desta cascata de sinalização (TUNON, GARCIA-MEDIAVILLA, SANCHEZ-CAMPOS *et al.*, 2009).

Embora não tenha sido estabelecido uma relação de estrutura:função para explicar como essas vias são moduladas, os estudos sugerem que, nos flavonoides, a presença de dupla ligação entre o carbono C2 e C3 e a presença de hidroxilas nos anéis aromáticos A e B da molécula são determinantes na atividade anti-inflamatória destes compostos (TUNON, GARCIA-MEDIAVILLA, SANCHEZ-CAMPOS *et al.*, 2009). No ácido caféico, a presença do grupamento hidroxila na porção catecol, tem sido reportada como essencial para inibir a formação do complexo entre NF-κB e DNA. A inibição ocorre por meio da ligação da porção catecol com os grupamentos sulfidrila dos resíduos de cisteína da porção p65 (KAZŁOWSKA, HSU, HOU *et al.*, 2010).

3.8. Linhaça

A linhaça é uma semente oleaginosa, proveniente da planta linho (*Linum usitatissimum L*.) que tem sido consumida há cerca de cinco mil anos como um ingrediente alimentar ou nutracêutico em razão de suas propriedades medicinais (OOMAH, 2001).

É cultivada principalmente em regiões frias, como no nordeste do Canadá, principal região mundial produtora da linhaça. No Brasil, o cultivo de linhaça é feito principalmente no Rio Grande do Sul (SOARES, PACHECO, BRITO *et al.*, 2009). A produção brasileira é baixa, 21 toneladas por ano, enquanto que a da Argentina, maior produtora da América Latina, é de cerca de 80 toneladas por ano (ALMEIDA, BOAVENTURA & GUZMAN-SILVA, 2009; SOARES, PACHECO, BRITO *et al.*, 2009).

A composição da semente de linhaça é de aproximadamente 41% de lipídeos, sendo 70% deles poli-insaturados, 20% de proteínas, 28% de fibras totais, 7,7% de umidade e 3,4% de cinzas. Essa composição sofre variações de acordo com genética, meio ambiente, forma de processamento da semente e método de análise utilizado (MORRIS, 2007).

Na semente de linhaça, 56 a 70% do conteúdo proteico é encontrado nos cotilédones e os outros 30% na casca e no endosperma (RABETAFIKA, VAN REMOORTEL, DANTHINE *et al.*, 2011). Os principais tipos de proteína que constituem a linhaça são a 11-12S globulina e a 1,6-2S albumina. A fração de alta massa molecular é representada pelas globulinas composta por 5 subunidades de massas moleculares 50,9; 35,3; 30,0; 24,6; e 14,4 kDa. Duas delas são ácidas (35,3; 30,0 kDa) e uma delas é básica (24,6 kDa). As albuminas são os principais componentes da fração de baixa massa molecular, composta por uma cadeia polipeptídica com massa molecular entre 16 e 18 kDa. Outros tipos de proteínas que podem ser encontradas na linhaça são as oleosinas, com

massa molecular entre 16 a 24 kDa, de caráter lipofílico, encontrada entre os fosfolipídios; as proteínas ligadoras ao cádmio e proteínas antifúngicas (WANASUNDARA & SHAHIDI, 2003).

A composição aminoacídica é similar à da proteína de soja, que é considerada uma das proteínas vegetais de melhor qualidade (WANASUNDARA & SHAHIDI, 2003; MORRIS, 2007). A proteína de linhaça é uma excelente fonte de Arg, Gln e His, que têm conhecido poder de estimular o sistema imune; também é rica em Cys e Met que potencializam o sistema antioxidante do organismo, por meio da estabilização do DNA durante a divisão celular, e contribuem para redução do risco de certos tipos de câncer de cólon. Os aminoácidos limitantes são Lys, Thr e Tyr (WANASUNDARA & SHAHIDI, 2003).

Em relação às vitaminas, pode-se destacar a vitamina E, presente na linhaça na forma de γ tocoferol o qual é um potente antioxidante que protege as proteínas celulares e os lipídios da oxidação, diminuindo a pressão arterial e os riscos do desenvolvimento de alguns tipos de câncer, doenças cardiovasculares e doenças degenerativas como mal de Alzheimer (MORRIS, 2007)

Dentre os compostos fenólicos encontrados na linhaça, destacam-se no grupo das lignanas o SECO-2 [2,3-di- (metóxi-4hidróxi-benzil) butano 1,4 diol], que é encontrado conjugado a um diglicosídeo SDG (secoisolariciresinol diglicosídeo), o lariciresinol, isolariciresinol e o matairesinol (STRUIJS, VINCKEN, VERHOEF *et al.*, 2007); no grupo dos ácidos fenólicos dos ácidos fenólicos os ácidos ferúlico (10,9 mg/g), o clorogênico (7,5 mg/g), e o gálico (2,8 mg/g) e entre o grupos dos flavonoides a C-flavona e o O-glicosídeo (SINGH, MRIDULA, REHAL *et al.*, 2011). Esses ácidos podem estar presentes nas formas livre ou ligada, principalmente a um glicosídeo (JOHNSSON, PEERLKAMP, KAMAL-ELDIN *et al.*, 2002). As lignanas são compostos polares e, por isso, podem se solubilizar em água, porém, por serem substâncias orgânicas, se solubilizam melhor em misturas de solventes orgânicos polares com água (ZHANG, LI, WANG *et al.*, 2007). De acordo com estudos de otimização de Zhang e colaboradores (2007) o melhor rendimento de extração das lignanas da linhaça é em etanol a 70%. Quando ligados a um glicosídeo, o SDG, ácido cumárico e ácido ferúlico, são liberados mais facilmente quando a linhaça é submetida à hidrólise alcalina (JOHNSSON, PEERLKAMP, KAMAL-ELDIN *et al.*, 2002). Todos esses compostos se destacam pela sua capacidade antioxidante e consequente efeito benéfico em doenças como diabetes, hipertensão aterosclerose e, além disso, estão positivamente relacionados com a prevenção de câncer de mama e próstata (STRUIJS, VINCKEN, VERHOEF *et al.*, 2007; CHETANA, SUDHA, BEGUM *et al.*, 2010).

A maioria das pesquisas tem como objetivo o estudo dos benefícios da linhaça na saúde focando principalmente na ação do ALA, das lignanas, dos compostos fenólicos e das fibras solúveis, enquanto que as proteínas, presentes em quantidades expressivas, são pouco estudadas (MARAMBE, SHAND & WANASUNDARA, 2008; UDENIGWE, LU, HAN *et al.*, 2009). Marambe e colaboradores (2008) verificaram que a proteína de linhaça hidrolisada por *Flavourzyme*, apresentou atividade anti-hipertensiva e antioxidante, capaz de sequestrar o radical hidroxila e no estudo de Udenigwe e colaboradores (2009), onde a proteína de linhaça foi hidrolisada por sete enzimas (Alcalase, Pancreatina, Pepsina, Termolisina, Papaína, Tripsina e Ficina), foi observado que o hidrolisado possui atividades anti-inflamatória e antioxidante, capaz de sequestrar radical hidroxila e superóxido. Em outro estudo, Marambe e colaboradores (2011), após submeter a proteína a simulação da digestão gastrintestinal, observaram a liberação de peptídeos inibidores da enzima conversora de angiotensina (ECA). Em nosso estudo, observamos que a proteína de linhaça hidrolisada com Alcalase é capaz de neutralizar íons férricos e peróxido de hidrogênio e que a simulação do processo digestório não provocou alterações nesta capacidade (SILVA, O'CALLAGAHAN, O'BRIEN *et al.*, 2013).

Não foram encontrados na literatura estudos que verificassem, *in vitro* ou *in vivo*, o sinergismo entre hidrolisado proteico e compostos fenólicos de linhaça na atividade antioxidante e anti-inflamatória.

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CAPÍTULO 4. ARTIGO 1. Influence of protein-phenolic complex on antioxidant capacity of flaxseed (*Linum usitatissimum*) products

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Este manuscrito foi submetido para a revista Journal of Agricultural and Food Chemistry

Abstract

This study evaluated the influence of the naturally-present phenolic compounds and/or proteins on the antioxidant capacity of flaxseed products- phenolic fraction, protein isolates and hydrolysates- before and after simulated gastrointestinal digestion. A chromatographic characterization was performed and the antioxidant capacity was determined. Four glycosylated phenolic compounds – secoisolariciresinol and ferulic, p-coumaric and caffeic acids – were identified in flaxseed products. Phenolic fraction exert the highest antioxidant capacity that increased by alkaline treatment and simulated gastrointestinal digestion. The action of Alcalase and digestive enzymes resulted in an increase of antioxidant capacity of whole and phenolic reduced protein samples. Protein/peptide-phenolic complexation, confirmed by fluorescence spectra, exerted a positive effect on antioxidant capacity, mainly in protein isolates. Principal component analysis showed that the preferential mechanism that proteinaceous samples with reduced phenolic compounds act as antioxidant is by H+ transfer, while the proteinaceous samples with phenolic compounds is similarly by electron donation and H+ transfer mechanisms.

Keywords

Peptides, phenolic compounds, protein: phenolic complex, bioactivity, simulated gastrointestinal digestion

4.1. Introduction

Several studies have shown the antioxidant potential of peptides released from enzymatic hydrolysis of different protein sources.[1-3] This source of antioxidants has attractive characteristics for the food industry, once it is non-toxic and has recognized nutritional value.[1] In food industry, these peptides can be an alternative to the use of synthetic antioxidants, preventing lipid peroxidation and maintaining the sensory characteristics of the products.[1] In the human body, they may assist the antioxidant defense system in the prevention or deceleration of the progression of diseases involving oxidative stress.[4,5]

It has been reported that procedures used to obtain protein plant protein isolates can also facilitate the extraction of polyphenols.[6,7] After enzymatic hydrolysis of isolates, both released peptides and phenolic compounds might be responsible for the antioxidant activity of hydrolysates.[1,8] In this respect, the antioxidant activity of plant-derived matrices has been associated with protein components, phenolic compounds and their complexes, although few studies on protein hydrolysates have considered the presence and contribution of phenolic compounds.[9]

Flaxseed is an oilseed widely studied for its beneficial health effects. It is a source of alpha linolenic fatty acids, phenolic compounds and soluble fiber, has anti-inflammatory and antioxidant capacities, and has been related to reduced risk of chronic diseases such as cancer, obesity, and diabetes.[10,11] Flaxseed is the richest source of plant lignans, due to its high content of secoisolariciresinol diglucoside (SDG).[12] In this seed, SDG along with non-lignan phenolic compounds, such as ferulic, p-coumaric and caffeic acids, are constituents of an oligomeric structure called lignan macromolecule.[13,14] These phenolic compounds have phytoestrogenic and antioxidant properties[12,14] and have potential health benefits.[15]

A previous study carried out in our laboratory with flaxseed protein isolates containing phenolic compounds have shown that simulated gastrointestinal digestion was equal or more effective than Alcalase hydrolysis to obtain antioxidant hydrolysates.[16] However, the specific contribution of phenolic compounds and peptides on the antioxidant capacity of flaxseed protein hydrolysates, as well as on other plant-derived hydrolysates, is not fully elucidated. Therefore, the aim of the present study was to evaluate the influence of naturally-present phenolic compounds and their complexes with proteins on the antioxidant potential of flaxseed products before and after simulated gastrointestinal digestion.

4.2. Materials and methods

4.2.1. Materials and reagents

Partially defatted brown flaxseed meal (FM) was obtained from Cisbra Ltd. (Panambi, RS, Brazil). Alcalase 2.4 L, pepsin, pancreatin, bile salts, Folin-Ciocalteu reagent, gallic acid, [(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid] (Trolox), 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ), 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH), sodium fluorescein, secoisolariciresinol, caffeic, p-coumaric and ferulic acids were purchased from Sigma (St. Louis, MO, USA). Trifluoroacetic acid (TFA) and acetonitrile were purchased from Merck (Hohenbrunn, Germany). All other chemicals and reagents were of analytical grade.

4.2.2. Preparation of defatted flours, protein isolates, protein hydrolysates, and phenolic fraction from flaxseed

To obtain the defatted flaxseed meal (DFM), FM was defatted with hexane in a ratio of 1:3 (w/v) for 24 h at room temperature. For polyphenols extraction, three consecutive steps with 63% ethanol solution (v/v) were performed.[13] Two first steps were performed for 4 h under stirring and room temperature, whereas the last one was made overnight. After each extraction phase, DFM was centrifuged at 2500 x g for 30 min, and filtered obtaining the phenolic-reduced DFM (phr-DFM) and the phenolic isolate (Phi).

Flaxseed protein isolate (FPI) and phenolic-reduced FPI (phr-FPI) were prepared from DFM and phr-DFM, respectively, following Dev & Quensel protocol.[17] The corresponding source sample was dispersed in deionized water at a product:water ratio of 1:10 (w/w), and after adjusting its pH to 9.0 with 0.5 M NaOH, the solution was stirred at room temperature for 30 min and centrifuged (2500 x g/30 min; 25 °C). The supernatant containing protein was filtered and its pH adjusted to 4.2 with 0.5 M HCl. The precipitated protein was then separated by centrifugation (2500 x g/30 min), washed three times with acidified water (pH 4.2), and suspended in deionized water adjusting its pH to 6.0 with 0.5 M NaOH. Flaxseed products were freeze-dried and stored at -20 °C until their use.

The hydrolysis of the FPI and phr-FPI was performed with Alcalase under the following conditions: protein concentration of 5% (w/v), 60 ° C, pH 8.5, and enzyme substrate ratio 1:90 (w/w). The hydrolysis reaction was monitored using the pH-stat method using an automatic titrator DL model Metler 21 (Schwerzenbach, Switzerland) with a stirring system coupled to a thermostatic bath. After 180 min, the reaction was stopped by heating at 90 °C for 10 min. Then, the pH of the hydrolysates was adjusted to 6.0, and they were freeze-dried, and stored at -20 °C. The degree of hydrolysis (% DH) of flaxseed protein hydrolysate (FPH)

and phenolic reduced flaxseed protein hydrolysate (phr-FPH) obtained from FPI and phr-FPI, respectively, was calculated according to the equation described by Adler Nissen.[18]

To assess whether the conditions for obtaining the protein hydrolysate may change the phenolic compounds present in the FPI, Phi (5% w/v) was subjected to the same conditions of temperature and time of the hydrolysis reaction, but without addition of Alcalase to obtain the phenolics hydrolysate (Phh). A flow chart of the preparation of flaxseed protein products and phenolic fraction is shown in Figure 4.1.

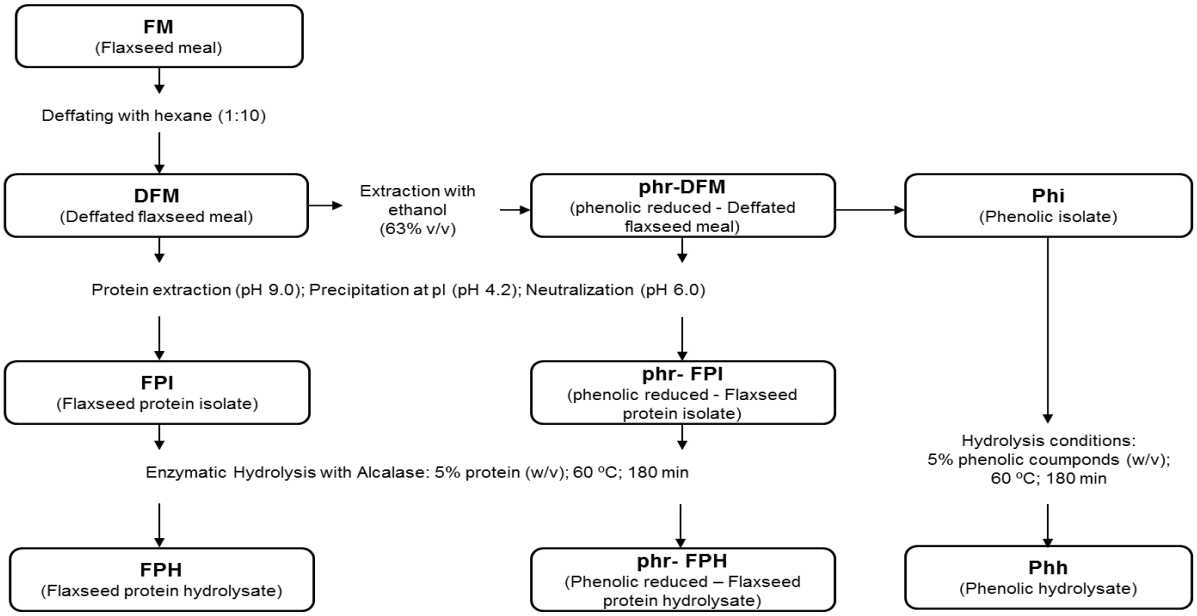

Figura 4.1. Preparation of flaxseed protein and phenolics products

4.2.3. Chemical composition

The chemical composition of flaxseed products was determined according to AOAC procedures.[19] Protein and lipid contents were determined according to Kjeldahl (N x 6.25)[19], and Bligh & Dyer[20] methods, respectively.

4.2.4. Characterization of samples by High-Performance Liquid Chromatography (RP-HPLC)

The chromatographic analysis (RP-HPLC) of flaxseed products was carried out using a high-performance liquid chromatography system with an automatic injector and a diode-array absorbance detector (Agilent, 1200 Series, Snoqualmie, WA, USA). Separation was carried out onto a Luna C18 column (250 mm x 4.6 mm, Phenomenex, Torrance, CA, USA) at a flow rate of 1 mL min^{-1}. The mobile phase was constituted by solvent A (0.04% TFA in water) and solvent B (0.03% TFA in acetonitrile). The gradient was from 0 to 80% of solvent

B over 40 min. The absorbance was measured at 214 and 280 nm. The samples were filtered through a 45-µm membrane, and 50 µL were injected. Sample concentration of flaxseed flours, isolates and hydrolysates was adjusted to 8 mg mL^{-1}. In the case of digested samples, concentration was adjusted to 4 mg mL^{-1}. The Star Chromatography Workstation software (Agilent) was used to record and process data.

4.2.5. Identification of phenolic compounds in hydrolysates and phenolic fraction by UPLC-MS/MS

The identification of ferulic, *p*-coumaric and caffeic acids and SDG was carried out using a UPLC-Q-Tof system comprised of an AcquityTM UPLC system coupled to a XevoTM G2-XS Q-Tof (Waters Corp., Milford, MA, USA), with an electrospray source ionization (ESI) in negative mode. Solutions containing the four standard compounds were freshly prepared and analyzed following the same procedure applied to the samples. Matching retention time, exact mass and fragmentation spectrum confirmed the presence of those compounds in the samples. In order to determine the presence of their glycosylated form, exact masses were screened in the LC-MS chromatograms and in cases peaks were observed, their fragmentation spectra were used for identity confirmation. The instrument control and data processing were performed by MassLynx software (Waters Corp.) version 4.1. Samples were analyzed in MSE mode, in which precursor and fragment information are collected from the same analysis. For the MS operating conditions the following parameters were set: capillary voltage 2.5 kV, cone voltage 30 V, source temperature 150 °C, desolvation temperature 550 °C, cone gas flow 50 L h^{-1} and desolvation gas flow 900 L h^{-1}. The instrument was previously calibrated with a sodium formate solution, and all runs were acquired with real time lockspray correction for mass accuracy (deprotonated rutin ion, mass/charge (m/z) 609.1456). Spectra were acquired every 0.1 s, on a m/z range of 100-1200. High energy spectra were acquired from m/z 50-1200 using a collision energy ramp from 20-30 eV.

The chromatographic separation was carried out on a BEH C18 column (50 mm × 2.1 mm × 1.7 µm) (Waters Corp.). Mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The gradient program was as follows: from 0-4 min B was ramped from 5-95%, followed by a 0.5 min ramp to 95% B. Immediately after that, mobile phase composition was restored to initial conditions for 0.5 min. Flow rate was set to 0.6 mL min^{-1}, the injection volume was 5 µL, and the column oven and sample manager were kept at 45 °C and 10 °C respectively. Each standard or sample was properly diluted in ultrapure water, and filtered through 0.45 µm polytetrafluoroethylene membrane before being injected onto the system.

4.2.6. Analysis of protein–phenolic complex by fluorescence spectroscopy

The intrinsic fluorescence analysis was performed to study the formation of the protein-phenolic complex among proteins/peptides and polyphenols according to Kanakis, et al. [21] with some modifications. FPI, phr-FPI, FPH and phr-FPH were dispersed in aqueous solutions at a protein concentration of 4 mg mL^{-1}. The fluorescence spectra of the Phi and Phh (4 mg sample mL^{-1}) were also recorded. All solutions were prepared at 24.0 ± 1 °C and kept in the dark. Fluorescence spectra were recorded at λ_{exc} = 280 nm and λ_{emi} from 290 to 500 nm, and data were acquired using an ISS PC1 Fluorimeter (Champaign, IL, USA).

4.2.7. Simulated gastrointestinal digestion

Simulated gastrointestinal digestion was performed as reported by Martos et al.[22] with modifications. The samples were dispersed in gastric juice (35 mM NaCl), and the pH was adjusted to 2.0 with 1 M HCl, thus obtaining a concentration of 5.9 mg protein mL^{-1} (DFM, phr-DFM, FPI, phr-FPI, FPH, phr-FPH) and 1.2 mg phenolic compounds mL^{-1} (Phi and Phh). Mixtures were left in a water bath at 3 °C for 15 min under constant stirring. Then, pepsin was added (E:S 1:20, w/w), and the pH was again adjusted to 2.0. The mixture was left in a thermostatic bath at 37 °C for 60 min under stirring. At the end of gastric phase, the pH of samples was adjusted to 6.8 with 1 M $NaHCO_3$, 1 M $CaCl_2$, and 9 mg mL^{-1} bile salt and they were maintained in a water bath at 37 °C for 15 minutes under stirring. Then, pancreatin was added (E:S 1:10, w/w), the pH was adjusted to 6.8 with 1 M $NaHCO_3$, and the volume was made up to 4 mL with deionized water. The intestinal digestion was carried out at 37 °C for 60 minutes under stirring. To stop the reaction, the digest was heated at 90 °C for 10 min under stirring and centrifuged (11000 x g) for 15 min. The supernatants (digests) were frozen and kept at -20 °C until further use.

4.2.8. Antioxidant capacity

The antioxidant capacity of the samples before and after simulated digestion was measured in the aqueous extracts. To obtain the extracts, lyophilized non digested samples (1% w/v) and digested samples (3% v/v) were suspended in deionized water, shaken for 30 min, centrifuged at 36000 x *g* for 30 min at 10°C, filtered through Nº1 Whatman qualitative filter paper, and stored at -20 °C in dark until use. All the antioxidant capacity assays were carried out using a Synergy™ HT Multi-Mode Microplate Reader (Biotek®, Winooski, VT, USA).

4.2.8.1. Folin-Ciocalteau reagent reducing substances (FCRRS)

The procedure was carried out according to Medina.[23] Briefly, 450 µL of deionized water and 50 µL of appropriately diluted samples, gallic acid standard solutions (0, 50, 100, 200, 300, 400, 500, 600 µg mL^{-1}) or deionized water for blank were added and mixed. The Folin-Ciocalteu reagent (50 µL) was added, mixed, and allowed to react for 5 min. Then, 500 µL of 7% Na_2CO_3 and 200 µL of deionized water were added and mixed. The mixture was left to react at room temperature in the dark for 90 min. The absorbance was measured at 760 nm and the results were expressed as mg gallic acid equivalent (GAE) per gram of sample (mg GAE g^{-1} sample).

4.2.8.2. Ferric reducing antioxidant power (FRAP)

The FRAP assay was carried out according to Benzie & Strain [24] with modifications. In the dark, 30 µL of sample extract, standard or blank was mixed with 90 µL of water and 900 µL of the FRAP reagent (450 µL of 0.3 M acetate buffer, pH 3.6, 225 µL of 10 mmol TPTZ in 40 mmol HCl and 225 µL of 20 mmol $FeCl_3$). The mixture was incubated at 30 °C for 30 min. The absorbance was measured at 595 nm and the results were expressed as µmol of Trolox equivalent (TE) per gram of sample (µmol TE g^{-1} sample).

4.2.8.3. Oxygen radical absorbance capacity (ORAC)

ORAC assay was performed according to Davalos et al.[25]. Briefly, 20 µL of sample extract and 120 µL of sodium fluorescein in potassium phosphate buffer (pH 7.4) (final concentration 0.378 µg mL^{-1}) were mixed in water with 60 µL of AAPH (final concentration 108 mg mL^{-1}). Potassium phosphate buffer was used as a blank. Trolox solutions (25-500 mM) were used as standard. Fluorescence was measured every minute for 80 min with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The antioxidant capacity was expressed as µmol TE g^{-1} sample, based on the area under the curve (AUC) for the decline in the fluorescence time.

4.2.8.4. Metal chelation activity

Fe^{2+}-chelating activity was determined by measuring the formation of the Fe^{2+}-ferrozine complex according to Carter [26] with adaptations. Samples were diluted (0.4-6.4 mg mL^{-1}) in 100 mM sodium acetate buffer (pH 4.9), stirred for 30 min and centrifuged at 27821 x *g*. Sample solution (250 µL) was mixed with 30 µL $FeCl_2$ (50 µg mL^{-1}), and incubated for 30 min at room temperature. Then, ferrozine (12.5 µL, 40 mM) was added. Ethylenediamine tetraacetic acid (EDTA) was used as a positive control at the same concentration used for

samples. The chromophore formed by binding of Fe^{2+} ions to ferrozine was measured at 562 nm. Iron chelating activity was calculated using the equation 1:

% Chelating Activity = $[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$ (eq.1)

4.2.9. Statistical Analysis

Data were presented as means and standard deviations. All antioxidant assays were carried out in triplicate. A paired t-test was performed to determine the difference between the FCRRS content and the antioxidant capacity before and after simulated gastrointestinal digestion. The FCRRS content and antioxidant capacity of all samples were analyzed by ANOVA followed by the Tukey´s test. The statistical analyses were carried out using the software SPSS 15.0 (SPSS Inc, Chicago, IL, USA). Principal component analysis (PCA) was performed to understand how the phenolic compounds and the digestive process can influence the antioxidant behavior of the samples. The FCRRS, and FRAP and ORAC values were considered as independent variables, while flours, isolates, and hydrolysates were the dependent variables. Data were autoscaled and analyzed using Pirouette Statistics version 3.11.

4.3. Results and Discussion

4.3.1. Chemical characterization of flaxseed products

The chemical composition (proteins, moisture, ash, lipids, and dietary fiber) of flaxseed products is shown in Table 4.1.

The basis product of this study (FM) contained 29.3% protein, 8.4% moisture, 4.2% ash, 14.9% lipids, and 35.1% dietary fiber. Defatting FM resulted in a reduction of lipids content and a slight increase in both proteins and dietary fiber. DFM was subjected to ethanol treatment in order to remove polyphenols. This process slightly increased the protein content of phr-DFM (from 33.4 to 35.7%), as well as of its moisture and fiber levels (Table 1). The resulting product, Phi, containing extracted polyphenols also contained a little amount of protein (5.21%), indicating that ethanol extraction also extracted some flaxseed protein from defatted meal. Protein extraction allowed obtaining two products, FPI and phr-FPI which protein contents were 73.9 and 82.1%, respectively, 2.2- and 2.3-times higher than those obtained for their source products, DFM and phr-DFM. However, these values were lower than that reported in the literature.[27] This fact could be due to the extraction method employed that did not include seed coat removal previous to the defatting procedure. The

levels of other components of FPI decreased after protein extraction, and they were similar to those reported in our previous study.[16]

FPI and phr-FPI were hydrolysed by Alcalase for 180 min. The DH for both products was 18.26 ± 0.2 and 17.20 ± 0.7%, respectively, with no significant differences between them (p <0.05). These results suggest that the phenolic fraction present in protein isolate did not interfere on Alcalase activity.

Table 4.1. Chemical composition (%) of flours, isolates and hydrolysates

Sample	Protein*	Moisture*	Ash*	Lipids*	Dietary Fiber
FM	29.3 ± 0.38	8.4 ± 0.00	4.2 ± 0.00	14.9 ± 0.87	35.1 ± 0.31
DFM	33.4 ± 0.94	8.2 ± 0.16	4.5 ± 0.03	6.8 ± 0.17	38.8 ± 0.32
phr-DFM	35.7 ± 0.01	11.9 ± 0.31	4.4 ± 0.04	6.4 ± 0.06	40.73 ± 0.49
FPI	73.9 ± 1.00	3.8 ± 0.12	1.6 ± 0.05	4.7 ± 0.17	13.0 ± 0.29
phr-FPI	82.1 ± 1.04	3.1 ± 0.22	1.6 ± 0.08	1.1 ± 0.05	14.91 ± 0.11
FPH	67.9 ± 0.22	ND	ND	ND	11.94 ± 0.27
phr-FPH	75.2 ± 0.31	ND	ND	ND	13.67 ± 0.08
Phi	5.21 ± 0.17	ND	ND	ND	ND
Phh	4.77 ± 0.13	ND	ND	ND	ND

*mean ± standard deviation of three replicates. FM: Flaxseed Meal; Defatted flaxseed meal (DFM); phenolic reduced defatted flaxseed meal (phr-DFM); Flaxseed protein isolate (FPI); phenolic reduced flaxseed protein isolate (phr-FPI); Flaxseed protein hydrolysate (FPH); phenolic reduced flaxseed protein hydrolysate (phr-FPH); Phenolic isolate (Phi) and Phenolic hydrolysate (Phh). ND: Not determined.

4.3.2. Chromatographic analysis of flaxseed products: effect of enzymatic hydrolysis

The chromatographic profiles of flaxseed products before and after Alcalase hydrolysis are shown in Figure 4.2. The chromatograms were obtained at 214 and 280 nm because these two wavelengths allow detecting both proteins/peptides and phenolic compounds.[28,29] The 214 nm-chromatogram of FPI (Figure 4.2A) showed numerous peaks eluting between 8 and 20 minutes which intensity was notably lower in phr-FPI and Phi products (Figure 4.2B and 4.2C). The intensity of a peak eluted at 15 minutes and detected at both 214 and 280 nm was much higher in the Phi than in FPI chromatogram, suggesting that it might correspond to a phenolic compound extracted with ethanol, as it was not observed in phr-FPI sample. Similarly, other components of lignan macromolecule present in FPI and Phi could elute as a wide peak with retention time between 20 and 23 minutes. This peak was also visible in the chromatographic profile of DFM product (data not shown). A previous analysis of flaxseed lignan macromolecule by size exclusion high performance liquid chromatography with diode array detection, had also described the elution of a wide peak at 280 nm.[14] The authors suggested that the heterogeneity of lignan macromolecule

could be responsible for this result, considering the sample as a mixture of molecules with similar molecular weight but a slightly different composition of individual phenolic compounds. According to Johnsson, et al.[30], and Struijs, et al.[31] SDG ester linked to hydroxymethyl-glutaric acid forms the backbone of the lignan macromolecule that is also comprised of the hydroxycinnamic acids, *p*-coumaric acid glucoside, and ferulic acid glucoside.

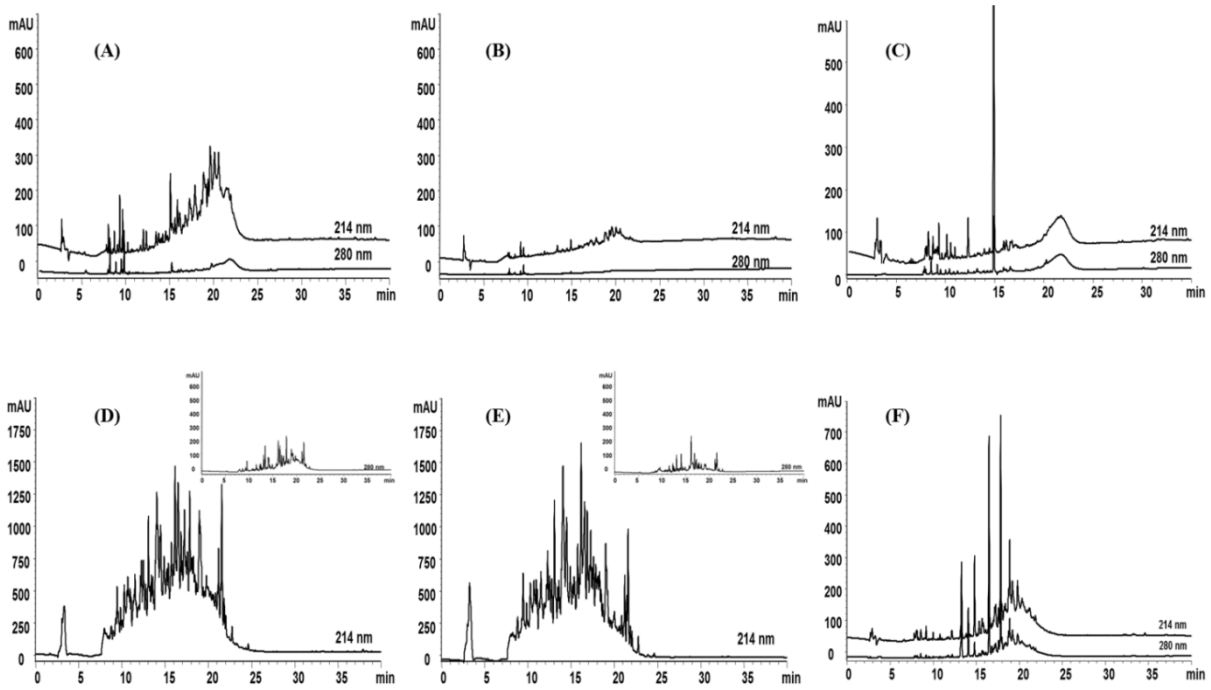

Figure 4.2. Chromatogram profiles (RP-HPLC) of flaxseed phenolic compounds and proteinaceus samples before simulated gastrointestinal digestion. Phenolic compounds isolate (Phi) (A). Phenolic compound hydrolysate (Phh) (B). Deffated flaxseed meal (DFM) (C) and phenolic reduced phr-DFM (D); Flaxseed protein isolate (FPI) (E) and phenolic reduced FPI (F); Flaxseed protein hydrolysate (FPH) recorded at 214 nm and (G) and reduced phenolic FPH recorded at 214 nm. The insert shows chromatograms profiles recorded at 280 and 320 nm for the FPH (G) and phr-FPH (H).

After hydrolysis with Alcalase, the chromatographic profiles of flaxseed products drastically changed in both appearance and intensity of eluted peaks. This was due to the release of peptides after the action of the microbial enzyme on flaxseed proteins. Profiles of FPH and phr-FPH (Figure 4.2D and 4.2E) were similar, indicating that phenolic compounds accompanying proteins in FPI did not affect Alcalase activity, as it had been observed measuring the DH. Notable changes were also observed for Phh compared with Phi that could be due to modifications in phenolic compounds resulting from partial hydrolysis of lignan macromolecule under conditions (pH 8.5 and 60 °C) used to simulate enzymatic hydrolysis.

In order to identify phenolic compounds, FPH, phr-FPH, Phi, and Phh products were subjected to UPLC-MS/MS analysis, injecting standards under the same experimental conditions. Figure 4.3A-4.3D shows the extracted ion chromatogram (EIC) of four phenolic compounds identified, ferulic, *p*-coumaric and caffeic acids, and SDG. Presence of these four compounds was confirmed in all samples except phr-FPH which is produced from a flaxseed product free of phenolic compounds. In the case of ferulic and *p*-coumaric acids (Figure 4.3A and 4.3B), more than one peak could be observed. Analysis of the mass spectra indicates that these peaks corresponded to modified versions of the phenolic compounds, mainly glycosylated forms, which fragment upon ionization conditions generated the same ions. These results strongly suggest that modified phenolic compounds are belonging to lignan macromolecule. According to Li, Yuan, Xu, Wang and Liu [15] phenolic compounds in lignan macromolecule are firstly esterified by ethanol and subsequently hydrolysed by alkali to produce SDG and other phenolic glycosides. These phenolic glycosides and SDG were more clearly detected in Phh.

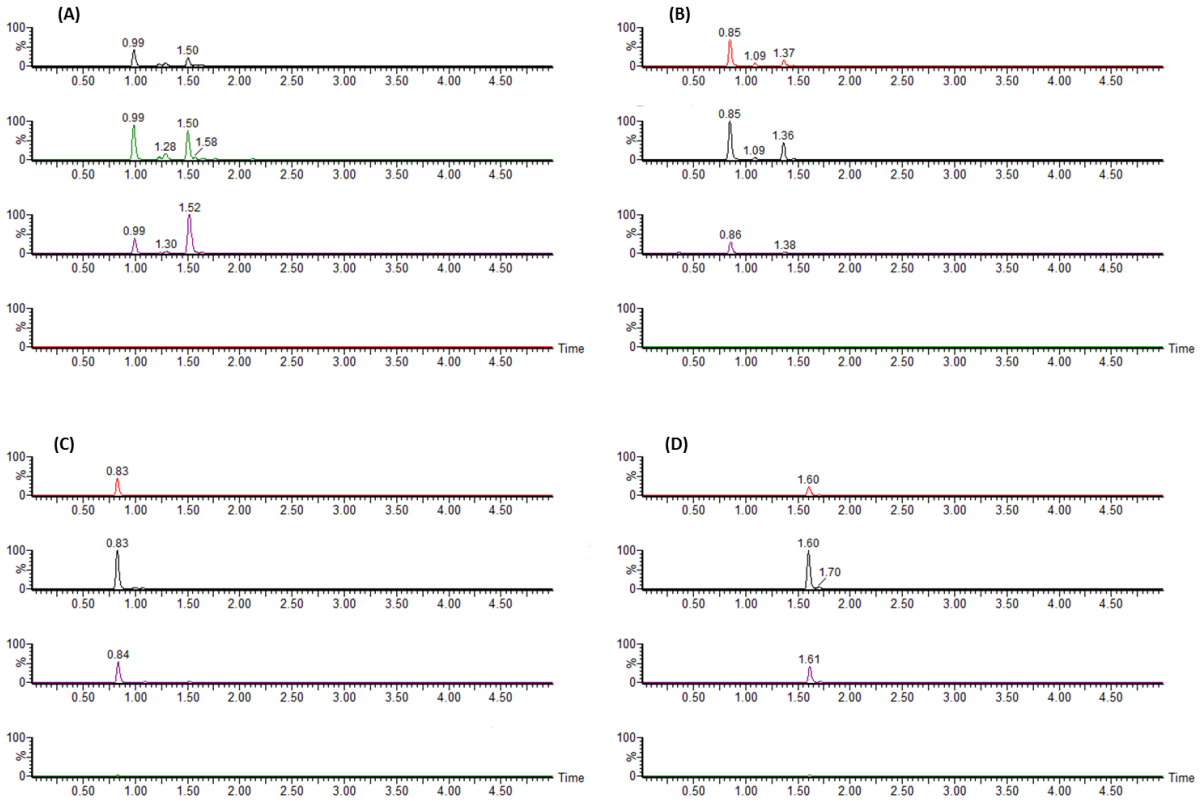

Figure 4.3. Extracted ion chromatograms (EICs) for ferulic acid (A, m/z 193.0501), p-coumaric acid (B, m/z 163.0395), caffeic acid (C, m/z 179.0344) and SDG (D, m/z 685.2708). From bottom to up, EICs correspond to reduced phenolic Flaxseed protein hydrolysate (phr-FPH), Flaxseed protein hydrolysate (FPH), Phenolic compounds hydrolysate (Phh) and Phenolic compounds isolate (Phi).

4.3.3. Protein-phenolic and peptide-phenolic complex

Once confirmed the presence of phenolic compounds in flaxseed products, potential interactions and the subsequent formation of complexes between proteins/peptides and polyphenols were investigated by fluorescence spectroscopy. Emission spectra from 290 to 500 nm of FPI, phr-FPI and Phi are shown in Figure 4.4A. Figure 4.4B shows spectra corresponding to FPH, phr-FPH and Phh samples.

The fluorescence of folded proteins is a result of the fluorescence from individual aromatic residues. Most of the intrinsic fluorescence emissions are due to excitation of Trp residues, with some emissions due to Tyr and Phe. Trp has an emission peak ranging from 308 to 350 nm depending on the local environment and the degree of solvent exposure of the chromophore.[32] In our study, the highest fluorescence intensity was measured for phr-FPI and phr-FPI products which did not contain phenolic compounds. However, presence of these components in FPI and FPH resulted in a notable reduction (~50%) of fluorescence intensity as well as a shift in the maximum emission peak (from 348 nm in FPI to 356 nm in phr-FPI, and from 358 nm in FPH to 360 nm in phr-FPH). This reduction could be due to modifications in protein/peptide chains caused by the formation of protein/peptide complexes with phenolic compounds in FPI and FPH products. Kanakis et al.[21] had reported that when proteins interact with other molecules, Trp fluorescence changes depending on the impact of the interaction on the spatial conformation of the protein structure. A negligible emission was observed for Phi and Phh samples (Figure 4.4A and 4.4B), which chemical characterization had demonstrated very low protein content.

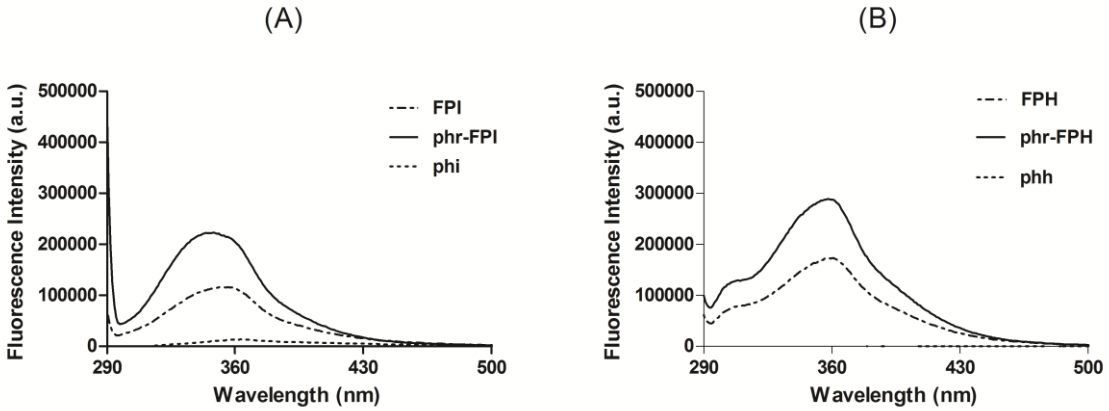

Figure 4.4. Fluorescence emission spectra. (A) protein isolates with phenolics and reduced phenolics (FPI and phr-FPI, respectively) and phenolic compounds isolated (Phi) and (B) protein hydrolysates with phenolics and reduced phenolics (FPH and phr-FPH, respectively) and hydrolysed phenolics (Phh).

4.3.4. Impact of simulated gastrointestinal digestion of peptide profile and antioxidant capacity

The chromatograms of flaxseed products after simulated digestion, obtained at 214 and 280 nm, are shown in Figure 4.5 (A-F). As it can be observed, the action of digestive proteases on FPI and phr-FPI led to a high number of peptides that eluted between 8 and 20 minutes (Figure 4.5A and 4.5B). Both samples after digestion showed similar profiles, indicating that phenolic compounds present in FPI did not affect the enzymatic action of pepsin and pancreatin. Only the wide peak eluting at 20-23 minutes, corresponding to lignan macromolecule, was still visible in the chromatogram of digested FPI (dFPI). The same behavior was observed for FPH and phr-FPH, samples resulting from Alcalase hydrolysis (Figures 4.5D and 4.5E). Moreover, these profiles were similar to those shown by their digested parent products (dFPI and dphr-FPI), suggesting that peptides visible in the chromatograms were released by the action of pepsin and pancreatin on flaxseed proteins that had been not previously degraded by the microbial enzyme.

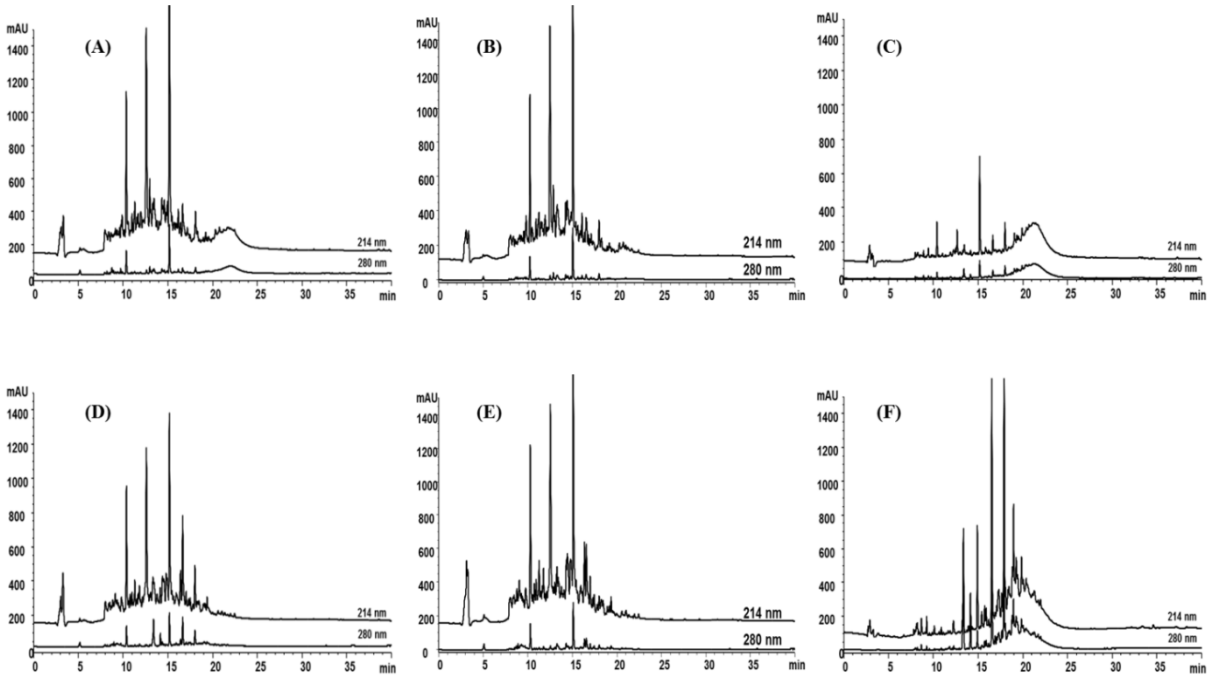

Figure 4.5. Chromatogram profiles (RP-HPLC) recorded at 214 nm and 280 nm of flaxseed products after simulated gastrointestinal digestion. (A) Digested flaxseed protein isolate (dFPI), (B) Digested phenolic reduced flaxseed protein isolate (dphr-FPI), (C) digested phenolic compounds isolate (dPhi), (D) digested flaxseed protein hydrolysate (dFPH), (E) digested reduced phenolic flaxseed protein hydrolysate (dphr-FPH), and (F) digested phenolic compounds hydrolysate (dPhh).

In the case of phenolic compounds fraction (Phi and Phh), different behavior was observed after their simulated digestion. Small differences in the profile of digested Phi (dPhi, Figure 4.5C) were detected comparing with non-digested sample (Phi, Figure 4.2C) that could be due to phenolics modifications caused by pH and temperature changes. Analysis of Phh and digested Phh (dPhh) showed similar chromatographic patterns (Figure 4.2F and 4.5F), thus indicating that phenolic compounds modified by alkaline conditions were not further affected by digestive conditions.

In order to evaluate the potential contribution of protein/peptide and phenolic compounds on the antioxidant capacity of flaxseed, different products were subjected to analysis using several measuring methods (ORAC, FRAP, FCRRS, and metal chelating activity). The results would also allow evaluating the impact of Alcalase hydrolysis and gastrointestinal digestion on the antioxidant activity. Figure 4.6 shows the results obtained from different assays before (Figure 4.6A, 4.6C, 4.6E, and 4.6G) and after simulated digestive process (Figure 4.6B, 4.6D, and 4.6F).

Before simulated digestion, Phi and Phh products showed the highest ORAC and FRAP values as well as the greatest chelating potential. This might be associated with the higher concentration of nucleophilic centers comparing with other flaxseed products, as well as with the possible synergisms among different phenolic compounds. The ability of the phenolic compounds to act as multifunctional antioxidant, as chain-breaking or metal chelating agent, can be explained by the nucleophilic character of the aromatic rings in its structure.[33] The ORAC value of Phh was 1.5-times higher than that of Phi, which can be related to the release of compounds of lower molecular weight and lower hydrophobicity resulting from alkaline conditions, as it was observed by chromatographic analysis (Figure 4.2C, 4.2F). Among these compounds, glycosylated ferulic, caffeic, and *p*-coumaric acids could be responsible for the increase in the ORAC value. These phenolic acids act as antioxidant mainly through a hydrogen atom (H^+) transfer mechanism due to the reactivity of their phenol moiety, although they also could act via electron donation.[34] The iron chelating ability of Phi and Phh was similar ($p > 0.05$) (Figure 4.6G), and about 2-times higher than that of FPH and phr-FPH. The potential of FPH and phr-FPH may be compromised by the dietary fiber content (Table 4.1) because these high molecular weight polysaccharides could interfere with the iron-peptides interaction, hindering the formation of the chelate.[35] No significant differences were observed between both hydrolysates, suggesting that their potential is due to peptides, especially those containing His, Glu, Asp, and Cys residues[36] rather to the presence of polyhydroxylated rings in phenolic compounds. Some studies have shown that iron chelating by peptides may facilitate absorption of this mineral by intestinal

cells[37-39] increasing its bioavailability, while some classes of phenolic compounds may exert an opposite effect.[40] However, it has been demonstrated that iron chelating by peptides or phenolic compounds may maintain the metal more stable and less prone to interactions,[41] which prevents free iron to catalyze human body reactions involving ROS, leading to the oxidation of unsaturated lipids and promoting oxidative damage in cells.[42] Thus, both peptides as phenolic compounds can have a beneficial effect due to its ability to chelate iron.

Alcalase hydrolysis was responsible for an increase in the antioxidant potential of flaxseed products up to 6 and 4 times, as determined by ORAC and FRAP assays, respectively, compared with non-hydrolysed products. Although the absolute values of the antioxidant capacity of samples containing protein and phenolic compounds were higher than those measured for products only containing proteins, the relative increase on the antioxidant capacity as a result of hydrolysis with Alcalase was similar. Therefore, this increase might be associated with the release of peptides during enzymatic hydrolysis rather than with changes in phenolic compounds.[1] FPH showed the highest FCRRS content (p <0.05) with a value 2- and 1.4-times higher than that measured in phr-FPH and Phi products, respectively (Figure 4.6E), indicating that exposure of both aromatic rings of the phenolic compounds and aromatic residues of proteins during hydrolysis with Alcalase was responsible for this antioxidant mechanism of action.

The antioxidant capacity of flaxseed products maintained or increased after gastrointestinal digestion (Figure 4.6B, 4.6D, and 4.6F), indicating that the digestive process might exert a beneficial effect on the bioactivity of released compounds, regardless of the mechanism evaluated. Even, this effect was higher than that demonstrated for Alcalase hydrolysis. In the case of the effect of digestion on the antioxidant capacity of Phi, a significant increase of FRAP value and FCRRS content was observed, while ORAC value did not change after the action of digestive enzymes. However, three antioxidant values were increased when simulated digestion was performed on Phh, indicating that alkaline conditions favored the access of nucleophilic sites of phenolic compounds to radicals in spite of chromatographic profiles of Phh and dPhh were similar. In literature, the effect of digestion on the phenolic compounds antioxidant capacity is contradictory and dependent on the digested product. Tarko, et al.[43] showed that the antioxidant capacity of the phenolic compounds from apple and plum increased, while those from pear and banana decreased after simulated digestion. The antioxidant activity of FPI and phr-FPI, without previous Alcalase hydrolysis, significantly increased after being subjected to simulated gastrointestinal digestion. Although the chromatographic profile of dFPI and dphr-FPI were similar, the antioxidant behavior was different, with highest capacity shown by product containing both

proteins and phenolic compounds. This was also observed for products previously hydrolysed by Alcalase (dFPH and dphr-FPH).

4.3.5. Impact of phenolic compounds on the antioxidant capacity

Principal component analysis (PCA) was performed to understand how the presence of phenolic compounds influenced the antioxidant profile of flaxseed products. PCA displays similarities and differences among samples from their spatial distribution. ORAC, FRAP and FCRRS values were considered as independent variables, while flaxseed deffated flours, protein isolates and hydrolysates, and their digests were the dependent variables. Samples were then distributed according to different oxidation assays, allowing knowing the predominant antioxidant mechanism for each sample (Figure 4.7).

Samples were centered on two main axes or principal components (PC), PC1 that explained 89.3% of the variance and PC2 that explained 9.7%. Thus, both components explained 99.0% of the antioxidant behavior of flaxseed products. As it can be observed in Figure 4.7, FPI and phr-FPI are located in the same quadrant, indicating that there are no differences between the antioxidant mechanisms of these samples. Same results were obtained for source flours, DFM and phr-DFM. However, once subjected to Alcalase hydrolysis, the behavior of the hydrolysates changed, and FPH and phr-FPH were located in opposite quadrants. Phr-FPH, only containing peptides, was located at the downright quadrant, indicating that its antioxidant activity was mainly mediated through protons transference and peroxyl radicals chelation. However, FPH, containing both peptides and phenolic compounds, was located at the upper right quadrant, suggesting that phenolic compounds were the main responsible for reducing ferric to ferrous iron (FRAP assay). After simulated gastrointestinal digestion, similar behavior was observed with peptides released from the action of digestive enzymes responsible for ORAC and FCRRS values, and phenolic compounds contributing to iron reduction.

The presence of polyphenols contributed positively, but in a variable way, on the antioxidant capacity of the majority of protein samples. Prior to digestion, the presence of phenolic compounds impacted on the protein isolate about 80% of antioxidant capacity measured by ORAC and the FRAP assays. For the hydrolysate, the presence of phenolic compounds also impacted on nearly 80% as measured by FRAP but only 15% as measured by ORAC. After the simulated digestion, the influence of the phenolic compounds on the antioxidant capacity of the samples determined by the FRAP remained high, between 70 and 80%, while lower influence was observed when the antioxidant capacity was measured by ORAC (Figure 4.6).

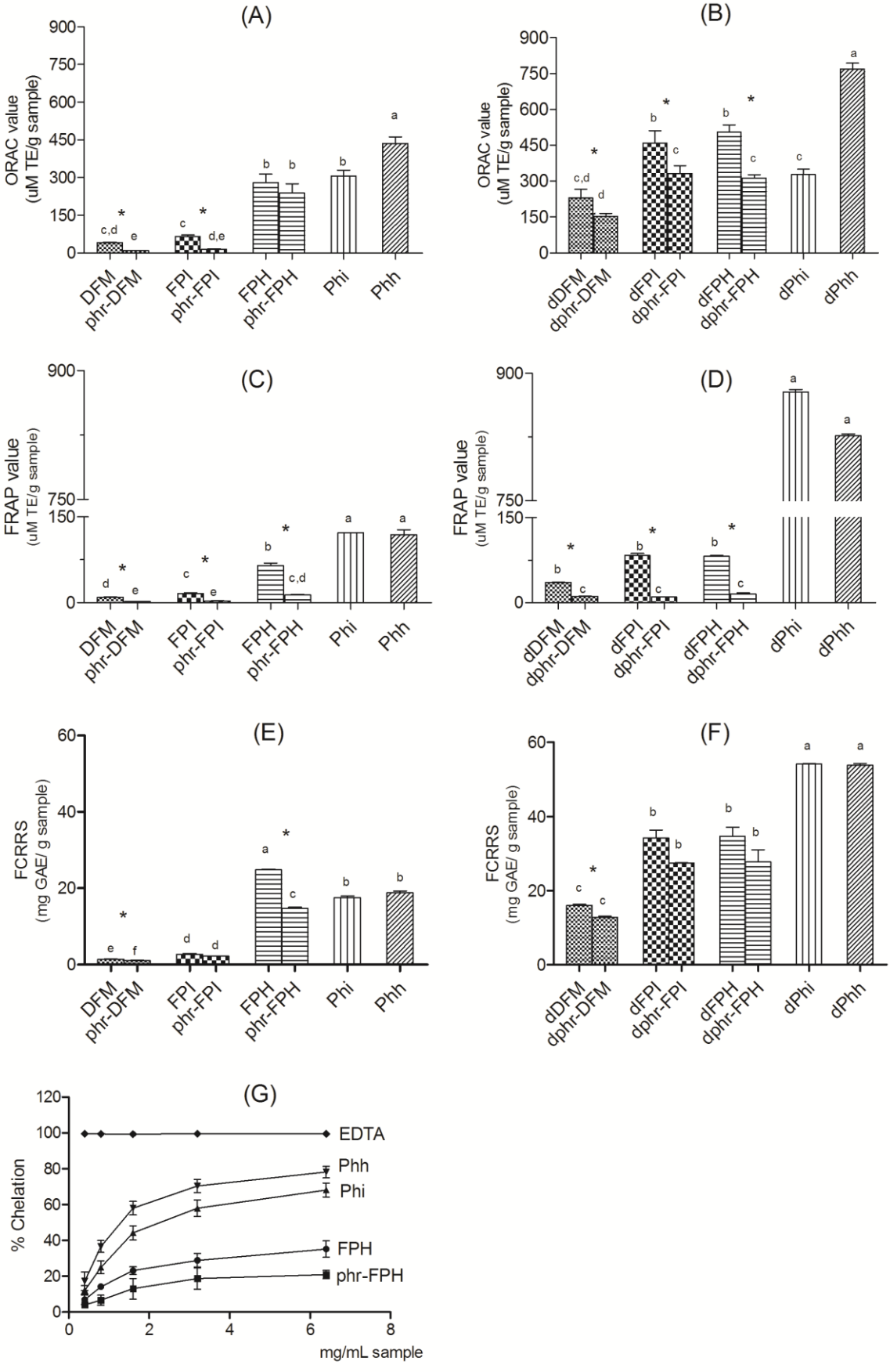
(A)
ORAC value (uM TE/g sample)
DFM
phr-DFM
FPI
phr-FPI
FPH
phr-FPH
Phi
Phh
(B)
ORAC value (uM TE/g sample)
dDFM
dphr-DFM
dFPI
dphr-FPI
dFPH
dphr-FPH
dPhi
dPhh
(C)
FRAP value (uM TE/g sample)
(D)
FRAP value (uM TE/g sample)
(E)
FCRRS (mg GAE/ g sample)
(F)
FCRRS (mg GAE/ g sample)
(G)
% Chelation
EDTA
Phh
Phi
FPH
phr-FPH
mg/mL sample

Figure 4.6. Antioxidant capacity by ORAC (A and B), FRAP (C and D), FCRRS levels (E and F) and quelating capacity (G), before (A, C, E, G) and after simulated digestion (B, D, F). Values expressed as mean of duplicates (each in triplicate) and ± standard deviation. Bars with different letters differ from each other by ANOVA, post hoc Tukey test ($p < 0.05$). Bars of the same sample with phenolics and reduced phenolics with '*' are different from each other by T test ($p < 0.05$). Defatted flaxseed meal (DFM); phenolic reduced defatted flaxseed meal (phr-DFM); Flaxseed protein isolate (FPI); phenolic reduced flaxseed protein isolate (phr-FPI); Flaxseed protein hydrolysate (FPH); phenolic reduced flaxseed protein hydrolysate (phr-FPH); phenolic compounds isolate (Phi) and phenolic compounds hydrolysate (Phh). Letter "d" before samples names means digested samples.

This impact can be explained either by synergism between the antioxidant compounds or formation of protein-phenolic complex. In proteinaceous samples, antioxidants compounds are composed by lignan macromolecule, SDG and caffeic, ferulic and p-coumaric acids, which have high potential to act as antioxidant[34], as well as flaxseed peptides, in Alcalase hydrolysates or in digested samples.[44] When together, such compounds may have their antioxidant potential increased, since a non-oxidized compound is able to regenerate the other which has been oxidized, in a similar way that synergism occurs between α-tocopherol and flavonoids or α-tocopherol and ascorbate.[45]

In the protein isolates, the positive influence of phenolic compounds on antioxidant capacity can be either due to their high amount of phenolic hydroxyl groups and/or by unfolding the protein structure due to protein-phenolic complex formation. In most cases, formation of complexes increases the exposure of nucleophilic centers formed by hydrophobic amino acid residues at the N-terminal portion, or the presence of His, Trp, Phe, Tyr, Cys in the protein moieties.[1] Although the formation of complex promotes the participation of protein as an antioxidant, it can, in turn, compromise the performance of polyphenols in this process, masking their bioactivity.[46] Thus the resulting antioxidant activity is due the increasing of antioxidant capacity of proteins and descreasing of the phenolic compounds.

On the other hand, in the hydrolysates, the formation of complex is hindered due to the small contact surface between peptides and phenolic compounds.[47] Thus protein hydrolysis can weaken the protein-phenolic complexes, by reducing the interactions between these compounds and increasing both the availability of the nucleophilic sites of peptides and phenolic compounds. This greater exposure of regions capable of neutralizing reactive species and the possible synergism between these two classes of compounds may also explain the higher antioxidant capacity of the hydrolysates when compared to the isolates.

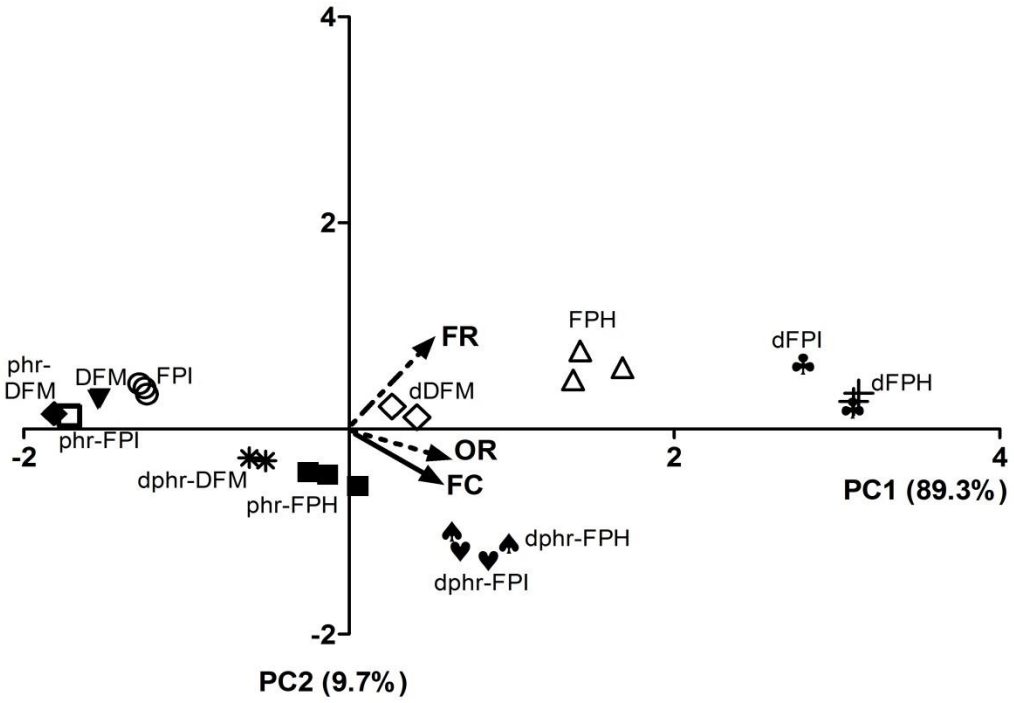

Figure 4.7. Principal component analysis on the measured parameters ORAC (OR), FRAP (FR) and FCRRS (FC) before and after in vitro digestion. Defatted flaxseed meal (DFM ▼); phenolic reduced Defatted flaxseed meal (phr-DFM ♦); Flaxseed protein isolate (FPI ○); phenolic reduced Flaxseed protein isolate (phr-FPI □); Flaxseed protein hydrolysate (FPH ∆); phenolic reduced Flaxseed protein hydrolysate (phr-FPH ■); Letter "d" before samples names means digested samples (dDFM ◊; dphr-DFM *; dFPI ♣; dphr-FPI ♥; dFPH +; dphr-FPH ♠).

4.4. Conclusion

The phenolic fraction showed the highest antioxidant capacity among the flaxseed products studied, which was enhanced by both alkaline hydrolysis and simulated gastrointestinal digestion, possibly by releasing SDG and *p*-coumaric, caffeic and ferulic phenolic acids by the hydrolysis conditions. The hydrolysis by both Alcalase and digestive enzymes also resulted in an increase of the antioxidant activity of protein isolates with/without phenolic compounds. Peptides released from enzymatic hydrolysis act through protons transference and peroxyl radicals chelation while phenolic compounds were mainly responsible for the iron reduction.

The formation of protein-phenolic complexes may have a positive effect on the antioxidant capacity of plant protein isolates. In our study, we have found that protein-phenolic complexes in flaxseed products favored the exposure of protein moieties capable of acting as an antioxidant, which would compensate the loss of antioxidant potential of phenolic compounds, with a positive relationship between these classes of compounds.

Another possible benefit of the formation of protein-phenolic and/or peptide-phenolic complexes refers to the action of proteins as phenolic compounds carriers, releasing them only in the intestinal lumen. This release has a dual effect of phenolic protection against oxidative degradation along the gastrointestinal tract and establishment of a positive antioxidant environment, since part of these liberated phenolic compounds can act as antioxidants and protect vitamins, lipids and proteins against oxidation. Animal models should be needed to evaluate the bioavailability of peptides and phenolic compounds as well as to confirm the *in vivo* antioxidant effects providing health benefits against oxidative stress-associated disorders.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors thank FAPESP (2010/52680-7) for the financial support, CNPq and UNICAMP (FAEPEX 607/11) for the scholarship granted to F.G.D. Silva, and the Laboratory of Spectroscopy and Calorimetry (Brazilian Biosciences National Laboratory) for the support with fluorescence analysis. We also want to thank Professor Márcia Miguel Castro Ferreira for the support with Principal Component Analysis and Waters Brazil (São Paulo, SP, Brazil) for supplying the UPLC-Xevo G2-XS QToF system employed in this study.

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CAPÍTULO 5. ARTIGO 2. Identification of peptides released from flaxseed (*Linum ussitatissimum*) protein by Alcalase hydrolysis: antioxidant activity

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Este artigo foi submetido à revista *LWT- Food Science and Techonology*

Abstract

In this study, the hydrolysis of a flaxseed protein isolate with Alcalase was performed as a strategy to generate antioxidant peptides. A chromatographic separation of the hydrolysate by RP-HPLC was conducted, and the antioxidant capacity of the initial sample and the six obtained fractions was determined using the ORAC and FRAP assays. The higher values corresponded to fractions comprising predominantly lower molecular peptides, as shown in the molecular weight profile determined by MALDI analysis. Four peptide sequences could be identified by using LC-MS/MS and one by Edman degradation. The last peptide, of sequence GFPGRLDHWCASE, was synthesised and showed a notable ORAC activity, 3.20 μmol Trolox equivalents/μmol of peptide, a value higher than that of butylated hydroxyanisole. Therefore, the contribution of this peptide to the activity of the fraction where it had been found was 61%. The identified sequences represent an advance in the molecular characterization of the flaxseed protein fraction.

Keywords

Flaxseed, peptides, antioxidant capacity, Alcalase hydrolysis, tandem mass spectrometry

5.1. Introduction

Flax is one of the most ancient crops, used until the nineteenth century for cloths and paper manufacturing. The major industrial product is flaxseed oil because it is the richest known plant source of α-linolenic acid, while the residual meal remaining after oil extraction is used in animal feed. At present time, flaxseed has new prospects in food because of the growing consumers' interest for functional food with health benefits (Shim, Gui, Arnison, Wang & Reaney, 2014). The presence of n-3 fatty acids, soluble fibers, vitamin E, lignans, and other phenolic and peptide compounds in flaxseed offers potential for it to exert diverse actions thought to benefit health (Cardoso Carraro, Dantas, Espeschit, Martino & Ribeiro, 2012). In the body tissues, free radicals are continually generated as byproducts of oxidative metabolism. Although they are essential on cellular processes, their accumulation and the absence of antioxidant endogenous defenses that neutralize their oxidative action can lead to oxidative stress status. Many studies associate this status with the occurrence and the progression of various chronic diseases such as diabetes, cancer, neurodegenerative and cardiovascular disorders, and aging (Fiaschi & Chiarugi 2012). Therefore, the interest of these compounds with the ability to prevent these disorders is evident.

In whole and ground/milled flaxseeds stored at room temperature limited oxidative deterioration was observed, which is indicative of an efficient antioxidant system. In accordance to the composition and existing research, this could be attributed to lignans, concretely secoisolariciresinol diglucoside and phenolic acids, i.e. p-coumaric, vanillic, sinapic and ferulic present in the seed (Hosseinian, Muir, Westcott & Krol, 2006; Johnsson et al., 2002). Barthet, Klensporf-Pawlik, & Przybylski (2014) studied the types of components involved in the antioxidant properties of the flaxseed meal. By the use of solvent extraction of different polarity such as water, acetone and methanol the results pointed to multiple components. It was found that lignans and phenolic acids are only partially responsible for the flaxseed antioxidative system, and the participation of some proteins was suggested. In accordance to these findings, we have shown that flaxseed protein products exert antioxidant capacity with different contribution to each mechanism, i.e. ferric reduction or oxygen radical scavenging, and this bioactivity was not impaired by simulated in vitro digestion (unpublished data).

In flaxseed, proteins represent 35–45% on dry oil-free matter basis, and 56–70% is concentrated in aleuronic grains in cotyledons (Rabetafika, Van Remoortel, Danthine, Paquot, & Blecker, 2011). Their solubility in various solvents reveals two major fractions namely globulin (linin, 11-12S; 64-73% of total seed protein) and albumin (conlinin, 1.6-2S;

27-42% of total seed protein) that have molecular masses of 252-298 and 16-17 kDa, respectively (Chung, Lei & Li-Chan., 2005; Marambe, Shand & Wanasundara, 2013). Moreover, with the use of electrophoretic techniques, other protein bands with molecular weights between 9 and 17 kDa have been detected (Sammour, 1999).

The proteinaceous materials, when hydrolysed, are known to yield hydrolysates with different bioactive and nutritional values. The strategies to recover and characterize bioactive components from flaxseed proteins include hydrolysis or simulated gastrointestinal digestion of protein isolates followed by size exclusion or cation exchange chromatographic separation (Marambe, Shand & Wanasundara, 2011; Udenigwe, Lu, Han, Hou & Aluko, 2009), electrophoresis (Liu, Shim, Poth & Reaney, 2016) or electrodialysis with ultrafiltration membranes (Doyen et al., 2014). The obtained peptide fractions have shown angiotensin converting enzyme (ACE) inhibitory and hydroxyl radical scavenging activity (Marambe et al., 2008), calmodulin inactivation with inhibition of CaM-dependent phosphodiesterase and renin inhibition (Udenigwe & Aluko, 2012), increased glucose uptake in L6 cells, and even antihypertensive activity on spontaneously hypertensive rats (Doyen, Udenigwe, Mitchell, Marette, Aluko, & Bazinet, 2014).

Due to the incomplete sequence description of flaxseed proteins, only in very few cases peptide identification has been achieved. However, the assessment of peptides linked to their accountability for the elicited effects is necessary because the observed activity cannot be only attributed to the degree of hydrolysis, as the decisive factor is the structure of the peptides and the sequence of amino acids that build them (Dryakova, Pihlanto, Marnila, Curda, & Korhonen, 2010). The aim of the present study was to evaluate the antioxidant behavior of fractions from a flaxseed protein isolate prepared by an optimized hydrolysis process with Alcalase, and to identify peptides responsible for this activity.

5.2. Materials and methods

5.2.1. Preparation of the phenolic reduced flaxseed protein hydrolysate (FPH)

The flaxseed protein isolate hydrolysate was prepared according to Silva, O'Callagahan, O'Brien, & Netto (2013) with modifications. Partially defatted brown flaxseed meal (Cisbra Ltd., Panambi, RS, Brazil) was defatted with hexane in a ratio of 1:3 (w/v). Then, three consecutive extractions with 63% ethanol (v/v) were performed to remove phenolic compounds. The phenolic reduced defatted flaxseed meal was dispersed in

deionized water at 1:10 (w/w) flour:water ratio, pH was adjusted to 9.0, stirred at room temperature for 120 min, and then centrifuged (2500 x g/30 min; 25°C). The pH of the supernatant was adjusted to 4.2, and the precipitated protein was separated by centrifugation (2500 x g/30 min), suspended in deionized water, and the pH adjusted to 6.0. It was then freeze-dried and stored at -20 °C until use. The hydrolysis of flaxseed protein isolate was performed with Alcalase 2.4 L purchased from Sigma (St. Louis, MO, USA) under the following conditions: protein concentration of 5% (w/v), 60°C, 180 min, pH 8.5, enzyme substrate ratio 1:90 (w/w) as it was reported by Silva et al. (2013). The hydrolysis reaction was monitored by a pH-stat method using an automatic titrator DL model Metler 21 (Schwerzenbach, Switzerland) with a stirring system coupled to a thermostatic bath. The pH of the hydrolysate was adjusted to 6.0, freeze-dried and stored at -20°C. The degree of hydrolysis (% DH) of FPH was 17.20 ± 0.7%, calculated using the equation described by Adler Nissen (1986).

For the subsequent analyses, an aqueous extract was obtained by suspension of FPH in deionized water (1% w/v), stirring for 30 min, centrifugation at 36,000 x g for 30 min at 10°C, filtration through Nº1 Whatman qualitative filter paper, and freeze-drying.

5.2.2. Fractionation of the flaxseed protein hydrolysate (FPH) by RP-HPLC

FPH, at a concentration of 5.0 mg/mL, was separated using a Waters Nova-Pak HR C18 (300 mm × 7.8 mm internal diameter) column, in a Waters 600 HPLC (Waters Corp., Milford, MA, USA) equipped with two pumps (module delta 600), a pump controller (module 600), an autosampler (module 717), and a diode array detector (module 996) in combination with an automatic fractions collector (module II). The digests were eluted by using 0.37% (v/v) trifluoroacetic acid (TFA) in water as solvent A and 0.27% (v/v) TFA in acetonitrile as solvent B, at a flow rate of 3.5 mL/min. The injection volume was 350 µL. Peptides were eluted with a linear gradient of solvent B in A going from 0 to 40% over 45 min. Detection was carried out at 214 nm. Data were processed by using Empower 2 Software (Waters Corp.). Six fractions were collected from separate RP-HPLC runs, pooled, lyophilized, and stored at -20°C until further analyses.

5.2.3. Characterization of the flaxseed protein hydrolysate (FPH) and its fractions

The protein content of the FPH and the collected fractions was determined by the bicinchoninic acid method (BCA) (Pierce, Rockford, IL, USA), using bovine serum albumin as standard protein.

Amino acids were analyzed in duplicate by RP-HPLC using a liquid chromatograph, consisting of a Waters 600 Controller programmable solvent module (Waters Corp.), a WISP 710B autosampler (Waters Corp.), and a HP 104-A fluorescence detector (Hewlett-Packard, Palo Alto, CA, USA). Samples were submitted to automatic precolumn derivatization with ortho-phthalaldehyde (OPA) in the presence of 2-mercaptoethanol following the method described by Moreno-Arribas, Pueyo, Polo & Martín-Álvarez (1998). Separation was carried out on a Waters Nova Pack C18 column (150 × 3.9 mm i.d., 60 A, 4 μm) column. Detection was performed by fluorescence (λexcitation = 340 nm, λemission = 425 nm), and chromatographic data were collected and analyzed with a Millenium 32 system (Waters Corp.). The FPH was previously hydrolyzed with 6 N HCl for 21 h at 110°C. Trp content was determined after alkaline hydrolysis of the sample with 4.2 N NaOH for 21 h at 110°C.

For analysis of peptide mass distribution both in the hydrolysate and its fractions, MALDI-TOF mass spectrometry (MS) was performed on a Bruker Autoflex Speed® (Bruker Daltonik GmbH, Bremen, Germany). Samples were spotted on a Bruker Anchorchip target with α-CHCA matrix in acetonitrile/water (30:70) containing 0.1% TFA. Mass spectra were acquired in positive reflectron mode by accumulating 1000 laser pulses on average. Calibration was performed with the Peptide Calibration Standard I and II (Bruker Daltonik). M/z signals appearing in more than two fractions were discarded.

5.2.4. Antioxidant activity of the flaxseed protein hydrolysate (FPH) and its fractions

5.2.4.1. Oxygen radical absorbance capacity (ORAC)

An oxygen radical absorbance capacity (ORAC)-fluorescein (FL) assay (ORAC-FL) was used based on that optimized for protein hydrolysates and peptides by Hernández-Ledesma, Dávalos, Bartolomé & Amigo (2005). Briefly, the reaction was carried out at 37ºC in 75 mM phosphate buffer (pH 7.4). The final assay mixture (200 μL) contained FL (70 nM), 2,2'-azobis (2-methylpropionamide)-dihydrochloride (AAPH, 14 mM) and antioxidant [6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 0.2-1.6 nmol) or sample (at different concentrations)]. Fluorescence was recorded during 137 min (104 cycles) in a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) with 485 nm excitation and 520 nm emission filters. The equipment was controlled by the FLUOstar Control ver. 1.32 R2 software for fluorescence measurement. Three independent runs were performed for each sample. Final ORAC-FL value was expressed as μmol Trolox equivalent per mg protein or μmol peptide.

5.2.4.2. Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power (FRAP) assay was carried out according to Benzie & Strain (1996) with modifications. In the dark, 30 µL sample extract, standard or blank was mixed with 90 µL of water and 900 µL of the FRAP reagent (450 µL of 0.3 M acetate buffer, pH 3.6; 225 µL of 10 mmol tripyridyltriazine (TPTZ) in 40 mmol HCl and 225 µL of 20 mmol FeCl3). The mixture was incubated at 30°C for 30 min. The absorbance was measured at 595 nm in a Synergy™ HT Multi-Mode Microplate Reader (Biotek®, Vermont, USA). Trolox was used as standard and the results were expressed as µmol of Trolox equivalent per mg of protein.

5.2.5. Peptide identification and synthesis

5.2.5.1 Peptide identification by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

FPH and its fractions were injected on an Acquity Ultrahigh Performance LC (UPLC) from Waters Corp., coupled to a Microtof-QII (Bruker Daltoniks). The LC-MS system was controlled by the HyStar 3.2 software (Bruker Daltonics). The column employed for the analyses was an Acquity UPLC BEH 130 C18 of 2.1 mm × 50 mm (Waters Corp.), with a particle size of 1.7 µm. The injection volume was 5 µL, and the flow was set at 0.2 mL/min. Freeze-dried samples were dissolved (2 mg/mL) in 0.1% (v/v) formic acid (FA). Solvent A was a mixture of Milli-Q water/FA (100:0.1, v/v), and solvent B contained acetonitrile (HPLC grade)/FA (100:0.1, v/v). The gradient was 45% of solvent B in 27 min, after which the percentage of solvent B increased to 70% in 2 min, and remained 3 min. Data processing was done by using Data AnalysisTM (version 4.0; Bruker Daltoniks). For peptide sequencing, the matched MS/MS spectra were interpreted by using BioTools from Bruker, MASCOT from Matrixscience and X! Tandem from the Global Proteome Machine Organization (GPM), using a homemade database that includes the main proteins of flaxseed.

5.2.5.2. Peptide identification by Edman degradation

The N-terminal sequence of the main compound of the most active fraction was identified by sequence analysis with a Perkin-Elmer/Applied Biosystems Procise 494 microsequencer (Uberlingen, Germany) running in pulsed liquid mode.

5.2.5.3. Peptide Synthesis

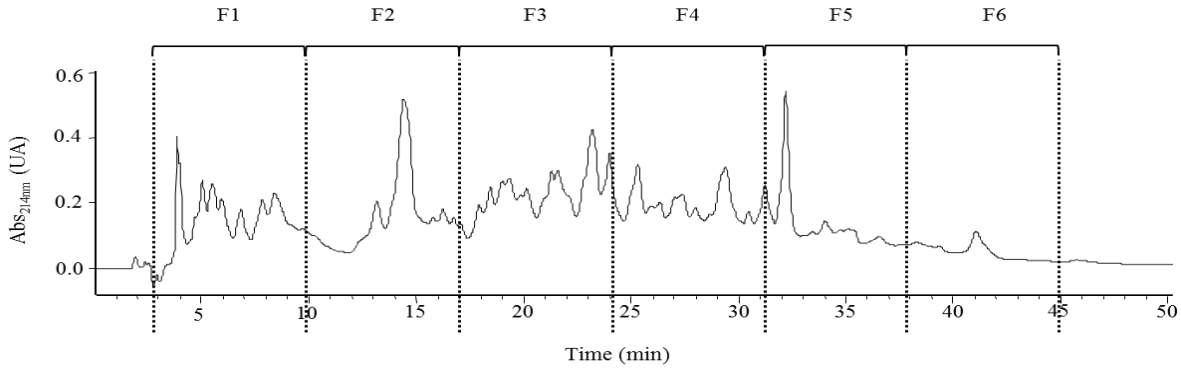
The sequence of the peptide determined by Edman degradation was prepared in our laboratory by the conventional Fmoc solid-phase synthesis method with a 433A peptide synthesizer (Applied Biosystems, Warrington, UK), and its purity was verified by LC-MS/MS (Sánchez-Rivera et al., 2014).

5.3. Results and discussion

5.3.1 Characterization of the hydrolysed product

Different flaxseed products are being studied for their potential to present antioxidant activity. Most research explored the role of polyunsaturated fatty acids and lignan in these properties while flaxseed protein has been studied less often. However, hydrolysis of protein concentrates from this source with several proteases and digestive enzymes has been attempted as an effective method to promote several biological activities, such as ACE inhibitory (Marambe, & Shand, 2008) or anti-inflammatory (Udenigwe et al., 2009). In the case of antioxidant properties, flaxseed hydrolysates with proteases (Alcalase, Pronase, Flavourzyme®) have shown scavenging capacity on different radicals. Concretely, Alcalase hydrolysates have been evaluated for scavenging 2,2-diphenyl-1-picrylhydrazyl radical, superoxide anion radical, electron-spin resonance-detected hydroxyl radical and nitric oxide with variable results for the different radicals (Udenigwe et al., 2009). The Alcalase hydrolysed product analysed in the present study was prepared under the conditions selected based on a mathematical model for obtaining the highest antioxidant capacity measured by ORAC and FRAP (Silva et al., 2013). The ORAC and FRAP values obtained for the hydrolysate were 0.93 ± 0.04 μmol Trolox equivalent/mg protein and 0.02 ± 0.00 μmol Trolox equivalent/mg protein, respectively). The differing values obtained with both assays can be attributed to the implicated mechanism, i.e. hydrogen atom transfer where the hydrogen of peptides neutralizes the radicals formed for ORAC, and ferric ion reducing by electron transfer for FRAP. A number of works have reported the ability Alcalase to release antioxidant peptides from other plant proteins, although the activity showed by the hydrolysates was dependent on the protein source. As an example, canola meal protein Alcalase hydrolysate showed an ORAC value of 2.20 μmol Trolox equivs/mg of protein, higher than that found in our flaxseed hydrolysate (Alashi et al., 2014) while Alcalase hydrolysate obtained from common bean (Phaseolus vulgaris) protein showed an ORAC value of 0.33 μmol Trolox equivs/mg of protein (Oseguera-Toledo, González de Mejia, and Amaya-Llano, 2015).

(A)



(B)

Fraction No.	Protein (mg/mL)	Antioxidant activity	
		ORAC (mmol Trolox equivs/mg protein)	FRAP (mmol Trolox equivs/mg protein)
F1	0.219 ± 0.004	2.268 ± 0.134	n.d.
F2	0.222 ± 0.009	2.411 ± 0.025	n.d.
F3	0.400 ± 0.014	1.709 ± 0.021	0.209 ± 0.013
F4	0.317 ± 0.014	1.430 ± 0.052	0.133 ± 0.006
F5	0.263 ± 0.011	1.311 ± 0.129	0.250 ± 0.014
F6	0.018 ± 0.001	3.581 ± 0.120	n.d.

Figure 5.1. RP-HPLC chromatographic separation of the flaxseed hydrolysate (A) and determined antioxidant activity in the collected fractions by ORAC and FRAP assays.

The RP-HPLC analysis confirmed the extensive hydrolysis of the product by this highly nonspecific endopeptidase, and allowed the separation of a remarkable number of peptides that were collected in six fractions of increasing hydrophobicity (Figure 5.1A). The antioxidant capacity of the fractions was determined by the methods above mentioned and the values were expressed as μM Trolox equivalent per mg of protein. All fractions showed activity in the ORAC assay (Figure 5.1B). Thus, taken as a whole, the separated peptides have a high potential to disrupt reactions that involve peroxyl radicals but the highest value corresponded to fraction F6 (3.58 ± 0.12 μmol Trolox equivs/mg protein). Some studies have revealed that high radical scavenging activities for the protein hydrolysates or peptides are usually associated with hydrophobicity or high hydrophobic amino acid content (Rajapakse,

Mendis, Byun, & Kim, 2005). In the FRAP assay, only fractions F3, F4 and F5 exhibited antioxidant capacity, and the values were below those found in the ORAC determination. When using these assays, other authors have found also different values. Thus, in ripe pistachio hulls, higher values in the FRAP than in the ORAC assay were found and the activity was attributed to the phenolic compounds content (Barreca, Laganà, Leuzzi, Smeriglio, Trombetta, & Bellocco, 2016). In contrast, in Palmaria palmate protein hydrolysates, the ORAC assay gave rise to higher values than FRAP. In this case, the peptides were found responsible for the antioxidant capacity (Harnedy, Soler-Vila, Edwards, & FitzGerald, 2014).

The peptide mass distribution profile of the FPH proved that Alcalase had digested the proteins in small peptide fragments with overall sizes under 4000 Da with 70% of peptides in the range 500 to 1500 Da (Figure 5.2A). Despite the higher abundance of low m/z signals in all samples, the individual analysis of the fractions showed different compositions (Figure 5.2B). The analysis revealed that some fractions comprised peptides with the whole range of molecular weights, i.e. fractions F2 and F5. In contrast, fractions F3 and F6 showed predominantly small size peptides. Fractions F1 and F4 showed an intermediate mass profile. In view of the antioxidant activity of the fractions, lower size peptides could be assigned to a higher activity. The results are in accordance with the statement that the majority of the antioxidative peptides derived from food sources have molecular weights between 500 and 1800 Da (Samaranayaka & Li-Chan, 2011). Onuh, Girgih, Aluko, & Aliani (2014) reported that the higher ORAC values of the small peptides may be due to increased ability to interact and donate electrons to the free radical when compared to bigger peptides that may have reduced ability to interact with the free radical.

The amino acid composition in protein hydrolysates is known to play an important role in the antioxidant capacity when it has to be attributed to peptides. Table 4.1 shows the amino acid distribution in the FPH. The hydrophobic amino acids represented 34.7% of the total amount determined, an important proportion that could increase the solubility of the peptides in lipids, facilitating a better interaction with free radicals. On the other side, the aromatic amino acids have the ability to donate protons to electron deficient radicals while maintaining their stability. Their content in the hydrolysate was also important, 18.5%. Therefore, the amino acid composition of the Alcalase hydrolysate was compatible with the presence of peptides with antioxidant capacity.

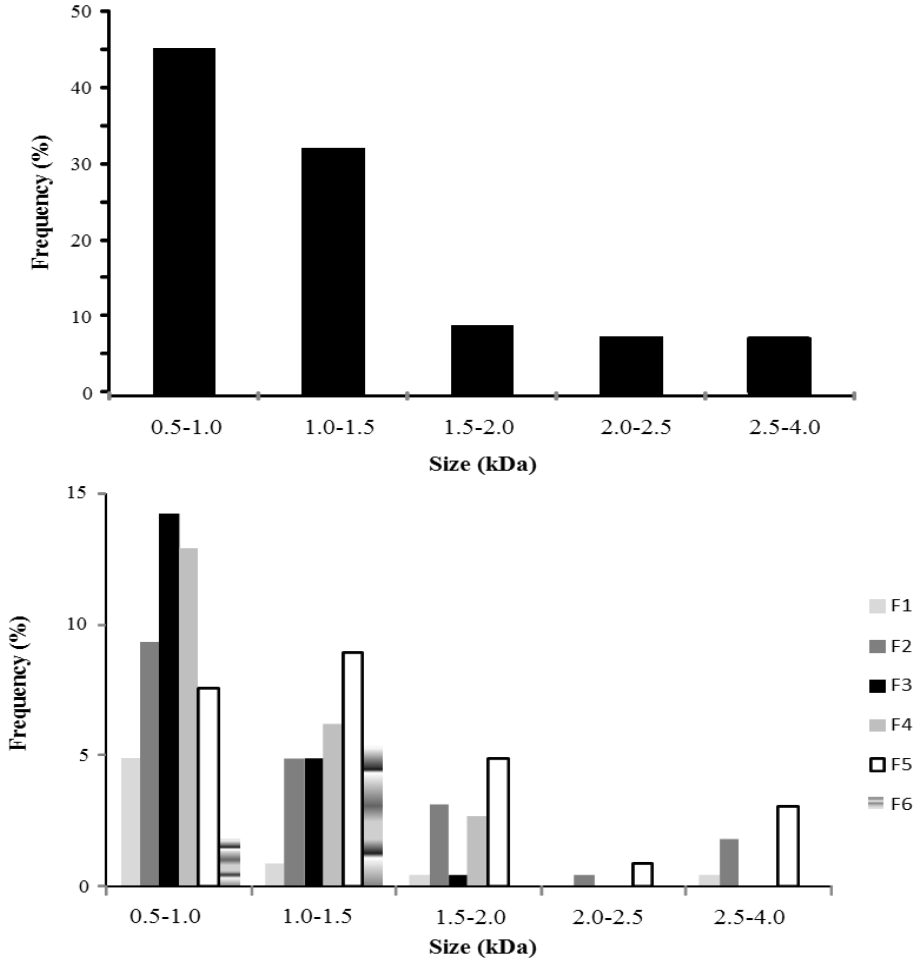


Figure 5.2. Peptide mass frequency distribution of the flaxseed hydrolysate (A) and the collected fractions (B).

5.3.2 Identification of peptides

The analysis by LC-MS/MS allowed identifying four fragments belonging to sequenced proteins from flaxseed (Table 5.2). The fragment found in fraction F2 corresponded to conlinin, one of the high abundant seed storage proteins, consisting of 168 amino acids. The fragment in fraction F5 belonged to cellulose synthase, a protein involved in the pathway of plant cellulose biotynthesis which consists of 1097 amino acids. The fragments found in fractions F5 and F6 corresponded each to the sequence of UDP-glycoslyltranferase-1, based on protein homology. Fraction F6 showed an ion signal that could not be assigned to any known protein although it was its main compound and had been also detected in the MALDI-MS analysis (data not shown). In order to identify the compound, fraction F6 was submitted to Edman degradation. A 17 amino acids sequence, GFPGRLDHWCASE, was identified although it could not be assigned to any of the

sequenced flaxseed proteins. It has to keep in mind that not all flaxseed proteins sequences are known to date. When the amino acid residues present in the sequence were analysed they anticipated antioxidant activity. Hence, His, Pro, Ala have been reported to contribute to the scavenging of free radicals in soybean conglycinin antioxidant peptides (Chen, Muramoto & Yamauchi, 1995). In addition, antioxidant behavior using the thiobarbituric acid method in sequences with Gly in the C-terminal end has been observed (Kim et al. 2001). Furthermore, Trp is an amino acid that has shown potent ORAC activity (Hernández-Ledesma, Amigo, Recio & Bartolomé, 2007).

Table 5.2. Peptides identified by HPLC-MS/MS in fractions collected by preparative RP-HPLC from flaxseed protein hydrolyzate by Alcalase

Fraction No.	Observed mass	Calculated mass	Peptide sequence	Source protein	Fragment
F2	1012.455 (+1)	1028.475	[a]QGRGGQGGQGQ	Conlinin	35-47
F5	634.600 (+3)	1900.880	NGSGYPGSDLDSSPPGAKVP	Cellulose synthase 6D	144-163
F5	1491.741 (+1)	1490.733	GREEIGNVMRSLM	UDP-Glycosyltransferase-1	426-438
F6	850.000 (+2)	1697.906	GVKVEGDGGLVRRDEI	UDP-Glycosyltransferase-1	367-382
F6	738.900 (+2)	1473.646	GFPGRLDHWCASE[b]	Unknown	Unknown

[a] Ammonia loss
[b] Identified by HPLC-MS/MS and Edman degradation

The identified sequence was synthesised and its ORAC activity was assayed. A value of 3.20 ± 0.24 µmol Trolox equivalents/µmol peptide was determined. This value indicates a potent antioxidant capacity in relation with the disruption of reactions involving peroxyl radicals. The antioxidant capacity of this peptide is higher than that of butylated hydroxyanisole (2.43 µmol Trolox equivalents/µmol), a synthetic antioxidant currently used in the food industry (Hernández-Ledesma et al., 2005). Taking into account the Trolox equivalents per mg of protein of fraction F6, the activity shown by the peptide would account for 61% of the total activity of the fraction.

Very few peptides released from flaxseed protein hydrolysis have been identified. Marambe et al., (2011) analysed selected ACE inhibitory fractions from a flaxseed hydrolysate, and provided two probable amino acid sequences that were assigned by the authors to the non-sequenced protein linin. In a next study, the chromatographic fractions of

the Alcalase hydrolysate were subjected to tandem MS peptide sequencing, and short flaxseed protein-derived peptides were provided although in most cases they were potential sequences comprising multiple options (Udenigwe & Aluko, 2012). Recently, several peptides corresponding to conlinin were identified by 2D electrophoresis and Maldi-Tof MS, and this protein was described to contribute to the emulsification properties of flaxseed gum (Liu et al., 2016). However, the identified peptides were not assayed for this property nor they can be assigned to a particular biological activity.

5.4. Conclusions

The specific contribution of the protein fraction to the antioxidant capacity of the whole flaxseed products is not fully elucidated. The present study showed that the protein fraction of flaxseed can be the source of peptides with a notable antioxidant capacity as determined by ORAC and FRAP assays, and the sequence of the identified peptides is compatible with the observed effects. This has allowed assigning, at least in part, the radical disruption activity of a flaxseed hydrolysate to the peptides generated with Alcalase. The sequences provided in this work, and especially the peptide GFPGRLDHWCASE which shows antioxidant activity itself represent an advance in the molecular characterization of the flaxseed protein fraction.

Acknowledgments

This work has received financial support from projects AGL2015-66886-R and FAPESP (2010/52680-7). F.G.D. Silva acknowledges the CNPq for the scholarship granted, and B. Hernández-Ledesma thanks the Ministry of Economy and Competitiveness (MINECO) for her “Ramón y Cajal” post-doctoral contract.

Conflict of interest

The authors declare that they have no competing interests.

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CAPÍTULO 6. ARTIGO 3. Protective effects of flaxseed protein hydrolysates and phenolic isolated fractions in a TNBS-induced colitis

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Este artigo será submetido na revista *Molecular Nutrition and Food Research*

Abstract

In an attempt to develop new therapeutic inputs for colitis, we evaluated the effects of phenolic compounds and protein hydrolysates fractions of flaxseed on the production of pro-inflammatory mediators by macrophages and on the course of experimental trinitrobenzene sulphonic acid TNBS-induced colitis, and tried to identify the mechanisms involved. We observed that both protein hydrolysates (FPH and phr-FPH) and phenolic compounds (Phi and Phh) reduced the in vitro production of pro-inflammatory cytokine (TNF-□) and nitric oxide (NO) in RAW 264.7 macrophages. The concomitant treatment with flaxseed phenolic and peptide fractions inhibited TNBS-induced colitis by protecting mice against weight loss, and downregulating, colonic inflammation, T cell proliferation, expansion of TH1 and TH17 cell and production of pro-inflammatory cytokines. Furthermore, the phenolic compound administration increased Treg lymphocytes in TNBS-induced colitis in BALB/c mice. Our results show that protein hydrolysates and phenolic fractions isolated from flaxseeds have a protective effect on the colitis, which may be at least partially attributed to the inhibition of the production of inflammatory mediators such as reactive species of nitrogen and cytokines by immune cells, as well as the expansion of regulatory T cells. Together, this study confirms our hypothesis that the use of phenolic compounds and protein hydrolysates isolated from flaxseeds can improve inflammatory intestinal disorders.

Keywords

Anti-inflammatory, Flaxseed, peptides, phenolic compounds, inflammatory bowel disease, RAW 264.7.

6.1. Introduction

Inflammatory bowel disease (IBD) encompasses two main clinical entities: Crohn's disease (CD), which involves any segment of the gastrointestinal tract, and ulcerative colitis (UC), that occurs in the large bowel or rectum [1, 2]. Both are characterized by epithelial injury and chronic, relapsing and remitting inflammation in the gastrointestinal tract [2, 3]. The pathogenesis of IBD is not completely elucidated, but it has been reported that a pivotal event is a dysregulated immune response against antigens of the intestinal microbiota [4, 5]. Several therapies are available for patients with IBD. However, these treatments are expensive and limited by side effects and poor clinical efficacy [2, 6].

A better understanding of the mechanisms involved in the pathogenesis of IBD has been achieved with animals models [5]. Most of these experimental models are based on chemical induction, immune cell transfer or transgenic or gene targeting mouse strains to induce the disease [1, 7]. Chemical induced models are the most commonly used because of their rapid onset of inflammation, reproducibility and technical simplicity [7]. Among the chemical substances frequently used for induction of IBD are oxazolone, dextran sulfate sodium (DSS) and trinitrobenzene sulphonic acid (TNBS) [8]. Colitis induced by TNBS mimics CD in humans, which has a predominant T helper (TH) 1 and TH17 profile [5, 6, 9]. New therapies has focused on decreasing of pro-inflammatory cytokines and pathogenic TH1 and TH17 cells and on increasing the suppressive functions of T regulatory cells (Treg) to prevent the initiation and progression of the intestinal disease [6, 10].

Flaxseed is an oilseed widely studied for its beneficial health effects. It is a source of alpha linolenic fatty acids, phenolic compounds and soluble fiber, has anti-inflammatory and antioxidant capacities, and has been related to reduce risk of chronic diseases like cancer, obesity, and diabetes [11, 12]. Flaxseed is the richest source of plant lignans, due to its high content of secoisolariciresinol diglucoside (SDG) [13]. These phenolic compounds have phytoestrogenic, anti-inflammatory and antioxidant properties [13, 14]. In vitro studies showed that flaxseed protein hydrolysates have biological activities such as antioxidant, anti-inflammatory and cholesterol-lowering ability [15, 16].

In a previous paper, our group demonstrated that the antioxidant capacity of flaxseed protein hydrolysates and phenolic compounds was maintained or increased after the simulated gastrointestinal digestion, suggesting that these samples may exert a protective role against the reactive species in the intestinal lumen [17]. Thus, the aim of the present study was, first of all, evaluate the inhibitory capacity of flaxseed protein hydrolysates and phenolic fractions on the production of inflammatory mediators by stimulated macrophages.

Further, we investigate the effects of flaxseed protein hydrolysate and phenolic fraction on TNBS-induced colitis in BALB/c mice. To this purpose, the severity of colitis was accompanied by clinical and histological analysis, and in vitro T cell proliferative response, T cell prolife and cytokine secretion.

6.2. Material and Methods

6.2.1. Material and Reagents

Partially defatted brown flaxseed meal (PDBFM) was obtained from Cisbra Ltd. (Panambi, RS, Brazil). The enzymes Alcalase 2.4 L, Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), RPMI 1640 medium, HEPES, lipopolysaccharides (LPS); 2,4,6-trinitrobenzenesulphonic acid (TNBS), Paraffin, Eosin and Hematoxylin were purchased from Sigma (St. Louis, MO, USA). Interferon-γ (IFN-γ) was purchased from eBioscience (Pharmingen, San Diego, USA). ELISA kits were purchased from BD Biosciences (Pharmingen, San Diego, USA). Bovine fetal serum was purchased from Nutricell (Campinas, SP, Brazil)

6.2.2. Preparation of flaxseed protein hydrolysates and flaxseed phenolic fractions

The samples were obtained as described in a previous study [17]. The flaxseed hydrolysates and phenolic compounds were obtained from defatting of brown partially defatted flaxseed meal with hexane in a ratio of 1: 3 (w / v) for 24 h at room temperature to yield the defatted flaxseed meal (DFM). For polyphenols extraction, three consecutive extractions with 63% ethanol solution (v/v) were performed [18] to obtain the polyphenols-reduced DFM and phenolic isolate (Phi).

Flaxseed protein isolates (FPI) and phenolic-reduced FPI (phr-FPI) were prepared from DFM and phr-DFM, respectively. The corresponding flaxseed meal was dispersed in deionized water at a flour: water ratio of 1:10 (w / w), pH was adjusted to 9.0 using 0.5 mol / L NaOH, and the system was stirred at room temperature for 30 min and then centrifuged (2500 xg / 30 min; 25 ° C). The supernatant containing protein was filtered and the pH adjusted to 4.2 with 0.5 mol / L HCl. The precipitated protein was separated by centrifugation (2500 xg / 30 min), washed three times with acidified water (pH 4.2), suspended in deionized water and the pH adjusted to 6.0 with 0.5 mol / L NaOH [19]. It was then freeze-dried and stored at -20 ° C until use.

The hydrolysis of the FPI and phr-FPI was performed with Alcalase under the following conditions: protein concentration of 5% (w / v), 60 ° C, pH 8.5, and enzyme

substrate ratio 1:90 (w/w). The hydrolysis reaction was monitored using the pH-stat method using an automatic titrator DL model Metler 21 (Schwerzenbach, Switzerland) with stirring system coupled to a thermostatic bath. After 180 min, the reaction was stopped by heating at 90 ° C for 10 min. To obtain the phenolic compounds hydrolysate (Phh), the isolated fraction (Phi) was subjected to the same hydrolysis conditions of the isolates, but without addition of Alcalase. All samples were freeze-dried and stored at -20 °C for further use on in vitro and in vivo assays.

6.2.3. Cell culture assays

6.2.3.1. Cell lines

Two murine macrophage cell lines were used in this study. L929 cells (ATCC) were used to conduct viability assay and RAW 264.7 cells (kindly provided by Dr. Gustavo Amarante, USP, Brazil) were used to assess the production of inflammatory mediators. Both cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2.

6.2.3.2. Preparation of samples

For use in culture assays, freeze-dried flaxseed extracts were suspended in ultrapure water (20.0 to 0.010 mg/mL), shaken for 30 min, centrifuged at 36000 xg for 30 min at 10 °C, filtered through Nº1 Whatman qualitative filter paper The extracts were diluted ten times in culture medium and then sterilized by membrane filtration (0,45µm; Millipore).

6.2.3.3. Viability assay

The cell viability assay was assessed by MTT method, as described elsewhere [20]. Briefly, cells were seeded into 96-well microplates at a density of 1.5 $x10^5$ cells/well and incubated for 24 h. After removing the supernatants, cells were incubated with flaxseed extracts in concentrations ranging from 2.0 to 0.001 mg / mL in complete RPMI medium, for 24 hours at 37° C. Ten microliters of 5 mg/ml MTT in phosphate buffered saline (PBS) pH 7.2 were then added to each well and the plates were incubated for another 4 h at 37 ºC. The supernatants were removed and formazan crystals formed by MTT reduction in living cells were dissolved in dimethylsulfoxide (DMSO). The optical density was measured using a Multiskan MS microplate reader (Labsystems, Helsink, Finland) at 540 nm. The results were expressed as relative cell viability (%) using the blank treatment (RPMI 1640 complete medium) as control.

6.2.3.4. Production of inflammatory mediators

Raw 264.7 cells were seeded into a 24 well culture plate at a density of 4 x 10^5 cells/well and incubated for 24h at 37 ºC. After removing the supernatants, cells were treated with flaxseed extracts, inflammatory stimuli or extracts combined to inflammatory stimuli. Based on preliminary experiments (data not shown), flaxseed extracts were added in different concentrations (1.0; 0.1; 0.01 mg/mL for flaxseed protein samples and 0.5; 0.05; 0.005 mg/mL for flaxseed phenolic samples). The inflammatory stimuli were IFN-γ (100 ng/mL) and LPS (1 µg/mL) added in combination.

6.2.3.5. Measurement of nitrite concentration

Nitric oxide (NO) released into the supernatants of the cell cultures was indirectly determined using a quantitative colorimetric assay based on Griess reaction (1% sulphanilamide, 5% phosphoric acid, and 0.1% naphthylethylenediamine). Briefly, 50 µL of supernatant was mixed with the same volume of Griess reagent in a 96 well microplate. After 10 min at room temperature, the optical density was measured at 540 nm in a microplate reader (Multiskan II, MS, Labsystem, Finland), and the concentration of nitrite in the samples was determined by comparison with a standard curve of sodium nitrite (5 to 320 µM). Culture medium alone was used as the blank control. Nitrite concentrations were calculated by comparison to a calibration curve obtained with sodium nitrite standard solutions. The experiment was performed in quadruplicate.

6.2.3.6. Cytokines determination by ELISA kit

The levels of mouse interleukin 1β (IL-1 β), interleukin 10 (IL-10), interleukin 12 (p40) (IL-12 p40) and tumor necrosis factor α (TNF-α) were measured in macrophage supernatants according to manufacturer instructions (BD-OptEIA Mouse Set; BD Biosciences Pharmingen, San Diego, USA). Absorbance was read at 450 nm in a microplate reader (Multiskan II, MS, Labsystem, Finland), with the wavelength of 540 nm used for correction.

6.2.4. Experimental colitis

6.2.4.1. Animals

BALB/c female mice (20–25 g) at four weeks of age were obtained from the breeding center of the University of Campinas (CEMIB/UNICAMP). This study was carried out in accordance with the ‘Guide for the Care and Use of Laboratory Animals’, as promoted by the Brazilian College of Animal Experimentation (COBEA), and was approved by the Ethics

Committee for Animal Experimentation of University of Campinas (Protocol nº 3737-1). Before starting experiments, mice were allowed to acclimate for two weeks. A total number of 108 mice were used in three independent experiments. The animals were randomized into six groups containing six animals in each group. Group 1: TNBS group; Group 2: phr-FPH + TNBS group; Group 3: TNBS + phr-FPH group; Group 4: Phi + TNBS group; Group 5: TNBS + Phi group; Group 6: Normal group. The experiment lasted 14 days and colitis was induced on day eight. Groups 2 and 4 received flaxseed phenolic extracts daily from day 1 to 7. Groups 3 and 5 received flaxseed protein hydrolysate extracts daily from day 8 to 14.

6.2.4.2. Preparation and administration of flaxseed extracts

For in vivo assays, freeze-dried flaxseed extracts were suspended in 0,15 M saline solution (20 mg/mL), shaken for 30 min, centrifuged at 36000 xg for 30 min at 10 °C, filtered through Nº1 Whatman qualitative filter paper and stored at -20°C in dark until use.

6.2.4.3. Induction of experimental colitis in mice

Colitis was induced by intracolonic administration of a single dose of 2,4,6-trinitrobenzenesulphonic acid (TNBS) on day eight, as described elsewhere [Neurath et al., (1995)] with modifications. Briefly, mice were anesthetized with halothane and then 100µL of 1.0mg/mL TNBS dissolved in 50% ethanol were instilled into the lumen of the colon by a cannula positioned 3.5–4.0 cm from the anus. To ensure the agent enter entire colon, mice were held in a vertical position for 30 s. The control group received 100 µL of 50% ethanol by intracolonic route. For treatments with flaxseed extracts, daily doses of 200mg/kg body weight were administered by gavage.

6.2.5. Parameters utilized to analyze the severity of TNBS-induced colitis

6.2.5.1. Body weight change

The weight of each animal was measured, starting on the day after TNBS treatment, and the change in weight was calculated as a percentage, the baseline (marked as 100 %) being taken as the weight on the day of the TNBS challenge.

6.2.5.2. Microscopic analysis

Two segments of colon, 1 cm each, were taken at 4 cm from the anus, fixed in 4% buffered formalin and dehydrated with grade ethanol solution. After embedded in paraffin, 5 µm slices were obtained, mounted on clean glass slides, deparaffinized and rehydrated.

Then the slices were dyed with hematoxylin and eosin (H&E). Slices were observed in an optical microscope (Eclipse E-800 Microscope, NIKON; Tokyo, Japan).

6.2.6. Spleen cell proliferation

Mice of all groups were killed and spleens were aseptically removed. The spleens were macerated individually, suspended in lysis buffer and pelleted by centrifugation. After washing, spleen cell suspensions were incubated with carboxyfluorescein succinimidyl probe ester (CFSE) at room temperature for 5 min according manufacturer's recomendations. Cells were then pelleted by centrifugation and suspended in complete medium. To determine the maximum value of incorporation of CFSE, aliquots of the cell suspensions were fixed with 1% formaldehyde in PBS and storage at 4º C until cytometer analysis. Labeled cells are then seeded in 96-well plates (Corning) at a density of 5.0 x 10^5 cells/well in the presence of 2.5 µg/mL ConA, in sextuplicate, and then incubated for 96 hours at 37 ºC. Cell cultures in absence of stimuli were used as control. Supernatants were collected for dosage of cytokines. The proliferation of T lymphocytes in cultures was assessed in gate of $CD4^+CFSE^+$ cells by flow cytometry (FACSCalibur flow cytometer, BD Becton Dickinson, San Jose, CA). The results were analyzed with the software FCSExpress, and expressed as proliferation index (fold change), calculated by dividing the percentages found in the experimental groups by the percentage found in the control group.

6.2.7. Phenotypic profile of T-cells by Flow Cytometry

The frequencies of T cell subsets were evaluated by flow cytometer, using anti-CD4-PE (Clone: GK1.5) and anti CD25-FITC (Clone: 7D4). Cells were permeabilized with fixation/permeabilization buffer (Cytofix/Cytoperm fixation/permeabilization kit, Becton-Dickinson, BD) and labeled with anti-Foxp3- APC (Clone: FJK-16s), anti-IL-17-APC (Clone: Ebio17B7) or anti-IL-17-Alexa Fluor (Clone: TC11-18410), anti-IFN-γAPC (Clone: XMG1.2), IL-10+ APC (Clone: JESS-16E3). All FACS reagents were purchased from eBioscience (San Diego, CA, USA) and used following manufacture's recommendation. Preparations were acquired in FACSCalibur flow cytometer (BD Becton Dickinson, San Jose, CA) and results were analyzed by the software FCS express v5.0 Plus Research Edition (FCS Express Launcher).

6.2.8. Determination of cytokines in spleen cells supernatants

The levels of IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ and TNF-α in spleen cell supernatants were measure using a BD CBA Mouse Th1/Th2/Th17 cytokine kit (BD

Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. The mean fluorescence intensity was detected using FACSCalibur flow cytometer. Data were analyzed by the software FCAP Array v3.0 software (BD Biosciences, San Jose, CA, USA).

6.2.9. Statistical Analysis

The statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The statistical significance of differences between control and experimental groups were determined by one-way ANOVA, followed by Bonferroni's test for multiple comparison. The results were expressed as mean ± standard deviation. Values were considered significant at $P > 0.05$. All data presented are representative of at least three independent experiments.

6.3. Results and Discussion

Most studies on bioactivity of phenolic compounds and protein hydrolysates mainly consider its antioxidant activity. However, these compounds can also present anti-atherogenic, anti-hypertensive and anti-inflammatory actions. Investigations into the mechanisms by which phenolic compounds have anti-inflammatory activity have shown that such effects are not due merely to its antioxidant activity, but is related to its ability to modulate intracellular pathways [21]. Similarly, the few studies concerning the anti-inflammatory activity of food derived peptides suggest that they may also modulate inflammatory pathways [22]. In this work, we demonstrated the in vitro anti-inflammatory activity of protein hydrolysates and phenolic fractions of flaxseed on RAW 264.7 cells stimulated by LPS+IFN-γ. We also find out that protein hydrolysates and phenolic fractions from flaxseed reduced the clinical and immunological parameters of TNBS-induced colitis in an experimental BALB/c model.

6.3.1. In vitro activity of protein hydrolysates and phenolic compounds of flaxseeds

As shown in Figure 6.1, protein hydrolysates and phenolic compounds from flaxseed from 0.001 to 2.0 mg/mL had no significant cytotoxic effects on L929 cells. These results indicated that the effects of protein hydrolysates and phenolic compounds of flaxseed on macrophages are not due to the reduction of cell viability. Thus, in the subsequent cell assays protein hydrolysates were used in concentrations ranging from 0.001 to 1.0 mg/mLand phenolic compounds from 0,005 to 0,5 mg/mL.

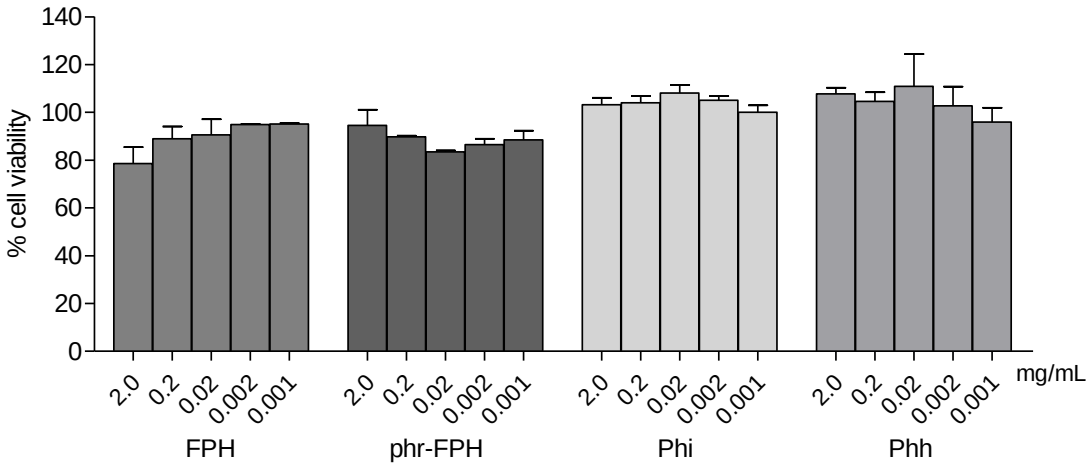

Figure 6.1. Flaxseed protein hydrolysates and phenolic fractions did not affect cell viability**.** Flaxseed protein hydrolysate (FPH), phenolic reduced Flaxseed protein hydrolysate (phr-FPH), Phenolic compounds isolate (Phi), Phenolic compounds hydrolysate (Phh). Values expressed as mean of quadruplicates ± standard deviation. These results are representative of two independent assays.

The effects of protein hydrolysates and phenolic compounds obtained from extracts of flaxseed on the production of inflammatory mediators induced by IFN-γ+LPS were examined in RAW 264.7 cells. IFN-γ is a pro-inflammatory cytokine that stimulates pro-inflammatory gene expression such as inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2). LPS is an endotoxin found in the membrane of Gram negative bacteria that stimulates inflammatory responses through Toll-like receptor (TLR-4) and promote the activation of intracellular signaling pathways like NF-κB, MAPK and Akt1 [23]. Our results show that, in the concentrations used here, IFN-γ+LPS were able to induce the production of NO and TNF-α in cultures of RAW 264.7 cells, but not IL-1β, IL-10 and IL-12 p40 (Figure 6.2).

The treatment with higher concentrations of protein hydrolysates (1.0 mg/mL) and phenolic compounds (0.5 mg/mL) from flaxseed showed an inhibitory effect with a reduction up to 40% on NO production (Figure 6.2 A). TNF-α secretion was inhibited in a dose-dependent manner by protein hydrolysates and phenolic compounds-treatments. The inhibition of TNF-α secretion by FPH, phr-FPH and Phh ranged from 20 to 30% while the treatment with Phi, the non-hydrolyzed phenolic fraction, presented the highest inhibition, ranging from 40 to 65% (Figure 6.2 B).

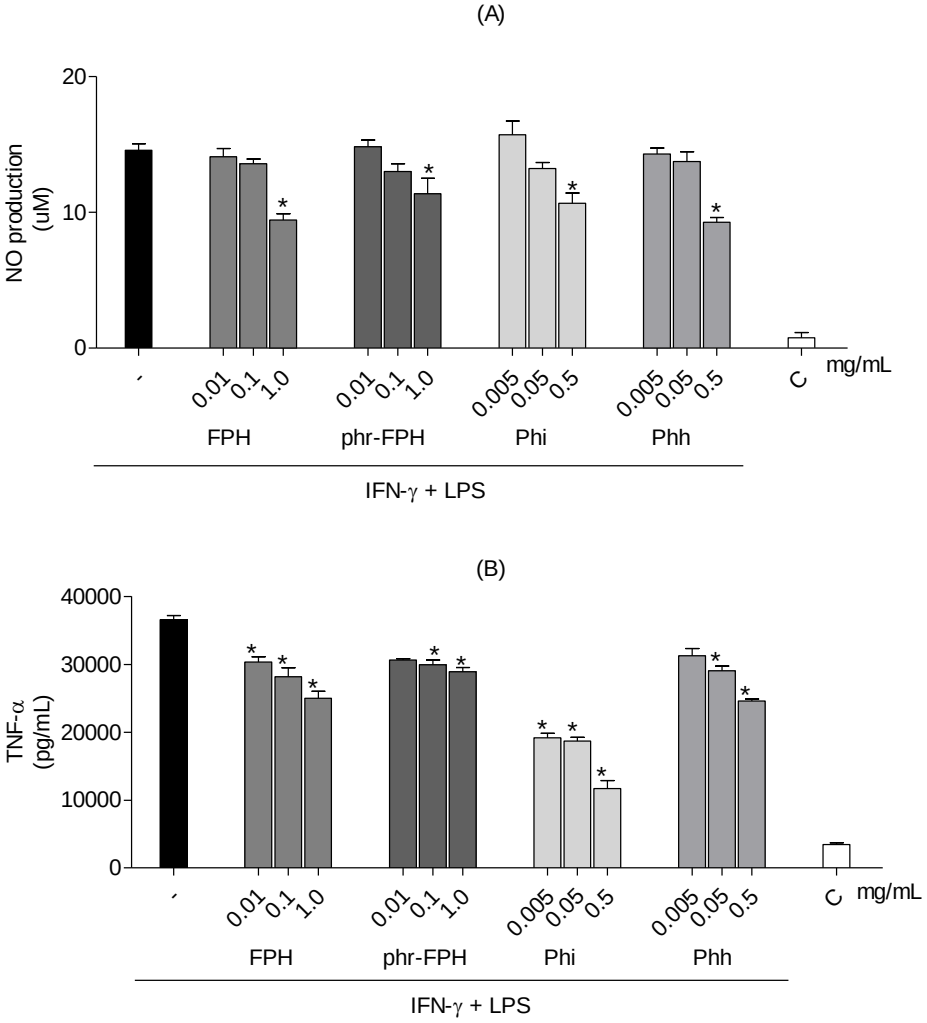

Figure 6.2. Treatment with flaxseed protein hydrolysates or phenolic compounds reduce NO and TFN secretion. Effects of protein hydrolysates (FPH and phr-FPH) or phenolic compounds (Phi and Phh) were evaluated on NO (A) and TNF-α (B) production by RAW 264.7 macrophages. Cells were stimulated by 24 hours with LPS+INFγ and treated with compounds at the indicated concentrations. The amount of NO released into the culture supernatants is expressed as nitrite. Values expressed as mean of quadruplicates and ± standard deviation (SD). Values marked with * are significantly different ($p < 0.05$) compared to INF-γ+LPS-stimulated cells without sample treatment. These results are representative of three independent assays. Flaxseed protein hydrolysate (FPH); phenolic reduced Flaxseed protein hydrolysate (phr-FPH); Phenolic compounds isolate (Phi); Phenolic compounds hydrolysate (Phh); Control (C), cell without treatment and stimuli.

Our results suggest that both protein hydrolysates and phenolic compounds have a potential anti-inflammatory activity, verified by their capability to inhibit NO and TNF-α production. Corroborating our data, literature has shown that compounds capable to inhibit NO and TNF-α production are potential anti-inflammatory agents, since inhibition of these mediators correlates to reduced oxidative stress, inflammatory response and risk of developing various chronic diseases such as cancer and heart diseases [24-26].

There is limited information in the literature, however, regarding the anti-inflammatory effects of flaxseed protein hydrolysates. A previous study by Udenigwe et al., (2009) reported that enzymatic hydrolysis of flaxseed proteins by various proteases resulted in inhibition of NO production in macrophages. Our results reinforce the anti-inflammatory potential of flaxseed peptides, since FPH and phr-FPH showed an inhibitory effect on the production of two important mediators of inflammation, i.e., nitric oxide and TNF-α. Similarly, the anti-inflammatory activity of Phi and Phh found here corroborate with several other studies with polyphenols and its inhibitory activity on NO and TNF-α production [27, 28].

In a previous work we have shown that FPH and phr-FPH are composed mainly by peptides. The differences between them are the presence of phenolic compounds in FPH. The similarity among inhibitory activities of these hydrolysates suggest that there is no synergist effect between peptides and phenolic compounds that could enhancement the anti-inflammatory activity of FPH in comparison to the phr-FPH. Moreover, the difference in the ability to inhibit TNF-α by Phi and Phh samples ($p > 0.05$) suggests that hydrolysis conditions (temperature and alkaline pH) could modify phenolic compounds by reducing its anti-inflammatory activity.

In a previous work we have also shown that FPH, phr-FPH, Phi and Phh have different antioxidant capacities, measured by FRAP and ORAC methods. In general, Phh has the highest FRAP and ORAC values while phr-FPH has the lowest FRAP and ORAC values. As antioxidant compounds, the anti-inflammatory activity of these flaxseed samples may be explained by its ability to neutralize reactive oxygen species (ROS). Excessive ROS production by inflammatory cells causes cellular and tissue damage, which in turn augments inflammation [29]. ROS could also trigger inflammatory signaling pathways such as nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) [30]. NF-κB is a redox-sensitive transcription factor that induces expression of genes associated with pro-inflammatory response, cell proliferation and apoptosis [31]. Both NK-κB and MAPK induced pro-inflammatory signaling transduction factors, producing cytokines such as IL-1β, IL-6, IL-8, TNF-α and IFN-γ [30]. Thereby, the scavenging ROS activity by flaxseed samples could decrease the activation of these pathways, leading in inactivation of inducible oxide nitric synthase (iNOS), responsible for increase NO production during inflammation process, and also in the inhibition of pro-inflammatory cytokines [21, 27]. However, the similarity between anti-inflammatory activity showed by FPH, phr-FPH and Phh ($P > 0.05$) indicates that this inhibitory effect on NO and TNF-α production is not directly related to its antioxidant activities, suggesting that will be other mechanisms responsible for such effect, possibly related to the proteinaceous nature or the phenolic nature of the samples.

Besides its antioxidant activity, FHP and phr-FPH may also reduce transcriptional activity of NF-κB by a variety of other signaling events. In un-stimulated cells, NF-κB is in an inactive form, bonded to inhibitor κB (IκB) proteins. Upon cell stimulation IκB proteins are phosphorylated by IκB kinases releasing the NF-κB, which is translocated to nucleus and induces the expression of various genes [27]. Thus, we suggest that the capability of flaxseed peptides to inhibit pro-inflammatory mediators may occurred through inactivation of IκB kinase complex or by inhibition the translocation of NF-κB p50 and p65 sub-units, since other food derived peptides have showed this ability [31-33].

There is no general rule concerning a structure/activity relation that could explain the mechanisms by which peptides exert anti-inflammatory activity. Protein hydrolysates are composed by peptides that differ by the amount, type and sequence of amino acids in its structure [34]. Based on the literature, all of them might be a potential anti-inflammatory agent, since different food derived-peptides with different sequences and molecular weight ranges present anti-inflammatory activity [32, 35-37].

In addition to reducing oxidative stress-mediated inflammation, phenolic compounds can also exert anti-inflammatory activity by modulation of NF-κB pathway by different molecular mechanisms [27, 38]. Possibly, one of these mechanisms is through inhibition of NF-κB DNA binding by the catechol moiety of caffeic acid, one of phenolic compounds identified in Phi and Phh samples. According to the literature, the catechol moiety may interact with the nucleophiles of cysteine sulfhydryl groups of p65 subunit of NF-κB, leading to the inhibition of this transcription factor binding to the DNA [39, 40].

Regarding IL-10, our data agree with those obtained by Millán-Linares et al., [36] for lupine protein hydrolysates and by Sergent et al., [41] for resveratrol, phenolic acids and curcumin. Results suggest that the mechanism by which flaxseed protein hydrolysates and phenolic compounds inhibit the production of pro-inflammatory mediators in IFN-γ+LPS induced RAW 264.7 cells does not involve the enhancement of anti-inflammatory cytokines, since the IL-10 was not detected in supernatants of treated cells. To the best of our knowledge, this work is the first to report the in vitro anti-inflammatory activity of flaxseed phenolic fractions.

Taken together, *in vitro* results suggest that protein hydrolysates and phenolic fractions from flaxseed are non-cytotoxic against the murine macrophage cells within the range of concentrations studied and might be helpful for preventing inflammatory diseases. However, this *in vitro* study has some limitations, since the anti-inflammatory activity from flaxseed samples may vary between cell types [28] and the stimuli applied [27]. In addition,

we have to consider that, *in vivo,* polyphenols and protein hydrolysates can suffer some chemical modifications during digestion and absorption which may compromise its antioxidant and anti-inflammatory potential [42]. Since all samples studied showed anti-inflammatory activity in INF-γ+LPS stimulated RAW 264.7 cells, we chose one sample with compounds of protein nature (phr-FPH) and one with mainly phenolic compounds (Phi). These samples were applied to further studies to investigate the anti-inflammatory activity in a TNBS colitis model.

6.3.2. In vivo anti-inflammatory activity

Intrarectal administration of a single dose of TNBS in BALB/c mice produces CD-like acute colitis, characterized by leukocyte infiltration, crypt destruction, and mucosal ulceration associated with diarrhea, wasting, rectal prolapse and rectal bleeding [43]. This model is considered valuable for studying not only epithelial disruption, but also T cell proliferation, differentiation and cytokines pattern, as well as the potential effect of new immunotherapies for human CD [1, 5, 44].

In this study, phr-FPH and Phi samples were administered before and after colitis induction in order to study the possible preventive or inhibitory effect on these samples experimental colitis. The administration of TNBS caused a dramatic decrease of body weight in all groups. Mice treated with Phi previous or concomitantly to colitis induction reduced weight loss and had better recovery in comparison to TNBS group (Figure 6.3).

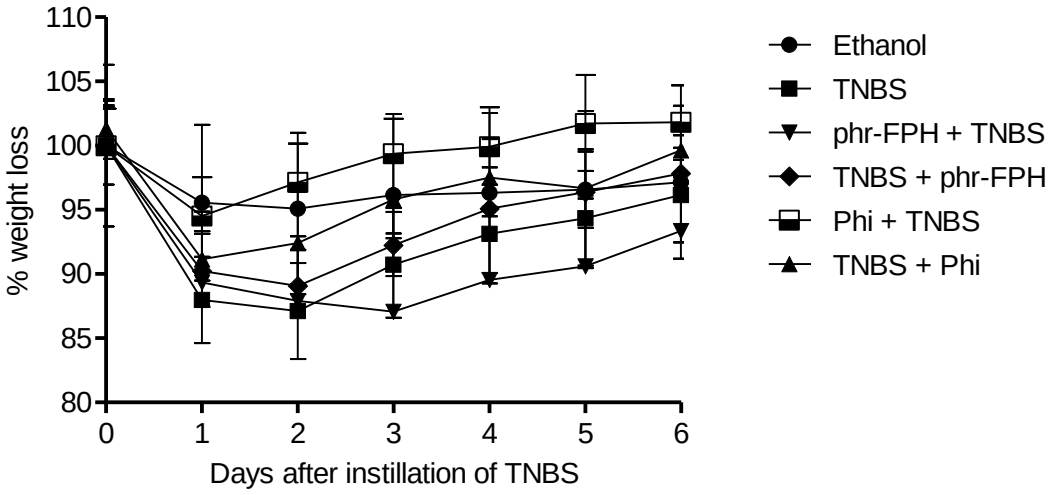

Figure 6.3. Flaxseed phenolic compounds reduced weight loss in experimental colitis model. Data are represented as means ± SD. The data presented are representative of three independent experiments with similar results.

Figure 6.4 shows the histological sections of intestinal mucosa after Hematoxylin and Eosin (HE) staining. Mice treated with TNBS (Figure 6.4B) presented high leukocyte infiltration and damage in colon tissue. All treatments analyzed, especially previous phr-FPH and Phi administration (Figure 6.4C and 6.4D), showed a reduction in morphological damage and preservation of mucosal structure in comparison with the TNBS group. Those treated groups presented decreased infiltration of inflammatory cells as well as reduced thickening of the colon wall and loss of goblet cells. These results suggested that both phr-FPH and Phi attenuated colonic injury in TNBS induced colitis in BALB/c mice.

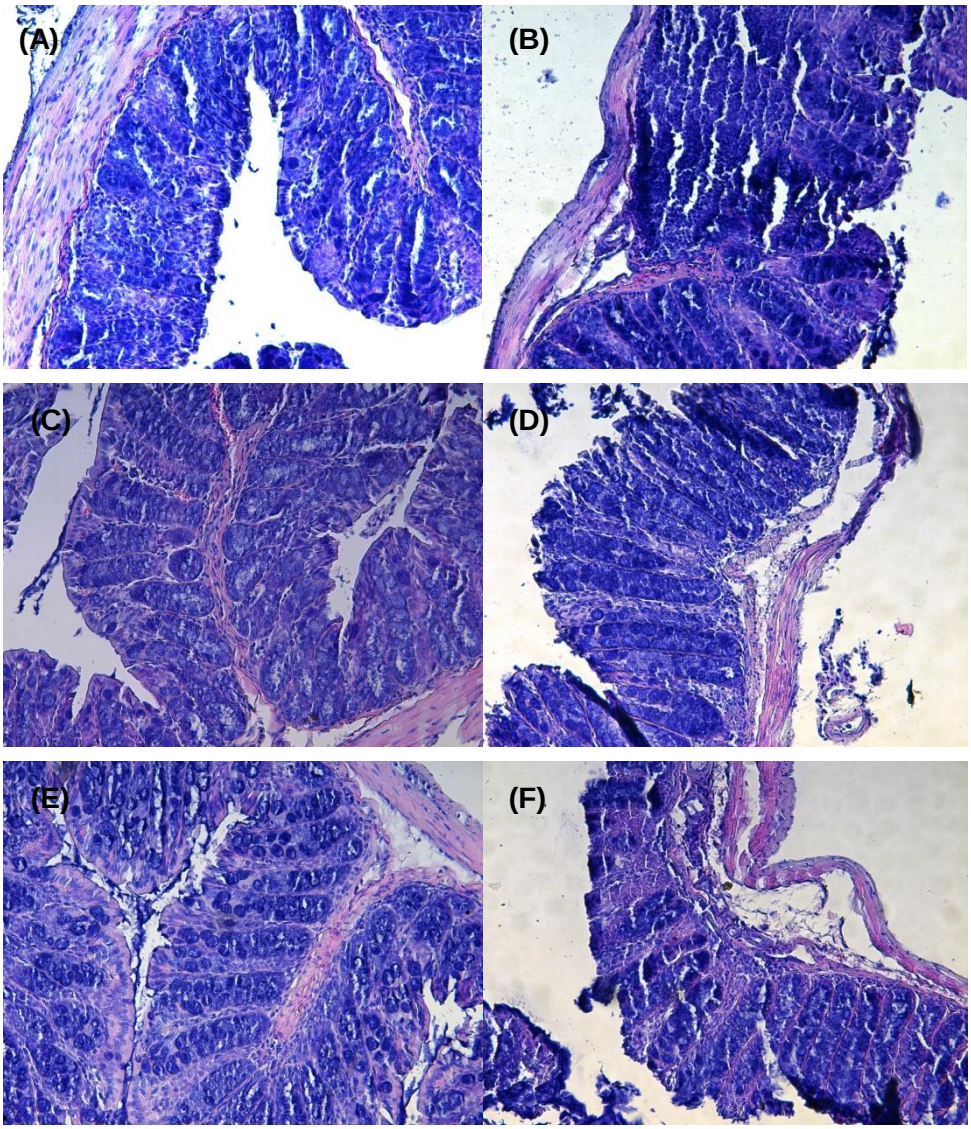

Figure 6.4. Treatment with flaxseed protein hydrolysates or phenolic preserve mucosal structure and decreased infiltration of inflammatory cells. Histological colonic sections (Hematoxylin and Eosin, 20x). The data presented are representative of three independent experiments with similar results. (A) Ethanol group; (B) TNBS group; (C) phr-FPH + TNBS group; (D) TNBS + phr-FPH group; (E) Phi + TNBS group; (F) TNBS + Phi group.

We next evaluate the effects of treatment with phr-FPH and Phi on $CD4^+$ T cells proliferation and phenotypic profile in spleen cells. As shown in Figures 6.5 and 6.6, the proliferation of $CD4^+$ T lymphocytes from TNBS+Phi group was significantly reduced in comparison to TNBS group, while the treatment with phr-FPH, before and after colitis induction, did not significantly alters T lymphocyte proliferation. In mice concomitantly treat with Phi, the frequency of $CD4^+CD25^+Foxp3^+$ cells was significantly increased while the frequency of $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}17^+$ decreased, in comparison to the TNBS group. We also observed that previous treatment with phr-FPH or Phi was able to decrease the frequency of $CD4^+IFN\text{-}\gamma^+$ cells.

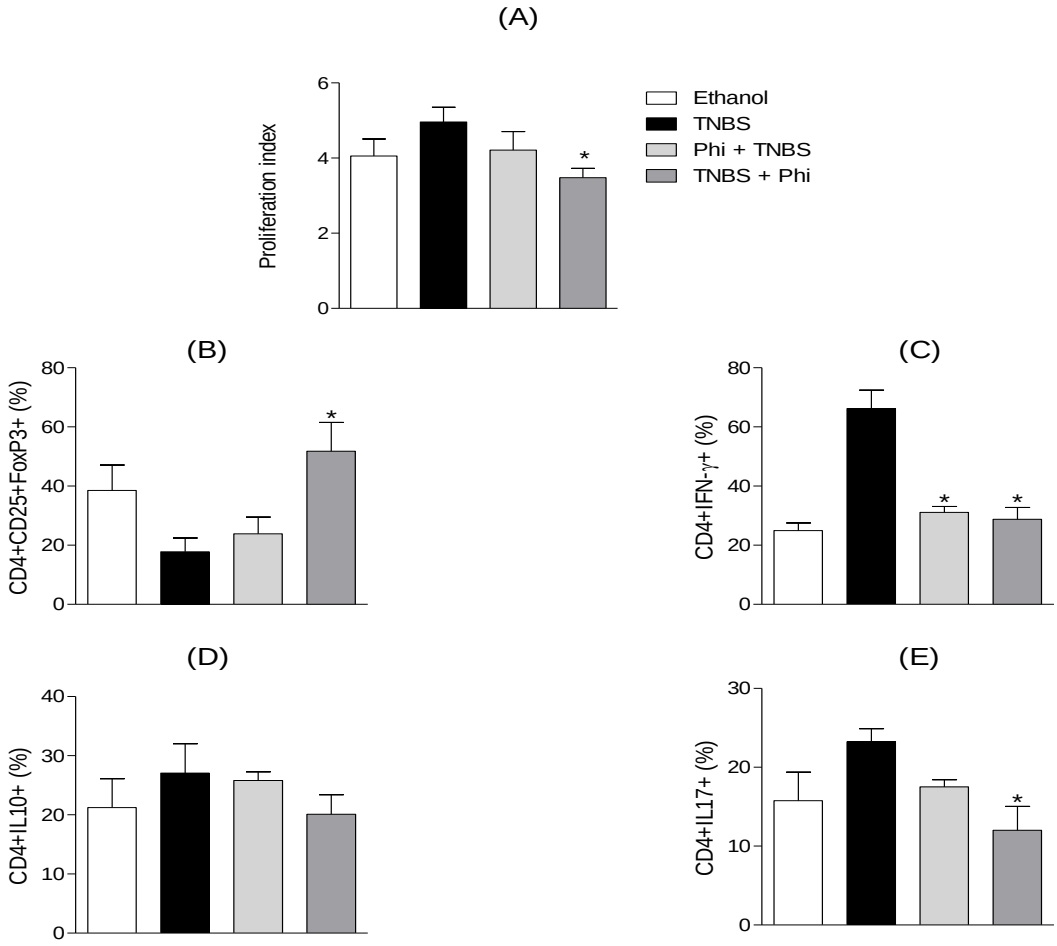

Figure 6.5. Suppression of proliferation of T lymphocytes, increased proportion of $CD4^+CD25^+Foxp3^+$ cells, decreased proportion of $CD4^+IFN\text{-}\gamma^+$ cells and $CD4^+IL\text{-}17^+$ producing-T cells after oral administration of Flaxseed phenolic isolate (Phi), either before or after TNBS-induced colitis in BALB/c mice. (A) T cell proliferation was determined using flow cytometry and assessed by fluorescence decay of the probe in the gate of $CD4^+$ cells. (B) The frequency of $CD25^+Foxp3^+$ cells and (C) $IFN\text{-}\gamma^+$, (D) $IL\text{-}10^+$ and (E) $IL\text{-}17^+$ producing cells were evaluated in the gate of $CD4^+$ cells and were evaluated in the gate of $CD4^+$ cells.. Data in % bar graphs represent the mean ± S.E.M. (n = 4) of cell frequencies obtained in three independent experiments. One-way ANOVA followed by Bonferroni's post-test were used to determine statistical significances; $P < 0.05$ in comparison with TNBS group.

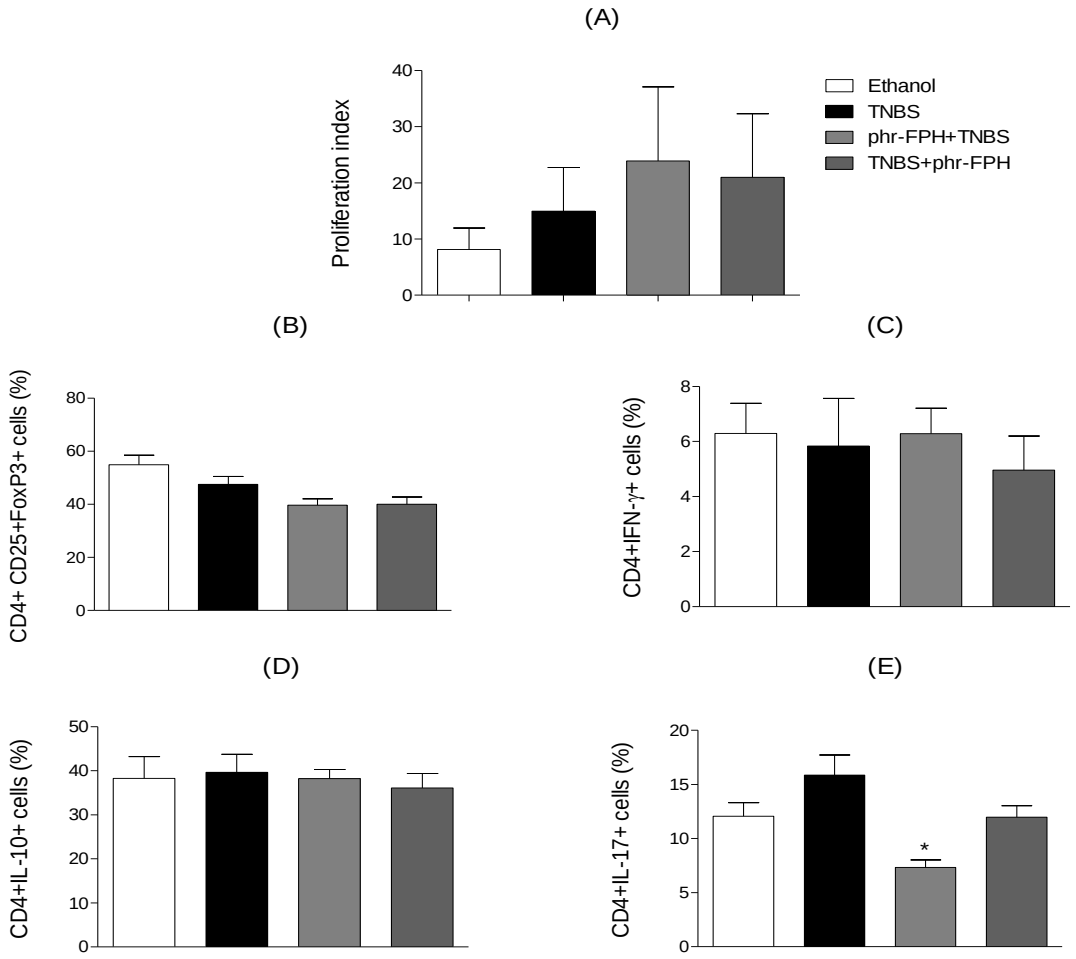

Figure 6.6. Suppression of $CD4^{+}IL\text{-}17^{+}$ producing-T cells after oral administration of Flaxseed protein hydrolysate (Phi) before TNBS-induced colitis in BALB/c mice. (A) T cell proliferation was determined using flow cytometry and assessed by fluorescence decay of the probe in the gate of $CD4^{+}$ cells. (B) The frequency of $CD25^{+}Foxp3^{+}$ cells and (C) IFN-γ^{+}, (D) IL-10^{+} and (E) IL-17^{+} producing cells were evaluated in the gate of $CD4^{+}$ cells and were evaluated in the gate of $CD4^{+}$ cells.. Data in % bar graphs represent the mean ± S.E.M. (n = 4) of cell frequencies obtained in three independent experiments. One-way ANOVA followed by Bonferroni's post-test were used to determine statistical significances; $P < 0.05$ in comparison with TNBS group.

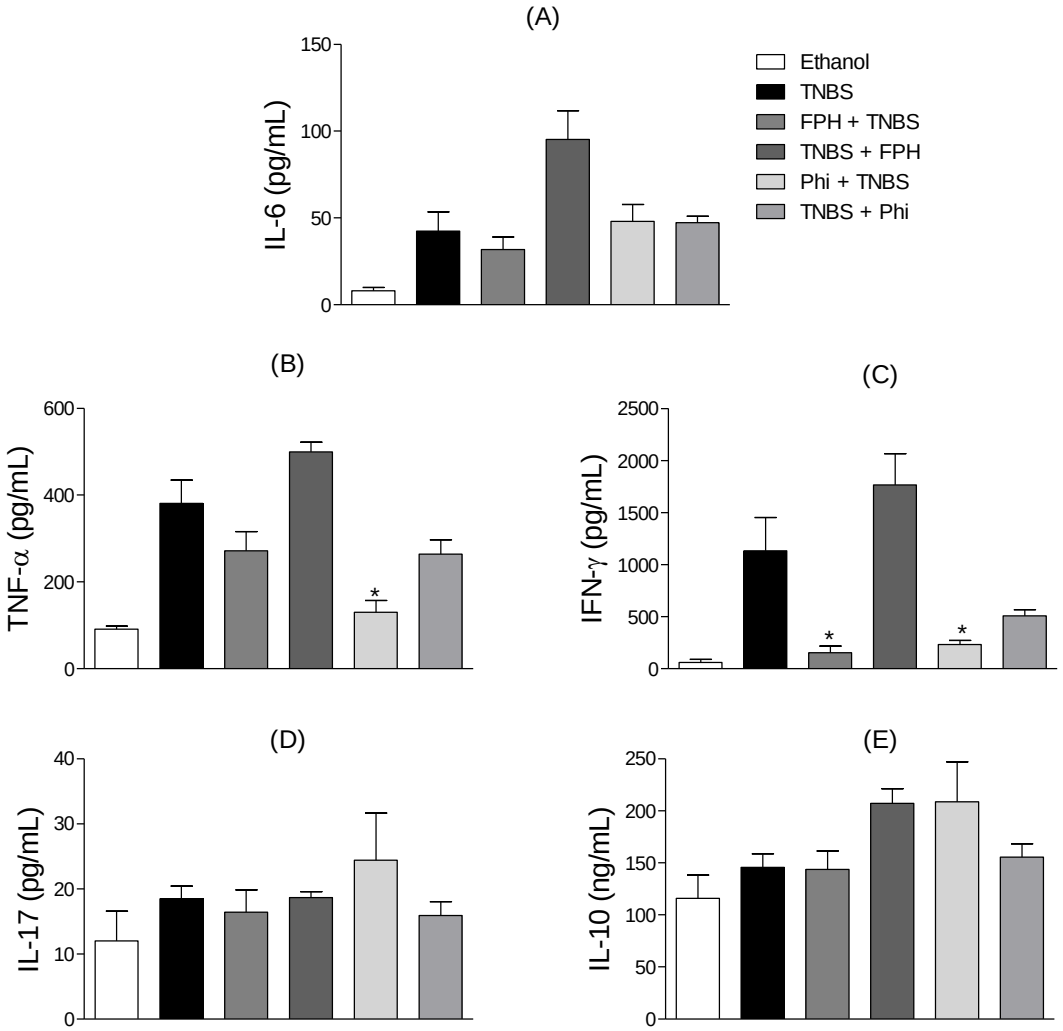

Figure 6.7. Oral treatment with flaxseed protein hydrolysate (Phi) reduced IFN-γ Secretion while phenolic compound reduced TNF-α and IFN-γ secretion in spleen cell cultures from colitic BALB/c mice. Culture supernatants were collected to dosage of IL-6, TNF-α, IFN-γ, IL-17, IL-10 (A-E, respectively) using commercial CBA Mouse $T_H1/T_H2/T_H17$ kit, according to manufacturer's instructions (e-Bioscience). Data in bar graphs represent the mean ± S.D. (n = 4) of cell frequencies obtained in three independent experiments. One-way ANOVA followed by Bonferroni's post-test were used to determine statistical significances; $P < 0.05$ in comparison with TNBS group.

Regarding to cytokine levels, the previous treatment with Phi reduced TNF-α and IFN-γ production while the previous treatment with phr-FPH reduced only IFN-γ production (Figure 6.7). The secretion of IL-2 and IL-4 were not detected in measurable levels in spleen cells supernatants (data not shown), confirming that these single administration of TNBS to BALB/c produces CD-like acute colitis characterized by dominant Th1 response.

In TNBS acute colitis, the inflammatory process is driven by a massive lymphocyte T helper (T_H) 1 / T_H17-mediated response, characterized by increased production of mainly IFN-γ and IL-17, respectively. IFN-γ stimulates macrophages to produce TNF-α, IL-1β, IL-2, IL-6 that together exacerbate inflammation and magnify T_H1 response in a positive feedback loop [2, 9, 45-47]. IL-17 secretion initiate and amplify the inflammatory process by promoting T_H1 and T_H17 responses and also activates anti-regulatory mechanisms on the intestinal epithelium [9]. Our previous treatment with Phi and with phr-FPH downregulated $CD4^+IFN\text{-}\gamma^+$ cells while treatment with Phi after colitis induction downregulated $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}17^+$ (Figures 6.5 and 6.6). In addition, levels of IFN-γ and TNF-α in spleen cells supernatants were reduced by both Phi and phr-FPH in previous treatment and exhibited similar trends in concomitants treatment with Phi (Figure 6.7). These results suggest that the protective effect of Phi and phr-FPH against TNBS-induced colitis is closely associated with the suppression of the T_H1 and/or T_H17 cells subsets, reducing their inflammatory potential and thus attenuating intestinal inflammation.

The treatment with Phi after colitis induction also increased the number of $CD4^+CD25^+Foxp3^+$ cells, known as T regulatory (T_{reg}) cells. These cells play an important role in maintaining immune tolerance and controlling the immune response by initiating suppressive effects on activated immune cells. According to Zhao et al., [10] dowregulation of T_{reg} cells led to an imbalance of T_{reg} and T_H17 cells and also of T_H1 and T_H2 cells. This imbalance results in an overexpression of pro-inflammatory cytokines and concomitant decreased in anti-inflammatory cytokines, such as IL-10, TGF-β and IL-35, resulting on inflammatory damages in the colonic mucosa. Thus, the suppression of the T_H1 and T_H17 cells subsets by concomitant treatment with Phi could be explained by the increase of the suppressive functions of T_{reg}, suggesting that the ingestion of phenolic compounds from flaxseed could regulate the inflammatory process in TNBS-induced colitis.

It is well established that modulating cytokine responses is crucial to control the inflammatory mechanisms underlying colitis [3]. In IBD, TNF-α is one of the most important pro-inflammatory cytokines and is the key regulator of the inflammatory process in TNBS-colitis model [48]. Besides TNF-α promotes activation of effector T cells and increase pro-

inflammatory cytokine production mentioned above, this cytokine also induces hypervascularization, angiogenesis, epithelial cells apoptosis and promotes tissue destruction [3, 48]. Due to its various inflammatory functions, blocking TNF-α activity has been the target of clinical therapies involving IBD [2, 3, 49]. Therefore, the inhibitory activity of Phi and phr-FPH treatment on TNF-α secretion (Figure 6.7) suggests that these compounds could be a promising alternative for the development of new immunotherapies against IBD.

Respect to anti-inflammatory cytokine IL-10, our treatment did not enhance its production compared with TNBS group (Figure 6.7). Corroborating our data, da Silva et al. [50] demonstrated that phenolic compounds from *Abarema cochliacarpos* was not able to increase IL-10 levels in TNBS colitis model. These *in vivo* results are in accordance with our *in vitro* results showed above and reinforce our hypothesis that the mechanism by which hydrolysates and phenolic compounds from flaxseed act as anti-inflammatory is not by enhancement of anti-inflammatory cytokines.

Oxygen reactive species (ROS) play a crucial role in the pathogenesis of intestinal inflammation [48]. The production of hydroxyl radical, superoxide, peroxide and others ROS by activated phagocytic leukocytes is thought to be a major cause of mucosal damage and tissue destruction [48]. Also, excessive ROS production has been associated with the initiation and progression of IBD [51, 52]. In colon tissue, ROS is responsible for disruption of tight-junction barrier, leading to an increase in intestinal neutrophil permeability and bacterial toxin infiltration [4]. The anti-inflammatory activity showed by Phi and phr-FPH-treatments could be related, in part, to the antioxidant activity of these compounds (Figures 6.5-6.7). As antioxidant compounds, they can decrease ROS production in the lumen, reducing its harmful action on the intestinal mucosa and therefore ameliorating chronic intestinal inflammation.

According to the literature, components of a dysregulated intestinal microbiota provide antigens that are necessary for initiating and perpetuating colonic inflammation in dextran sulfate sodium (DSS) and TNBS-induced colitis models as well as in the development of human IBD [10, 53]. Several studies in mice and humans have demonstrated that polyphenols and its metabolites can modify microbiota components, increasing the composition and activity on nonpathogenic bacteria [42, 54-56]. Moreover, phenolic compound metabolites can pass the enterocyte barrier and modulate intestinal immune responses [57]. According to Corsini et al., [57], enterolignans, an metabolite derived from microbial metabolism of secoisolariciresinol (SDG), one of phenolic compounds identified in

Phi, can decreased TNF-α secretion by preventing the I-κB degradation and NF-κB activation.

6.4. Conclusions

Flaxseed protein hydrolysates and phenolic fractions inhibited the production of anti-inflammatory cytokines in cell culture. However, the presence of phenolic compounds in flaxseed protein hydrolysate did not increase its anti-inflammatory potential in compare to phenolic reduced-flaxseed protein hydrolysate. The concomitant treatment with flaxseed phenolic or peptide fractions inhibit TNBS-induced colitis in mice by downregulating T_H1 and T_H17 responses, immune cell proliferation and inflammatory cytokine levels, systemic indicators of colonic mucosal injury and inflammation were inhibited in flaxseed treated mice.. Furthermore, the phenolic compound administration increased T_{reg} cells lymphocytes on TNBS-induced colitis in BALB/c mice. Nevertheless, the treatment with these flaxseed products decreased inflammatory cytokine production in spleen cell supernatants.

Thereby, we could demonstrate our initial hypothesis that consumption of flaxseed-based diets would beneficially attenuate TNBS-induced colitis severity due to its anti-inflammatory effects based on its peptides and phenolic compounds. In fact, our results provide strong evidence that flaxseed phenolic compounds and protein hydrolysate are able to ameliorate or prevent intestinal inflammation in TNBS-colitic mice and may yield a novel immunotherapy for IBD.

Possibly, the protective effect showed by Phi treatment, before and after colitis induction, could be partially attributed to the modulation of intestinal microbiota and the anti-inflammatory activity of its metabolites. Further studies will be developed to evaluate the role of the microbiota in our model. To the best of our knowledge, this is the first study that shows the anti-inflammatory activity of flaxseed protein hydrolysates and phenolic fractions *in vivo*.

Acknowledgments

F.G.D. Silva acknowledges the CNPq and L.N. Payatto acknowledges the FAPESP (#2014/ 08591-0) for the scholarship granted. This work has received financial support from FAPESP (grant# 2013/20258-3).

Conflict of interest

The authors declare that they have no competing interests.

6.5. References

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CAPÍTULO 7. DISCUSSÃO GERAL

7.1. Discussão Geral

Em estudos anteriores em nosso laboratório verificamos que farinha, concentrado e principalmente o hidrolisado proteico de linhaça apresentaram capacidade antioxidante *in vitro.* Depois de submetidos à simulação da digestão gastrintestinal, observamos que não houve redução da capacidade antioxidante desses produtos e que a atividade do concentrado proteico foi superior a do hidrolisado (SILVA, O'CALLAGAHAN, O'BRIEN *et al.*, 2013). No entanto, não foi possível elucidar se a atividade antioxidante encontrada nesses produtos proteicos é devida aos peptídeos antioxidantes, aos compostos fenólicos ou ao complexo proteína:fenólico resultante da interação entre estes compostos.

Dessa forma, esse trabalho teve como objetivo estudar a interação de proteínas e fenólicos na bioatividade do hidrolisado proteico de duas formas. A primeira como um redutor em meio biológico, capaz de atuar junto ao sistema de defesa antioxidante minimizando os possíveis efeitos deletérios que o estresse oxidativo pode provocar no organismo. A segunda, como um agente anti-inflamatório, capaz de modular a resposta inflamatória por meio da sinalização redox.

Isolado e hidrolisado proteico com fenólico, FPI e FPH respectivamente, foram obtidos a partir da DFM. Para obtenção destes mesmos produtos proteicos com teor reduzido em fenólico, phr-FPI e phr-FPH, respectivamente, a DFM foi submetida a uma etapa adicional para extração dos compostos fenólicos, obtendo-se a fração fenólica isolada denominada de Phi. Para avaliar se os compostos fenólicos presentes no FPI sofreriam alguma modificação decorrente do processo de hidrólise com Alcalase, a Phi foi submetida às mesmas condições de pH e temperatura utilizados na hidrólise, obtendo-se a Phh.

As amostras Phi e Phh apresentaram os maiores valores de FRAP e ORAC e também o maior potencial quelante (Figura 4.6 A, C, E, G). Essa capacidade dos fenólicos de atuarem como antioxidante multifuncional, que pode agir como *chain-breaking* ou quelante de metais como o ferro, pode ser explicada pelo caráter nucleofílico dos anéis aromáticos presentes na sua estrutura (KHOKHAR & OWUSU APENTEN, 2003).

Dentre as amostras proteicas, os hidrolisados apresentaram a maior capacidade antioxidante, seguida dos isolados e das farinhas (Figura 4.6 A, B, C). A hidrólise dos isolados proteicos com Alcalase foi responsável por um incremento no potencial antioxidante de até 6 e 4 vezes, quando determinado por ORAC e FRAP, respectivamente. O aumento se deve principalmente à liberação de peptídeos, cuja capacidade antioxidante é maior que

das proteínas intactas (SAMARANAYAKA & LI-CHAN, 2011). Embora os valores absolutos da capacidade antioxidante dos produtos proteicos com fenólicos, FPI e FPH sejam superiores aos de seus respectivos produtos com teor fenólico reduzido, phr-FPI e phr-FPH, o aumento relativo na capacidade antioxidante como resultado da hidrólise com Alcalase foi semelhante. Possivelmente, o aumento deve-se mais à formação de peptídeos do que às alterações nos compostos fenólicos. Estes resultados podem também indicar que a presença dos fenólicos não foi capaz de provocar alterações no padrão de liberação de peptídeos, seja pela formação de complexo com a proteína ou com a Alcalase.

Após a simulação da digestão, a capacidade antioxidante da maioria das amostras aumentou, sugerindo ação benéfica das condições da simulação da digestão gastrintestinal na bioatividade dos compostos, independente do mecanismo avaliado. A capacidade antioxidante dos isolados se igualou aos dos seus respectivos hidrolisados, indicando que a digestão é tão ou mais eficaz que a Alcalase na liberação de compostos com atividade antioxidante (Figura 4.6 D, E, F). Também foi possível inferir que os polifenóis da linhaça não atuaram como fator anti-nutricional, pois, aparentemente, não comprometerem a ação das enzimas gastrintestinais a e bioatividade das amostras proteicas, diferentemente de outros compostos fenólicos (GAWLIK-DZIKI, DZIKI, BARANIAK *et al.*, 2009; TAGLIAZUCCHI, VERZELLONI, BERTOLINI *et al.*, 2010; HELAL, TAGLIAZUCCHI, VERZELLONI *et al.*, 2014).

A presença dos polifenóis nas amostras proteicas contribuiu positivamente, porém de maneira variável, na capacidade antioxidante da maioria das amostras, antes e após a simulação da digestão (Figura 4.7 e 4.8). Este impacto pode ser explicado tanto pela elevada capacidade antioxidante dos fenólicos (Figura 4.6), pelo sinergismo entre fenólicos e compostos de origem proteica e ainda pela presença do complexo proteína:fenólico, confirmada pelo espectro de fluorescência (Figura 4.5).

A presença de diferentes compostos antioxidantes numa mesma amostra pode resultar numa atividade antioxidante maior que a soma das atividades antioxidantes de cada composto isolado. Isso ocorre porque o composto não oxidado é capaz de regenerar o outro que tenha sido oxidado, exarcebando a capacidade antioxidante da amostra (MARINOVA, TONEVA & YANISHLIEVA, 2008). Esse sinergismo acontece entre α-tocoferol e flavonóides e entre α-tocoferol e ascorbato (MARINOVA, TONEVA & YANISHLIEVA, 2008). Assim, parte do impacto observado pela presença dos fenólicos nas amostras proteicas pode ser explicada pelo possível sinergismo entre compostos de origem fenólica e proteica.

A presença dos fenólicos nas amostras proteicas, FPI e FPH, favoreceu a formação do complexo proteína:fenólico (Figura 4.5). Nos isolados, a formação do complexo proteína:fenólico é favorecida pelo pH alcalino empregado na extração da proteína e pela massa molecular das proteínas presentes no FPI. Quanto maior a massa molecular da proteína, maior a superfície de contato, maior será a quantidade de interações com os compostos fenólicos, maior a estabilidade do complexo (OLIVEIRA, ALEXANDRE, COELHO *et al.*, 2015). Por outro lado, nos hidrolisados, as interações entre fenólicos e peptídeos são limitadas devido à baixa superfície de contato entre os compostos. A formação do complexo altera a conformação nativa da proteína e pode resultar no aumento da exposição dos centros nucleofílicos presentes na estrutura proteica. Como a atividade antioxidante da proteína está diretamente relacionada com a quantidade e grau de exposição destes centros nucleofílicos (SAMARANAYAKA & LI-CHAN, 2011), quanto maior a abertura da estrutura proteica provocada pela interação com os fenólicos, maior será a contribuição do complexo na atividade antioxidante da amostra. Assim, a formação do complexo proteína:fenólico parece exercer um efeito positivo na capacidade antioxidante das amostras, principalmente no isolado proteico, o que em parte explicaria o maior impacto da presença dos fenólicos no isolado em relação ao hidrolisado.

No entanto, embora a formação do complexo possa facilitar a atuação antioxidante de compostos de origem proteica, ela pode mascarar a atividade antioxidante dos fenólicos, uma vez que os anéis polihidroxilados da estrutura dos polifenóis, principal porção responsável pela atividade antioxidante, são os mesmos que interagem com a proteína para a formação do complexo (BANDYOPADHYAY, GHOSH & GHOSH, 2012). Nossos resultados sugerem que o aumento da exposição de porções da proteína capazes de atuarem como antioxidante compensou a perda de parte do potencial antioxidante dos fenólicos provocada pela formação do complexo, tornando positiva a associação entre essas classes de compostos. Outro possível benefício da formação do complexo se dá pela ação das proteínas como carreadores de fenólicos, liberando-os somente na luz intestinal. Essa liberação tem um duplo efeito: proteção dos fenólicos da degradação oxidativa ao longo do trato gastrintestinal e criação de um meio antioxidante no lúmen, já que, devido à digestão das proteínas, os fenólicos são liberados do complexo e podem atuar como antioxidantes, reduzindo a formação de EROS.

A análise de componentes principais (Figura 4.7) permitiu conhecer, dentre os mecanismos antioxidantes estudados, FRAP, ORAC e FCCRS, qual o que mais influencia no comportamento antioxidante das amostras. De forma geral, as amostras com fenólicos tem seu mecanismo influenciado principalmente pelo método avaliado por FRAP, enquanto

que o mecanismo determinado por ORAC é o que mais influencia no comportamento antioxidante das amostras com teor de fenólicos reduzido.

A sobreposição das amostras digeridas FPH e FPI e a proximidade destas com FPH mostram que essas amostras se assemelham em relação ao comportamento antioxidante assim como os phr-FPI e phr-FPH (Figura 4.7). Estes resultados, associados à semelhança nos perfis cromatográficos e aos valores de FRAP e ORAC (Figuras 4.2 e 4.4) sugerem que a hidrólise com a Alcalase ou com enzimas do trato gastrointestinal libera peptídeos com semelhantes massa molecular, capacidades e comportamento antioxidante.

A fim de elucidar as características estruturais dos peptídeos da linhaça responsáveis pela atividade antioxidante, a amostra phr-FPH foi separada em 6 frações por cromatografia (CLAE-FR). A análise das frações por LC-MS/MS permitiu identificar quatro fragmentos pertencentes às proteínas da linhaça (Tabela 5.2). O fragmento encontrado na fração 2 pertence à conlinina, uma das principais proteínas de armazenamento da linhaça. O fragmento pertencente à Fração 5 é derivado da celulose sintase, uma enzima envolvida na biossíntese de celulose. Os fragmentos identificados das frações 5 e 6 pertencem a UDP-glicosiltransferase. O fragmento obtido da fração 6, GFPGRLDHWCASE, cuja identificação foi feita a partir da reação de degradação de Edman, não pode ser atribuído a nenhuma proteína da linhaça. No entanto, isso não significa que este fragmento não pertença à linhaça, uma vez que nem todas as proteínas desta semente foram sequenciadas.

Todas as sequências de peptídeos obtidas nesse estudo são compatíveis com as de peptídeos com atividade antioxidante e confirmam a origem proteica da atividade antioxidante determinada para o phr-FPH.

A atividade antioxidante do peptídeo sequenciado, GFPGRLDHWCASE corresponde a 61% da atividade antioxidante da Fração 6, sugerindo que há outros peptídeos com atividade antioxidante nesta fração. Entretanto, este fragmento possui elevada capacidade antioxidante (3.20 ± 0.24 µmol TE/ µmol peptídeo), superior ao do 2,3-terc-butil-4-hidroxianisol (BHA), um antioxidante sintético amplamente utilizado na indústria alimentícia (HERNÁNDEZ-LEDESMA, DÁVALOS, BARTOLOMÉ *et al.*, 2005).

Considerando todos os resultados relativos à capacidade antioxidante dos produtos de linhaça, seu possível efeito protetor na luz intestinal e a relação entre estresse oxidativo e inflamação, as amostras FPH, phr-FPH, Phi e Phh foram escolhidas para avaliação de seu potencial anti-inflamatório. Esta atividade foi estudada *in vitro*, por meio da

indução da produção de mediadores inflamatórios em células RAW 264.7 e, posteriormente, *in vivo* por meio da indução de colite experimental em animais.

Nenhuma das amostras interferiu significativamente na viabilidade celular quando comparada ao controle (Figura 6.1). A partir destes resultados, foram escolhidas as faixas de concentração de hidrolisados (0,001 a 1,0 mg/mL) e fenólicos (0,005 a 0,5 mg/mL) para realizar o ensaio de produção de citocinas inflamatórias.

A produção de óxido nítrico, determinada pela quantidade de nitrito, foi reduzida em até 40% por todas as amostras nas maiores concentrações (Figura 6.2 A). A produção de TNF-α foi inibida de forma dose-dependente por todos os tratamentos. O tratamento com Phi foi o mais eficaz, sendo capaz de inibir de 40 a 65% a produção de TNF-α, enquanto que as demais amostras inibiram cerca de 20 a 30% (Figura 6.2 B). Estes resultados mostram que tanto hidrolisados quanto compostos fenólicos da linhaça exercem atividade anti-inflamatória, uma vez que foram capazes de inibir a produção de NO e TNF-α. De acordo com a literatura, compostos capazes de inibir a produção de mediadores inflamatórios podem ser considerados anti-inflamatórios, uma vez que essa inibição está relacionada com a redução do estresse oxidativo, inflamação e redução do risco de desenvolvimento de doenças crônicas não transmissíveis (MIDDLETON, KANDASWAMI & THEOHARIDES, 2000; OSEGUERA-TOLEDO, DE MEJIA, DIA *et al.*, 2011; NDIAYE, VUONG, DUARTE *et al.*, 2012).

A semelhança entre a atividade inibitória das amostras FPH e phr-FPH sugere que não houve efeito sinérgico entre os peptídeos e os fenólicos presentes na FPH que pudesse potencializar a atividade anti-inflamatória. Por outro lado, a amostra Phi exibiu atividade anti-inflamatória superior a Phh, sugerindo que as condições de hidrólise (temperatura e pH) podem ter modificados os fenólicos e comprometido seu potencial anti-inflamatório.

As amostras FPH, phr-FPH e Phh apresentaram atividade anti-inflamatória semelhante, porém inferior à Phi (Figura 6.2). Em relação à atividade antioxidante, Phh apresentou os maiores valores de FRAP e ORAC, seguida da Phi, FPH e phr-FPH (Figura 4.6 A, C, E). Estes resultados sugerem que a atividade anti-inflamatória das amostras não está diretamente relacionada com a atividade antioxidante, e que, possivelmente, essas amostras são capazes de atuar como anti-inflamatórios por mecanismos que não envolvam somente a neutralização de espécies reativas.

Um desses possíveis mecanismos pelos quais hidrolisados proteicos poderiam atuar como anti-inflamatórios é por meio da redução da atividade do NF-κB, já que

peptídeos derivados de proteínas alimentares possuem a capacidade de inativar a enzima IκB quinase, uma enzima envolvida na translocação do NF-κB para o núcleo da célula (DE MEJIA & DIA, 2009; VERNAZA, DIA, GONZALEZ DE MEJIA *et al.*, 2012; MONTOYA-RODRÍGUEZ, MILÁN-CARRILLO, DIA *et al.*, 2014). Em relação aos fenólicos, sua ação anti-inflamatória pode ser devida à presença do grupamento catecol, derivado do ácido cafeico, um dos fenólicos da linhaça (Figura 4.3). Este grupamento é capaz de impedir a ação do NF-κB ao se ligar à porção p65 e bloquear a formação do complexo com o DNA (KAZŁOWSKA, HSU, HOU *et al.*, 2010).

Uma vez que todas as amostras estudadas exibiram atividade anti-inflamatória, foram escolhidas duas amostras, uma composta majoritariamente por peptídeos (phr-FPH) e outra de origem fenólica (Phi) para avaliar seu efeito na modulação da inflamação em animais com colite experimental induzida por TNBS.

Os animais que foram tratados com phr-FPH ou Phi antes da indução da colite apresentaram redução da frequência das células $CD4^+IFN\text{-}\gamma^+$, enquanto que o tratamento com Phi após a indução da colite, além de reduzir a frequência de células $CD4^+IFN\text{-}\gamma^+$, também reduziu a das células $CD4^+IL\text{-}17^+$ (Figuras 6.5 e 6.6). Além disso, os níveis de IFN-γ e TNF-α nos sobrenadantes dos esplenócitos foram reduzidos pelo tratamento prévio com phr-FPH ou Phi e apresentaram a mesma tendência no tratamento concomitante com Phi (Figura 6.7). Estes resultados sugerem que o efeito protetor contra a colite experimental induzida por TNBS exercido por ambas as amostras está intimamente relacionado à supressão das subpopulações T_H1 e/ou T_H17, reduzindo seu potencial inflamatório e, consequentemente, atenuando a inflamação intestinal.

O tratamento concomitante com Phi também provocou aumento na frequência das células $CD4^+CD25^+Foxp3^+$, conhecida como células T_{reg}. De acordo com Zhao e colaboradores (2013), a supressão de células T_{reg} provoca um desequilíbrio entre células T_H1 e T_H2 e entre T_{reg} e T_H17. Esse desbalanço entre as células resulta numa produção exacerbada de citocinas pro-inflamatórias, redução da produção de citocinas anti-inflamatórias e aumento do dano tecidual na parede do intestino. Assim, a supressão das células T_H1 and T_H17 pelo tratamento concomitante com Phi pode ser explicado pelo aumento da atividade das células T_{reg}, sugerindo que a ingestão de compostos fenólicos pode modular o processo inflamatório na colite experimental induzida por TNBS.

TNF-α é uma das principais citocinas pro-inflamatórias envolvidas no desenvolvimento e progressão das DII (HUR, KANG, JUNG *et al.*, 2012) e, por isso, bloquear a atividade dessa citocina por meio de drogas tem sido umas das estratégias para

controlar a doença (OUSSALAH, DANESE & PEYRIN-BIROULET, 2010; NEURATH, 2014; DE MATTOS, GARCIA, NOGUEIRA *et al.*, 2015). Dessa forma, a atividade inibitória sobre a secreção de TNF-α provocada pelo tratamento prévio com phr-FPH ou Phi, sugere que esses compostos possam ser utilizados no desenvolvimento de novas drogas anti-inflamatórias contra a DII.

A atividade anti-inflamatória das amostras phr-FPH e Phi pode ser atribuída, ao menos parcialmente, à capacidade antioxidante destas amostras (Figura 4.6). Na DII, as EROS estão associadas ao desenvolvimento e progressão do processo inflamatório e também às lesões no epitélio intestinal (CHO, SHIN, NOH *et al.*, 2011; DE FARIA, LUIZ-FERREIRA, SOCCA *et al.*, 2012). Atuando como antioxidantes, phr-FPH e Phi podem neutralizar as espécies reativas liberadas pelos leucócitos, contribuindo para redução dos danos provocados pelo processo inflamatório.

Outro possível mecanismo de atuação dos fenólicos na redução da inflamação intestinal é por sua capacidade de modular a microbiota, em favorecimento do crescimento de bactérias não patogênicas (DUDA-CHODAK, 2012; CARDONA, ANDRÉS-LACUEVA, TULIPANI *et al.*, 2013; PARKAR, TROWER & STEVENSON, 2013; MARTIN & BOLLING, 2015). Essa modificação da microbiota intestinal reflete na inflamação, pois componentes de uma microbiota patogênica fornecem antígenos que podem atuar no desencadeando e perpetuando a inflamação intestinal (ALBENBERG, LEWIS & WU, 2012; ZHAO, HUANG, ZUO *et al.*, 2013).

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CAPÍTULO 8. CONCLUSÃO GERAL

Dentre todas as amostras estudadas, a fração fenólica e os hidrolisados proteicos apresentaram a maior capacidade antioxidante, antes e após a simulação da digestão. A formação do complexo proteína: fenólico favoreceu a capacidade antioxidante dos produtos proteicos, principalmente do isolado. A redução do teor de fenólicos nos produtos proteicos de linhaça não alterou o padrão de peptídeos liberados após hidrólise com Alcalase e / ou enzimas gastrointestinais, mostrando que a formação do complexo proteína: fenólico não interferiu na ação dessas enzimas. Em relação ao mecanismo antioxidante, os fenólicos da linhaça parecem atuar tanto como doadores de elétrons quanto de prótons, enquanto que as proteínas exercem sua atividade redutora preferencialmente pela doação de H^+.

A identificação de peptídeos presentes nas frações do hidrolisado proteico de linhaça com reduzido teor de fenólicos mostrou que elas são compatíveis com as de peptídeos com atividade antioxidante e confirmam a origem proteica da atividade antioxidante determinada para o phr-FPH. Os peptídeos presentes na fração de maior atividade antioxidante foram os que apresentaram maior hidrofobicidade e menor massa molecular. Dentre todas as sequências identificadas, GFPGRLDHWCASE, apresentou capacidade antioxidante superior a do BHA, antioxidante sintético bastante utilizado na indústria de alimentos.

Hidrolisados proteicos e fenólicos da linhaça demonstraram capacidade de inibir a produção de TNF-α e NO *in vitro*. Diferentemente do observado para a atividade antioxidante, a presença dos compostos fenólicos junto aos peptídeos não resultou em aumento da atividade anti-inflamatória. No estudo da modulação da colite experimental induzida por TNBS, o tratamento concomitante com hidrolisado proteico reduzido em teor fenólico ou com a fração fenólica da linhaça resultou em inibição do processo inflamatório pela supressão da atividade das subpopulações T_H1 e T_H17 e pela redução da produção de TNF-α e IFN-γ pelos esplenócitos.

Este trabalho contribui com informações relevantes quanto a interferência do complexo proteína: fenólico na bioatividade de amostras proteicas e com novas sequencias de peptídeos antioxidantes da linhaça. Além disso, é um dos pioneiros em estudar *in vivo* a ação anti-inflamatória de peptídeos e fenólicos da linhaça. Mais estudos são necessários para avaliar a interferência do complexo proteína:fenólico na qualidade nutricional da proteína, na biodisponibilidade de peptídeos e fenólicos, e a relação destes compostos com

a microbiota intestinal para conhecer a real capacidade destes produtos em fornecer benefícios ao organismo.

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ANEXO 1. CERTIFICADO CEUA

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

CERTIFICADO

Certificamos que o projeto "**Capacidade antioxidante da lignana e de hidrolisados proteicos de linhaça (Linum usitatissimum L.) na forma livre e incorporado à emulsão na modulação da inflamação em camundongos BALB/c com retocolite ulcerativa**" (protocolo nº **3737-1**), sob a responsabilidade de **Profa. Dra. Wirla Maria da Silva Cunha Tamashiro / Fernanda Guimarães Drummond e Silva**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **09 de março de 2015**.

Campinas, 09 de março de 2015.

Prof. Dr. Alexandre Leite Rodrigues de Oliveira
Presidente

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ANEXO 2. TABELA DE AUTO-VALORES DAS VARIÁVEIS DA ANÁLISE DE COMPONENTES PRINCIPAIS

Tabela 1. Auto-valores das variáveis da Análise de Componentes Principais (PCA)

Variáveis	Auto-valores (PC1)	Auto-valores (PC2)
DFM	0,2930	-1,5308
	0,2919	-1,5305
	0,2816	-1,5438
phr-DFM	0,1438	-1,8158
	0,1442	-1,8078
	0,1412	-1,8148
FPI	0,3343	-1,2431
	0,4351	-1,2872
	0,3953	-1,2575
phr-FPI	0,1191	-1,7144
	0,1118	-1,7211
	0,1332	-1,7248
FPH	0,7552	1,4205
	0,4727	1,3778
	0,5917	1,6812
phr-FPH	-0,4471	-0,1307
	-0,4231	-0,2521
	-0,5562	0,0529
dDFM	0,1095	0,4180
	0,2135	0,2656
dphr-DFM	-0,3137	-0,5122
	-0,2830	-0,6129
dFPI	0,2326	3,0980
	0,6600	2,7942
dphr-FPI	-1,2428	0,8585
	-1,1536	0,6853
dFPH	0,3408	3,1324
	0,2613	3,1006
dphr-FPH	-1,0779	0,9828
	-0,9642	0,6320
ORAC	-0,3486	0,5955
FRAP	0,8377	0,5443
FCRRS	-0,4203	0,5909