**FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA**
**PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA**

# AVALIAÇÃO DOS EFEITOS ANTIOXIDANTES E ANTI-GENOTÓXICOS *IN VIVO* DA SEMENTE DE *Linum usitatissimum* L.

**DISSERTAÇÃO DE MESTRADO**

**Luísa Zuravski**

**Uruguaiana, RS, Brasil.**
**2013**

**LUÍSA ZURAVSKI**

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Dissertação apresentada ao programa de Pós-graduação *Stricto Sensu* em Bioquímica da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Mestre em Bioquímica.

Orientadora: Prof[a]. Dr[a]. Vanusa Manfredini

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Área de concentração: Bioprospecção Molecular

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Ao meu esposo, Michel, e à minha família,
maiores incentivadores e fontes inesgotáveis
de apoio, amor e compreensão.

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*As grandes ideias surgem da observação*
*dos pequenos detalhes.*
Augusto Cury

**RESUMO**

Dissertação de Mestrado
Programa de Pós-Graduação em Bioquímica
Fundação Universidade Federal do Pampa

**AVALIAÇÃO DOS EFEITOS ANTIOXIDANTES E ANTI-GENOTÓXICOS *IN VIVO* DA SEMENTE DE *Linum usitatissimum* L.**

AUTORA: Luísa Zuravski
ORIENTADORA: Vanusa Manfredini
Data e Local da Defesa: Uruguaiana, 24 de maio de 2013.

As plantas são consideradas a melhor fonte de antioxidantes naturais. Entre elas, um alimento funcional largamente utilizado é a linhaça (*Linum usitatissimum* L.). A linhaça é composta basicamente por minerais, vitaminas, proteínas e lipídeos. Adicionalmente, contêm quantidades significativas de outros componentes bioativos como polifenóis, ácidos graxos poliinsaturados, fibras e lignanas. Relata-se que uma refeição contendo linhaça tem um elevado potencial nutricional, não somente pelo teor de proteínas, mas também pela quantidade de fibras solúveis e lignanas. O uso popular está em plena ascensão, entretanto, ainda existem poucos trabalhos envolvendo humanos. Assim, o objetivo deste estudo foi avaliar a variedade (linhaça marrom ou dourada), forma de apresentação mais eficaz (farinha de linhaça ou grãos) e os reais benefícios da linhaça sob o status oxidativo, nível glicêmico e perfil lipídico em voluntários humanos saudáveis. Para isso, 48 voluntários foram selecionados e orientados a ingerirem 40g diárias de linhaça *in natura* pela manhã, durante 14 dias. Coleta de sangue venoso em jejum, medidas antropométricas e de pressão arterial foram realizadas antes e após o período de suplementação. Os resultados demonstraram que os parâmetros clínicos não modificaram significativamente no período de 14 dias de suplementação, mas mudanças significativas nos marcadores de estresse oxidativo foram observadas. Observou-se um efeito significativo da intervenção dietética com grãos de linhaça dourada ou marrom em proteger as proteínas plasmáticas e os lipídeos de membrana da ação danosa das espécies reativas. Este efeito positivo não foi observado na suplementação com farinha de linhaça marrom ou dourada. Esta ausência de resultado pode estar relacionada com o conteúdo de flavonoides que se apresenta maior no grão e mostra uma notável capacidade de neutralizar espécies reativas. A concentração de polifenóis plasmáticos diminuiu significativamente após 14 dias de suplementação. Este resultado, possivelmente, é consequência direta da neutralização de espécies reativas geradas pela metabolização dos ácidos graxos linoleico e linolênico, presentes na linhaça. Os teores de vitamina C aumentaram após 14 dias de ingestão de linhaça marrom ou dourada, mostrando a presença desta vitamina na semente. Os dados não mostraram nenhuma alteração na análise de danos ao DNA pelo teste cometa e frequência de micronúcleos. A defesa antioxidante enzimática, *Superoxido dismutase*, *Catalase* e *Glutationa peroxidase*, não mostraram alterações significativas em suas atividades após o período de suplementação. Assim, os resultados indicam que a semente de linhaça, na forma de grão, em ambas as variedades, pode ser um valioso suplemento, auxiliar no tratamento de doenças que apresentam como base os radicais livres.


*Palavras chave: Linum usitatissimum*, linhaça, antioxidante, parâmetros clínicos.

## ABSTRACT

Dissertation of Master's Degree
Program of Post-Graduation in Biochemistry
Federal University of Pampa

**EVALUATION OF ANTIOXIDANTS AND ANTI-GENOTOXIC EFFECTS OF *Linum usitatissimum* L. SEEDS *IN VIVO*.**

AUTHOR: Luísa Zuravski
ADVISOR: Vanusa Manfredini
Date and Place of Defense: Uruguaiana, May 24rd, 2013.

Plants are considered the best source of natural antioxidants. Among them, a functional food widely used is flaxseed (*Linum usitatissimum* L.). Flaxseed is composed mainly of minerals, vitamins, proteins and lipids. Additionally, contain significant amounts of bioactive compounds such as polyphenols, polyunsaturated fatty acids, fibers and lignans. It is reported that a meal containing flaxseed has a high nutritional potential, not only for protein content, but also by the amount of soluble fiber and lignans. The popular use is on the rise, however, there are few studies involving humans. The objective of this study was to evaluate the variety (brown or golden flaxseed), presentation more effective (flax flour or grains) and the real benefits of flaxseed on oxidative status, glucose level and lipid profile in healthy human volunteers. For this, 48 volunteers were selected and instructed to ingest 40g of flaxseed daily fresh in the morning for 14 days. Venous blood was collected in fasting, anthropometric and blood pressure measurements were performed before and after the supplementation period. The results demonstrate that clinical parameters did not change significantly within 14 days of supplementation, but significant changes in markers of oxidative stress were observed. There was a significant effect of dietary intervention with grain flaxseed golden or brown in protecting plasma proteins and membrane lipids of harmful action of reactive species. This positive effect was not observed in supplementation with flour flaxseed golden or brown. This lack of result may be related to the content of flavonoids which shows a remarkable ability to neutralize reactive species. The plasma concentration of polyphenols significantly decreased after 14 days of supplementation. This result is possibly a direct consequence of the neutralization of reactive species generated by metabolism of linoleic and linolenic fatty acids present in flaxseed. The vitamin C increased after 14 days of taking flaxseed golden or brown, showing the presence of this vitamin in the seed. The data showed no change in the analysis of DNA damage by comet assay and micronucleus frequency. The antioxidant defense enzymes, superoxide dismutase, catalase and glutathione peroxidase, showed no significant changes in their activities after the supplementation period. Thus, the results indicate that the flaxseed in the form of grain in both varieties can be a valuable supplement assist in treatment of diseases that have as base free radicals.


*Keywords: Linum usitatissimum*, flaxseed, antioxidant, clinical parameters.

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# LISTA DE ABREVIATURAS

**ANOVA -** Análise de variância

**ARG -** Arginina

**BHT** - Butil-hidroxitolueno

**CAT** - Catalase

**CEP** - Comitê de ética em pesquisa

**CLAE** - Cromatografia líquida de alta eficiência

**CO -** Grupamentos carbonílicos

**CV** - Coeficiente de variação

**DNA** - Ácido desoxirribonucleico

**DNPH** – 2,4 dinitrofenilhidrazina

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

**DTNB** - Ácido 5,5'-ditio-bis(2-nitrobenzóico)

**ERNs** - Espécies reativas de nitrogênio

**EROs** - Espécies reativas de oxigênio

**FAO -** Organização de agricultura e alimentos

**GPx -** Glutationa peroxidase

**GSH -** Glutationa reduzida

**GSSG -** Glutationa oxidada

**$H_2O_2$ -** Peróxido de hidrogênio

**HDL-C** - Lipoproteína transportadora de colesterol de alta densidade (do inglês *High Density Lipoprotein*)

**$HNOO^{\bullet-}$** - Peroxinitrito

**HOMA-IR** – Modelo de avaliação da homeostase

**HPLC** – *High Performance Liquid Chromatography*

**IMC -** Índice de massa corporal

**LDL-C** - Lipoproteína transportadora de colesterol de baixa densidade (do inglês *Low Density Lipoprotein*)

**LIS -** Lisina

**MDA** – Malondialdeído

**NADPH** - Nicotinamida adenina dinucleotídeo fosfato reduzida

**$NADP^+$** - Nicotinamida adenina dinucleotídeo fosfato

**$N_2O$ -** Óxido nitroso

**NO** - Óxido nítrico

**$O_2$** - Oxigênio molecular

**$O_2^{\bullet-}$** - Ânion superóxido

**$OH^{\bullet}$** - Radical hidroxila

**OMS** - Organização mundial da saúde

**PRO** - Prolina

**RLs** - Radicais livres

**$RO^{\bullet}$** - Radical alcoxila

**$RO_2^{\bullet}$** - Radical peroxila

**ROOH** - Peróxido orgânico

**SDG** - Secoisolariciresinol diglicosídeo

**SOD** - Superóxido dismutase

**TBARS** - Espécies reativas ao ácido tiobarbitúrico

**TCA** - Ácido tricloroacético

**TRE** - Treonina

# SUMÁRIO

# APRESENTAÇÃO

A presente dissertação foi dividida em três partes principais. Na **parte I** encontram-se a **INTRODUÇÃO, REFERENCIAL TEÓRICO** e **OBJETIVOS**. Os resultados que fazem parte desta dissertação estão apresentados sob a forma de manuscritos, os quais se encontram no item **MANUSCRITOS, parte II** deste trabalho. As seções materiais e métodos, resultados, discussão dos resultados e referências bibliográficas, encontram-se nos próprios manuscritos e representam a íntegra deste estudo. Os itens **DISCUSSÃO** e **CONCLUSÃO** encontram-se na **parte III** desta dissertação, apresentam interpretações e comentários gerais sobre os resultados apresentados nos manuscritos deste trabalho. O item **REFERÊNCIAS** refere-se somente às citações que aparecem nos itens introdução, referencial teórico, discussão e conclusão desta dissertação. No item **PERSPECTIVAS**, estão expostos os possíveis estudos para dar continuidade a este trabalho.

# PARTE I

## 1. INTRODUÇÃO

Os radicais livres (RLs) são agentes oxidantes altamente reativos (HALLIWELL, 2007). Em condições fisiológicas, os RLs podem desempenhar importante papel na regulação da resposta imunológica, participando do processo de defesa contra infecções e atuando como fatores de transcrição na sinalização intracelular, induzindo a apoptose (VALKO et al., 2007). No entanto, o aumento na sua produção e/ou a redução na sua eliminação gera um desequilíbrio fisiológico, caracterizando o estresse oxidativo (PÉREZ-MATUTE et al., 2009; WILKING et al., 2012). Os RLs podem ser originários de várias fontes como poluição, drogas, radiações, altas ingestas de ácidos graxos poliinsaturados, exercício físico extenuante e fumo; em altas concentrações os RLs podem danificar macromoléculas celulares, como membranas lipídicas, proteínas e ácidos nucléicos. Por isso, o estresse oxidativo tem sido relacionado na patogênese de diversas doenças, que variam de cardiovasculares, neurodegenerativas, alguns tipos de câncer, doenças neurológicas, pulmonares, autoimunes e vasculares, diabetes, problemas de visão bem como no processo de envelhecimento precoce (TOUR'E e XUEMING, 2010; TAKASHIMA et al., 2012).

Por definição, uma substância antioxidante é aquela capaz de diminuir ou inibir a oxidação mesmo quando presente em baixas concentrações em relação a seu substrato. Assim, estes compostos protegem o sistema biológico dos efeitos deletérios da oxidação de macromoléculas (PÉREZ-MATUTE et al., 2009; WILKING et al., 2012). Para se proteger do estresse oxidativo, o organismo dispõe do sistema de defesa contra radicais livres. O sistema enzimático é a primeira linha de defesa antioxidante, evitando o acúmulo do ânion radical superóxido e do peróxido de hidrogênio. É formado por diversas enzimas, destacando-se a *Glutationa peroxidase* (GPx), *Catalase* (CAT) e a *Superóxido dismutase* (SOD) que atuam na redução das espécies reativas de oxigênio (EROs) e, consequentemente, evitam a oxidação de estruturas biológicas. Já o sistema não enzimático compreende os antioxidantes hidrofílicos (*Glutationa reduzida* (GSH), vitamina C e polifenóis) e lipofílicos (bioflavonas, vitamina E e carotenóides) (URSO e CLARKSON, 2003; VALKO et al., 2007; WILKING et al., 2012).

A Organização Mundial da Saúde (OMS) relata que 82% da população mundial utiliza alguma forma de medicina tradicional para seus cuidados primários de saúde, como uso de extratos de plantas ou de seus princípios ativos (MORRIS et al., 2012).

Estudos científicos têm sugerido que os alimentos, além das suas propriedades energéticas, podem conter compostos secundários que agem sobre a fisiologia e saúde humana. Muitos alimentos apresentam elevada concentração e variedades de moléculas do grupo dos polifenóis, que possuem atividades antioxidante, anticarcinogênica, antiaterogênica, cardioprotetora, antimicrobiana e anti-inflamatória previamente comprovadas (FAN e LOU, 2004; GUENDEZ et al., 2005; ARON e KENNEDY, 2008; MARTÍN et al., 2013; BOLCA et al., 2013)

Entre as fontes naturais de antioxidantes, destaca-se a semente de linhaça (*Linum usitatissimum* L.) (RHEE e BRUNT, 2011). É relatado que uma refeição contendo linhaça tem um alto potencial nutricional, não somente pelo seu elevado teor de proteínas, mas também pelo seu conteúdo de fibras solúveis e lignanas (MUELLER et al., 2010). Potentes benefícios preventivos ou terapêuticos, como por exemplo, redução dos níveis de glicemia, triglicerídeos e aumento dos níveis de colesterol HDL, têm sido demonstrados em animais de laboratório após o uso de linhaça ou derivados na dieta, aumentando a atenção da comunidade científica para o seu uso como alimento funcional (MOLENA-FERNANDES et al., 2010).

Como é comum no meio vegetal, existem muitas variedades na mesma família. No que se refere à linhaça, a marrom e a dourada são as mais conhecidas. Ambas as variedades são praticamente idênticas nas propriedades nutricionais e terapêuticas, sendo mínimas as diferenças constitucionais, as quais, de um modo geral, são resultantes das condições de cultivo (MOLENA-FERNANDES et al., 2010).

# 2. REVISÃO BIBLIOGRÁFICA

## 2.1 Considerações gerais sobre *Linum usitatissimum* L.

### 2.1.1 Origem

A linhaça, semente do linho (*Linum usitatissimum* L.), pertence à família Linaceae (MORRIS e VAISEY-GENSER, 2003; TRUCOM, 2006) e é utilizada para consumo humano e animal. Registros históricos da linhaça informam seu aparecimento em 9000-8000 A.C. no Irã (HELBAEK,1969), Turquia (VAN ZEISTE, 1972), Síria (HILIMAN, 1975) e Jordânia (HOPF, 1983; ROLLEFSON et al., 1985). O seu primeiro uso como alimento pode ter sido como ingrediente de pães e laxante (JUDD, 1995).

### 2.1.2 Caracterização

Planta herbácea que varia de 30 cm a 130 cm de altura, o linho apresenta talos eretos, folhas estreitas e flores de coloração azul (**Figura 01**). O fruto consiste de uma cápsula globosa, da qual são obtidas as sementes brilhantes e planas, denominadas de linhaça (GILL e YERMANOS, 1967).

**Figura 01: *Linum usitatissimum* L. (Linho). Disponível em http://www.flickriver.com/7208148, acessado em 26/03/2013.**

As sementes de linhaça apresentam duas variedades, marrom e dourada. A cor das sementes é determinada pela quantidade de pigmentos presentes e suas dimensões variam de aproximadamente 3,0-6,4 mm de comprimento, 1,8-3,4 mm de largura e 0,5-1,6 mm de espessura (FREEMAN, 1995). A semente apresenta dois cotilédones achatados, que constituem a maior parte do embrião, o qual é revestido pela casca e camada aderente de endosperma. Casca e cotilédones podem ser utilizados separadamente como ingredientes funcionais (OOMAH e MAZZA, 1997; WIESENBORN et al., 2002).

A variedade marrom tem sido cultivada em regiões de clima quente e úmido, como o Brasil, e a dourada em regiões frias como o norte dos Estados Unidos e o Canadá. O plantio da linhaça ocorre no outono e a colheita na primavera e verão (TRUCOM, 2006).

A produção mundial de linhaça encontra-se entre 2.300.000 e 2.500.000 toneladas anuais. O Canadá destaca-se por liderar o *ranking* dos maiores produtores seguido pelos Estados Unidos, Índia, China, Ucrânia, Rússia, Bélgica, França e Alemanha. Na América do Sul, o maior produtor é a Argentina. No Brasil, a linhaça é produzida, principalmente, no noroeste gaúcho, sendo utilizada para a fabricação de tecidos, cosméticos, medicamentos, alimentação animal e humana. Até 2005, o Brasil produzia somente a variedade marrom, mas, no final de 2006, ocorreu a primeira colheita da variedade dourada (TRUCOM, 2006).

As expectativas no segmento da alimentação humana são promissoras, acredita-se que o consumo de semente de linhaça no Brasil cresça cerca de 10% ao ano. Em países de grande produção, como os EUA e o Canadá, calcula-se que a demanda por esse alimento seja de dez a vinte vezes maior que no Brasil. Supõe-se que somente 2% dos brasileiros conheçam os benefícios da linhaça para a manutenção da saúde (TRUCOM, 2006).

Conforme a Organização Mundial da Saúde (OMS)/Food Agriculture Organization (OMS/FAO, 1995), Morris e Vaisey-Genser (2003) a linhaça é comercializada, principalmente, como semente inteira ou moída grosseiramente na forma de farinha. Também, pode ser utilizada como ingrediente em vários produtos alimentícios como bolos, pães, massas, biscoitos, barras de cereal e produtos cárneos (LEE et al., 2004; MACIEL et al., 2008; VALENCIA et al., 2008).

### 2.1.3 Composição Química

As sementes marrom e dourada são praticamente idênticas, sendo mínimas as diferenças na composição química e, provavelmente, resultantes de distintas condições de cultivo (CANADIAN GRAIN COMMISSION, 2001).

A linhaça, semente oleaginosa, é rica em gordura, fibra dietética e proteínas. Sua composição em média é de 41% de gordura, 28% de fibra alimentar total, 20% de proteína, 7,7% de umidade, 3,5% de cinzas e 1% de açúcares simples (OOMAH e MAZZA, 1998; MORRIS e VAISEY-GENSER, 2003). Geadas precoces ou tardias, calor ou períodos de seca podem ter efeitos negativos sobre a qualidade da semente, gerando teores de óleo significativamente menores (GUBBELS et al., 1994).

O ácido graxo α-linolênico (ω-3) (**Figura 02**), responsável por 40% a 60% dos ácidos graxos da linhaça, torna esse alimento uma das mais ricas fontes deste nutriente (MADHUSUDHAN, 2009). O ácido graxo linoleico (ω-6) (**Figura 02**) está presente em quantidade menor (16%) (MORRIS e VAISEY-GENSER, 2003).

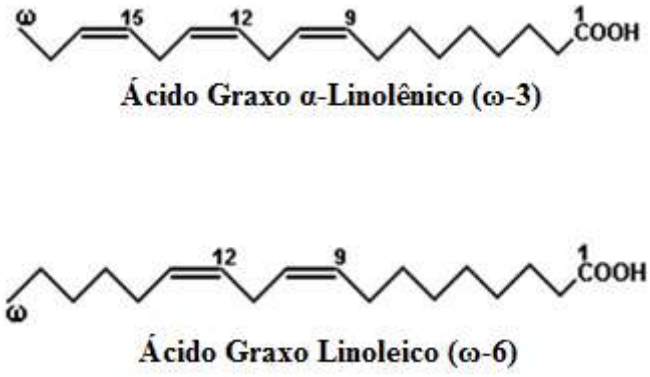


**Figura 02: Estrutura química dos Ácidos Graxos Linolênico (ω-3) e Linoleico (ω-6). Disponível em http://pt01.wkhealth.com/pt/re/chf/addcontent.11085486.htm, acessado em 29/03/2013.**

Em relação ao perfil de aminoácidos, a linhaça é composta por teor relativamente elevado de metionina e cisteína. A lisina, treonina e tirosina são consideradas os aminoácidos limitantes desse alimento (OOMAH e MAZZA, 2000).

Embora a composição relativa de monossacarídeos na linhaça varie conforme o cultivar, o ácido galacturônico, a galactose, xilose e ramnose representam os

carboidratos majoritários desta semente, enquanto que a fucose, arabinose e glicose são constituintes minoritários (OOMAH et al., 1995b).

As fibras dietéticas, no total, respondem por 28% do peso seco de linhaça, e as proporções das fibras solúveis e insolúveis na semente variam entre 20:80 e 40:60. A linhaça dourada contém menor quantidade de fibra total em relação à linhaça marrom, entretanto apresenta maiores teores de proteínas (BELL e KEITH, 1993).

A linhaça apresenta em sua constituição consideráveis teores de minerais como ferro, zinco, potássio, magnésio, fósforo e cálcio, além de vitaminas A, B, C e E, diferindo conforme a variedade, cultivo, meio ambiente e métodos de análise (COSKUNER e KARABABA, 2007).

Os compostos fenólicos são amplamente distribuídos nas plantas. Em sementes oleaginosas, os compostos fenólicos ocorrem na forma de derivados hidroxilados dos ácidos benzoico e cinâmico, cumarinas e compostos flavonoides. A linhaça contém 35 a 70 mg de flavonoides/100 g de linhaça (OOMAH e MAZZA, 1998). Oomah e colaboradores, (1995a) relataram que o total de ácido fenólico em oito cultivares canadenses variou de 790 a 1030 mg/100 g de linhaça. Entre os compostos fenólicos, a lignana predominante e mais biologicamente ativa na linhaça é o *Secoisolariciresinol Diglicosídeo* (SDG) (**Figura 03**) (SMEDS et al., 2007; MUELLER et al., 2010; MOLENA-FERNANDES et al., 2010).

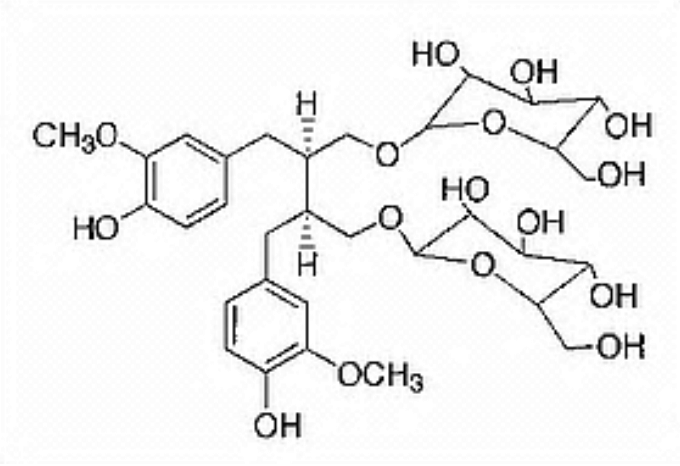


**Figura 03: Estrutura do Secoisolariciresinol Diglicosídeo (SDG). Adaptado de SMEDS et al., 2007.**

### 2.1.4 Atividades biológicas descritas

Em 1999, Yuan e colaboradores avaliaram o efeito da linhaça em enzimas antioxidantes em ratos jovens durante 26 dias. Os animais receberam doses diárias de 10% de linhaça ou 3mg de SDG associadas à ração. Os resultados dos grupos tratados não mostraram alteração na atividade das enzimas GPx e CAT em relação ao grupo controle.

Em 2001, Ford e colaboradores relataram que o SDG (**Figura 03**) é a principal lignana presente na linhaça, e suas concentrações podem atingir 60 a 700 vezes mais que em qualquer outra fonte descrita. Além disso, já são relatadas para o SDG, atividades antioxidante, redutora de aterosclerose e preventiva para desenvolvimento de diabetes em modelos experimentais. Este mesmo trabalho relata também que o SDG apresenta atividade quimiopreventiva contra câncer de mama, colo e próstata.

Em 2005, Stuglin e Prassad investigaram o efeito do uso de linhaça sob a pressão arterial, perfil lipídico, hematológico e parâmetros bioquímicos em humanos, por curtos períodos de tempo. Os voluntários consumiram três *muffins* contendo 32,7g de linhaça diariamente durante 4 semanas. Os resultados mostraram ausência de alterações após a dieta com linhaça, para parâmetros de pressão arterial, perfil hematológico e perfil lipídico (com exceção dos níveis de triglicerídeos que se elevaram). Parâmetros bioquímicos, como bilirrubinas, aspartato aminotransferase, fosfatase alcalina, proteínas, albumina, glicose e uréia não sofreram alterações, mas os níveis de creatinina decresceram.

Em 2006, Rajesha e colaboradores reportaram que o uso de linhaça em ratos por 14 dias, em doses de 0,75 e 1,5 g/kg, preveniu a redução das atividades das enzimas antioxidantes causada pela toxina tetracloreto de carbono ($CCl_4$), além de diminuir o dano oxidativo a lipídeos na dose de 1,5 g/kg.

Em 2008, Dodin e colaboradores avaliaram o efeito da linhaça em marcadores de risco de doença cardiovascular em mulheres saudáveis na menopausa. Oitenta e cinco voluntárias ingeriram 40g diariamente durante 12 meses. Após este período observaram-se modestas reduções nos níveis de colesterol total e na fração HDL e não significativas para colesterol LDL e glicose.

Em 2010, Molena-Fernandes e colaboradores compararam os efeitos da farinha de linhaça dourada ou marrom sobre o perfil lipídico e evolução ponderal em ratos Wistar. Os resultados mostraram que a farinha de linhaça, como suplemento dietético no

período de 35 dias promoveu redução significativa dos níveis de triglicerídeos séricos e da razão colesterol total / colesterol HDL, demonstrando efeito cardioprotetor.

Em 2011, Marques e colaboradores verificaram as possíveis atividades biológicas causadas pelo consumo diário de linhaça em diferentes condições de preparo (ração com 16% de grãos de linhaça cru; ração com 16% de grão de linhaça assado; ração com 7% de óleo de linhaça) em ratos Wistar machos recém-desmamados. Os resultados mostraram reduções significativas nos níveis de glicose, colesterol total, triglicerídeos e proteínas totais após 23 dias de suplementação.

Em 2011, Rhee e Brunt, determinaram a atividade antioxidante da linhaça e o seu papel no processo inflamatório e na resistência à insulina em indivíduos obesos intolerantes a glicose, após consumo diário de 40g de farelo de trigo ou linhaça, na forma de grão ou pão, pelo período de 12 semanas. A suplementação com linhaça decresceu o nível de peroxidação lipídica e o índice HOMA-IR (Homeostatic Model Assessment for Insulin Resistance), porém não provocou alterações nos biomarcadores inflamatórios plasmáticos.

## 2.2 Radicais livres

Os radicais livres (RLs) são agentes oxidantes caracterizados como espécies atômicas ou moleculares que possuem um ou mais elétrons desemparelhados na sua órbita externa, tornando-as espécies altamente reativas que agem como eletrófilos (GILLHAN et al., 1997).

Dentre os oxidantes mais importantes envolvidos em processos patológicos estão as espécies reativas de oxigênio (EROs) e as de nitrogênio (ERNs). As principais EROs distribuem-se em dois grupos, as radicalares: ânion superóxido ($O_2^{\bullet -}$), radical hidroxila ($OH^{\bullet}$), peroxila ($ROO^{\bullet}$) e alcoxila ($RO^{\bullet}$); e as não radicalares: oxigênio singleto ($O_2$), peróxido de hidrogênio ($H_2O_2$) e ácido hipocloroso (HOCl). Dentre as ERNs incluem-se óxido nítrico ($NO^{\bullet}$), óxido nitroso ($N_2O$) e peroxinitrito ($ONOO^-$), dentre outros (GILLHAM et al., 1997). A maioria destes compostos apresenta tempo de vida médio muito curto, como pode ser observado na **Tabela 1**.

**Tabela 1. Espécies reativas e suas respectivas meias-vidas.**

| Espécie Reativa | | Meia-vida (segundos) |
|---|---|---|
| **$HO^{\bullet}$** | Radical Hidroxila | $10^{-9}$ |
| **$HOO^{\bullet}$** | Radical Hidroperoxila | $10^{-8}$ |
| **$RO^{\bullet}$** | Radical Alcoxila | $10^{-6}$ |
| **$ROO^{\bullet}$** | Radical Peroxila | 7 |
| **$ONOO^{-}$** | Peroxinitrito | 0,05 – 1 |
| **$H_2O_2$** | Peróxido de Hidrogênio | Variável |
| **$O_2^{\bullet -}$** | Radical Superóxido | Variável |
| **$O_2$** | Oxigênio Singleto | $10^{-5}$ |
| **$NO^{\bullet}$** | Radical Óxido Nítrico | 1 – 10 |
| **HOCl** | Ácido Hipocloroso | Estável |

**Obs: R é um lipídeo, por exemplo, o linoleato.**

Adaptado de JORDÃO et al., 1998.

As espécies reativas podem ser formadas no organismo de diversos modos. Durante a fosforilação oxidativa, mecanismo usado pelas células para produzir energia química (ATP), parte dos elétrons é transferida para o oxigênio, dando origem ao $O2^{\bullet -}$ (JUNQUEIRA e RAMOS, 2005). Na **Figura 04** é demonstrado a formação e interação das principais EROs no organismo a partir do oxigênio molecular. Eles podem ainda ser produzidos durante a oxidação de ácidos graxos, reações do citocromo P450 e de células fagocíticas, entre outros. Algumas enzimas também são capazes de gerar EROs, sob condições normais ou patológicas. Fontes exógenas como tabaco, radiações, luz ultravioleta, solventes e alguns fármacos, dentre outros, também geram EROs (BIESALSKI, 2002).

Em condições fisiológicas normais, as EROs podem desempenhar importante papel fisiológico na regulação da resposta imunológica, participando do processo fagocítico de defesa contra infecções e atuando como fatores de transcrição na sinalização intracelular, induzindo a apoptose (HALLIWELL, 1994; BIESALSKI, 2002). No entanto, o aumento na sua produção e/ou a redução na sua eliminação gera um desequilíbrio fisiológico, caracterizando o estresse oxidativo (FINKEL e HOLBROOK, 2000; GUTTERIDGE e HALLIWELL, 2000)

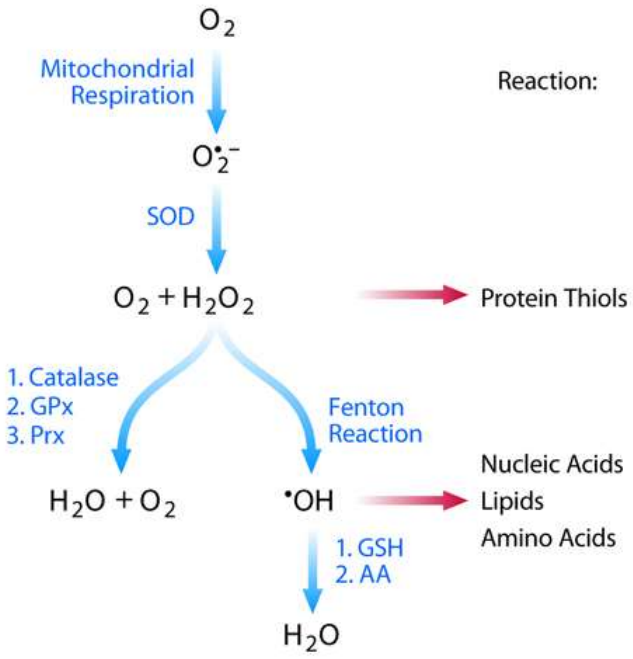


**Figura 04. EROs: produção e proteção. Figura mostrando as principais EROs associadas com a respiração celular. As setas azuis indicam o mecanismo de detoxificação, enquanto as setas vermelhas indicam a reatividade de cada EROs. Adaptado de Merksamer et al., 2013.**

## 2.3 Antioxidantes

Por definição, uma substância antioxidante é aquela capaz de diminuir ou inibir a oxidação mesmo presente em baixas concentrações em relação a seu substrato. Desta forma, estes compostos protegem os sistemas biológicos contra os efeitos deletérios dos processos ou das reações que levam à oxidação de macromoléculas ou estruturas celulares. Dessa forma, os antioxidantes podem, teoricamente, prolongar a fase de iniciação ou então inibir a fase de propagação, mas não podem prevenir completamente a oxidação (JORDÃO et al., 1998; GUTTERIDGE e HALLIWELL, 2000; BARREIROS et al., 2006). Para a proteção dos danos deletérios causados pelo estresse oxidativo, o organismo dispõe de um elaborado sistema de defesa contra radicais livres. Para uma melhor distinção entre os vários tipos de antioxidantes, esses compostos são classificados conforme a sua estrutura em enzimáticos e não enzimáticos *(*URSO e CLARKSON, 2003).

### 2.3.1 Sistema enzimático

O sistema enzimático é a via primária de defesa antioxidante, sendo representado principalmente pelas enzimas antioxidantes GPx, CAT e SOD (GUTTERIDGE e HALLIWELL, 2000; BELLÓ, 2002). Desta forma, o sistema antioxidante enzimático diminui as EROs e, consequentemente o dano às estruturas biológicas (BELLÓ, 2002).

#### *2.3.1.1 Superóxido dismutase (SOD)*

Pertence ao grupo das metaloenzimas, sendo encontrada principalmente sob duas isoformas, mitocondrial (MnSOD) e citosólica (CuZnSOD). A SOD catalisa a dismutação do O2•- em $H_2O_2$ e $O_2$ (**Figura 05**).

#### *2.3.1.2 Catalase (CAT)*

A CAT é uma hemeproteína citoplasmática que catalisa a redução do $H_2O_2$ a $H_2O$ e $O_2$ (**Figura 05**) (FRIDOVICH, 1998). É encontrada no sangue, medula óssea, mucosas, rim e fígado (INOUE, 1994).

#### *2.3.1.3 Glutationa peroxidase (GPx)*

A GPx é uma selenoenzima que catalisa a redução do $H_2O_2$ ou hidroperóxidos lipídicos a $H_2O$. Esta reação ocorre na presença de GSH que atua como doadora de elétrons e é convertida à glutationa oxidada (GSSG). A GSH é regenerada pela glutationa redutase por intermédio da oxidação de NADPH a NADP+ (**Figura 05**) (VANCINI et al., 2005).

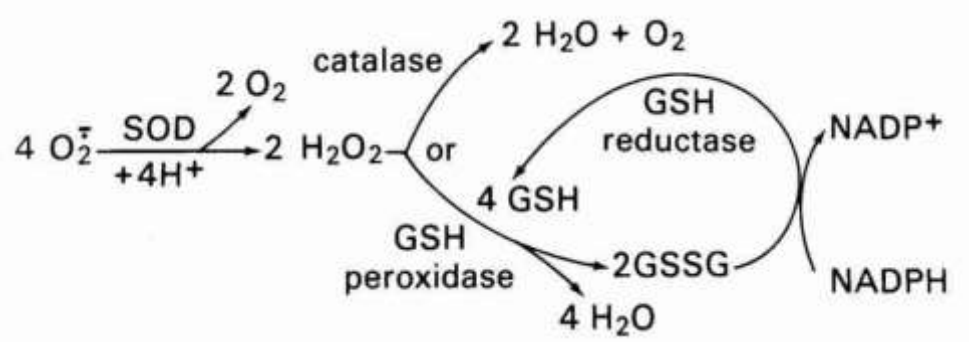


**Figura 05: Representação esquemática das inter-relações entre as enzimas antioxidantes. Disponível em http://www.drproctor.com/rev/84/84rev.htm, acessado em 29/03/2013.**

### 2.3.2 Sistema não enzimático

As substâncias que representam o sistema de defesa antioxidante não enzimático podem ter origem endógena ou serem obtidas através da dieta, e subdividem-se em antioxidantes hidrofílicos (GSH, vitamina C, indóis, catecóis e polifenóis) e lipofílicos (bioflavonas, vitamina E e carotenóides). Assim, o consumo de alimentos que conferem valor nutritivo associado à atividade antioxidante, garante proteção ao sistema biológico por atuarem na neutralização de espécies reativas e consequentemente redução de dano oxidativo (NOZAL et al.,1997; GUTERIDGE e HALLIWELL, 2000).

#### *2.3.2.1 Glutationa reduzida (GSH)*

A GSH (tripeptídeo, L-γ-glutamil-L-cisteinil-glicina), é encontrada na maioria das células e é o tiol (-SH) mais abundante no meio intracelular. Sua capacidade redutora é determinada pelo grupamento -SH, presente na cisteína. A GSH pode ser considerada um dos agentes mais importantes do sistema de defesa antioxidante da célula, atuando como doadora de elétrons para neutralizar o $H_2O_2$ e lipoperóxidos e sequestradora de EROs e ERNs. Além disso, atua como transportadora e reservatório de

cisteína e é requerida para a síntese de DNA, de proteínas e de algumas prostaglandinas (NOZAL et al., 1997).

*2.3.2.2 Polifenóis*

Os polifenóis são metabólitos secundários de plantas, encontrados amplamente em alimentos de origem vegetal. Atuam pela deslocalização de elétrons do anel aromático, reagindo com os RLs, formando radicais estáveis, e com isso neutralizando diretamente essas espécies reativas (**Figura 06**) (KATZ, DOUGHTY e ALI, 2011; CHO et al., 2003). São representados principalmente pelos flavonoides, lignanas e taninos. Os flavonoides são componentes comuns em nossa dieta e apresentam efeitos bioquímicos e farmacológicos previamente descritos, incluindo a atividade antioxidante (IGNAT, VOLF e POPA, 2011).

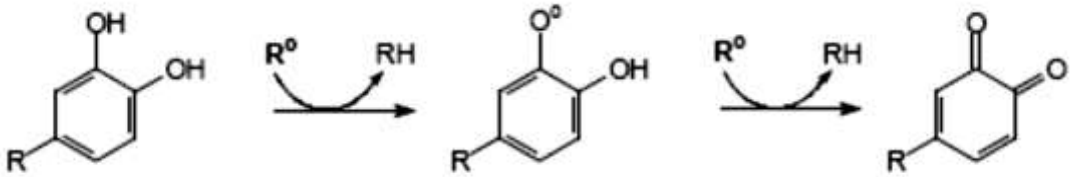


**Figura 06: Estabilização dos radicais livres pelos polifenóis. Adaptado de CHO et al., 2003**.

## 2.4 Biomarcadores de estresse oxidativo

Os radicais livres produzidos pelo metabolismo oxidativo têm alta reatividade, e uma meia-vida curta (JORDÃO et al., 1998; VOSS e SIEMS, 2006). Por esse motivo, sua determinação *in vivo* não é viável. Em contrapartida, os lipídeos, proteínas, carboidratos e ácidos nucléicos, após serem modificados pelas espécies reativas, têm uma meia-vida maior, o que os tornam marcadores ideais do estresse oxidativo (DURSUN et al., 2005; GONZÁLES RICO et al., 2006; VOSS e SIEMS, 2006; VASCONCELOS et al., 2007).

Os antioxidantes enzimáticos e não enzimáticos também são considerados biomarcadores de dano oxidativo em sistemas biológicos. As concentrações de GSH,

ceruloplasmina, transferrina, vitaminas hidro e lipossolúveis fazem referência ao sistema não enzimático, enquanto que as atividades das enzimas SOD, CAT, GPx e glutationa redutase são utilizadas como marcadores do sistema de defesa antioxidante enzimático (BARREIROS et al., 2006; VASCONCELOS et al., 2007).

### *2.4.1 Dano a lipídeos de membrana*

O dano oxidativo a lipídeos é um processo complexo que envolve a interação de EROs com ácidos graxos poliinsaturados, componentes das membranas celulares (REED, 2011). Este processo resulta em desorganização estrutural e consequente perda da seletividade das membranas, podendo levar à morte celular (GUTTERIDGE e HALLIWELL, 2000). Nessa reação ocorre liberação de produtos de degradação de ácidos graxos, como o malondialdeido (MDA), e a quantificação deste composto têm sido utilizada para avaliar a extensão do dano oxidativo (OHKAWA et al., 1979; MARNETT, 1999).

### *2.4.2 Dano a proteínas plasmáticas*

As proteínas são alvos imediatos para a modificação oxidativa ocasionada por EROs, alterando sua estrutura, provocando perda de função e fragmentação protéica. A formação e quantificação da proteína carbonil pode se usada para mensurar a extensão do dano oxidativo. Os grupamentos carbonílicos (CO) são produzidos pela oxidação da cadeia lateral de aminoácidos suscetíveis, como prolina (PRO), arginina (ARG), lisina (LIS) e treonina (TRE), ou pela clivagem oxidativa das proteínas (BERLETT e STADMAN, 1997; BEAL, 2003; DALLE-DONNE et al., 2003).

### *2.4.3. Material genético*

EROs, principalmente $HO^{\bullet}$, atacam o açúcar desoxirribose (principalmente em 4’ e/ou 5’) e as bases purínicas (adenina e guanina) e pirimidínicas (timina, citosina e uracila), com ataque preferencial à guanina, gerando 8-hidroxi- ou 8-oxoguanina, mutagênicas. Como resultado, ocorre quebra da cadeia de DNA, ligação cruzada entre

as fitas e modificações nas suas bases gerando mutações e apoptose (VASCONCELOS et al., 2007).

# 3. OBJETIVOS

## 3.1 Geral

Avaliar os efeitos antioxidantes e anti-genotóxicos *in vivo* da semente de *Linum usitatissimum* L.

## 3.2 Específicos

Avaliar os efeitos *in vivo* das variedades marrom e dourada da semente de *Linum usitatissimum* L., na forma de grão e farinha, em voluntários humanos saudáveis antes e após 14 (quatorze) dias de suplementação, através da determinação dos seguintes parâmetros:

- Glicemia de jejum;
- Perfil lipídico (colesterol total, colesterol HDL, colesterol LDL e triglicerídeos);
- Perfil hematológico;
- Medidas antropométricas;
- Medidas de pressão sistólica e diastólica;
- Atividade das enzimas antioxidantes (*Catalase, Superóxido dismutase* e *Glutationa peroxidase*);
- Níveis de polifenóis plasmáticos;
- Níveis de vitamina C plasmáticos;
- Dano oxidativo em lipídeos, proteínas e DNA.

# PARTE II

## MANUSCRITO I

**Effects of brown flaxseed (*Linum usitatissimum* L.) in oxidative and clinical parameters in healthy subjects.**

*Luísa Zuravski, Ritiele Pinto Coelho, Jonathaline Apollo Duarte, Manoelly Oliveira Rocha, Bruna Cocco Pilar, Leandro Leal Galarça, Juliana Mezzomo, Margareth Linde Athayde, Aline Augusti Boligon, Michel Mansur Machado, Vanusa Manfredini.*

**Submetido a Human & Experimental Toxicology**

**Confirmação de Submissão**

De: Leila.Ahlstrom@ttl.fi <Leila.Ahlstrom@ttl.fi>
Assunto: Human and Experimental Toxicology - Manuscript ID HET-13-0214
Para: vanusamanfredini@unipampa.edu.br
Data: Quarta-feira, 17 de Abril de 2013, 21:39

17-Apr-2013

Dear Prof. MANFREDINI:

Your manuscript entitled "Effects of brown flaxseed (Linum usitatissimum L.) in oxidative and clinical parameters in healthy subjects." has been successfully submitted online and is presently being given full consideration for publication in Human and Experimental Toxicology.

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**Effects of brown flaxseed (*Linum usitatissimum* L.) in oxidative and clinical parameters in healthy subjects.**

Luísa Zuravski [a], Ritiele Pinto Coelho [b], Jonathaline Apollo Duarte [b], Manoelly Oliveira Rocha [b], Bruna Cocco Pilar [a], Leandro Leal Galarça [b], Juliana Mezzomo [a], Margareth Linde Athayde [c], Aline Augusti Boligon [c], Michel Mansur Machado [d], Vanusa Manfredini [a, b, *].

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**Abstract**

It is reported that a meal containing flaxseed has a high nutritional potential, not only for its elevated protein content, but also by its amount of soluble fiber and lignans. The objective of this study was to evaluate the presentation form more effective of brown flaxseed (grain or flour) and the real benefits under the oxidative status, glycemic level and lipid profile in healthy human volunteers. In order to, 24 volunteers were selected and instructed to ingest daily 40 grams of flaxseed *in natura* for 14-days. Collection of fasting venous blood, anthropometric and blood pressure measurements were performed before and after the supplementation period. Our results demonstrated that clinical parameters do not change significantly for periods of 14-days supplementation, but significant changes in markers of oxidative stress were observed. The results indicated that the flaxseed could be a valuable adjunct in treating diseases that presents as base the production of free radicals.

*Keywords: Linum usitatissimum*, flaxseed, antioxidant, clinical parameters.

## 1. Introduction

Evidence has suggested that food beyond its energetic properties may contain secondary compounds that act on the physiology and human health. Most foods present a great concentration and variety of molecules from the group of polyphenols acting as antioxidant, anticarcinogenic, antiatherogenic, cardioprotective, anti-inflammatory and antimicrobial.[1]

Free radicals are highly reactive oxidizing agents. In excess can react with lipid membranes, proteins and nucleic acids that can cause cancer, neurological, pulmonary, autoimmune, vascular, diabetes and vision problems and premature aging.[2] An antioxidant substance is that capable of decreasing or inhibits oxidation even when present in low concentrations relative to its substrate.

In this context arises the flaxseed (*Linum usitatissimum* L.), a promising antioxidant.[3] It is reported that a meal containing flaxseed has a high nutritional potential, not only for its high protein content, but also by its content of soluble fiber and lignans.[4] Potent preventive or therapeutic benefits, such as reduced levels of glucose, triglycerides and increased HDL cholesterol levels, have been demonstrated in laboratory animals after the use of flaxseed or its derivatives in the diet, increasing the attention of the scientific community for the use of this seed as a functional food.[5] Studies show that supplementation with flaxseed reduces blood glucose levels, glycosylated hemoglobin and increases insulin sensitivity in obese people glucose intolerant.[3]

As is common among plant, there are many varieties in the same family. With regard to flaxseed, golden and brown are the most recognized. Both varieties are practically identical, been minimal the differences in the chemical composition, what are, probably, result of culture conditions. The golden flaxseed develops in very cold climates such as Canada (world's largest producer of flaxseed) and the northern United States and brown flaxseed may develop in regions of hot and humid climate, such as Brazil.[5] In abundance in Brazil, brown seeds are sold at more affordable prices than the golden flaxseed, thereby making its use more common in the country.

Scientific studies of natural products have social objectives aimed at validating the use of them so that they can ensure efficacy when used as functional foods. Although some studies reported effects of flaxseed, there are few studies with humans aiming to prove the effects of flaxseed under oxidative status and the best presentation

form. The objective of this study was to evaluate the presentation of brown flaxseed more effective (grain or flour) and the real benefits under the oxidative status, glycemic level and lipid profiles in healthy human volunteers.

## 2. Material and Methods

### *2.1 Chemicals*

All the chemicals were of analytical grade. Solvents for HPLC analysis were purchased from Merck (Darmstadt, Germany). All the others reagents were acquired from Sigma Chemical Co. (St. Louis, MO, USA).

### *2.2 Plant material*

Flaxseed was purchased at local supermarket in Uruguaiana (State Rio Grande do Sul, Brazil) on February of 2012.

### *2.3 Phytochemical analysis*

For the phytochemical analysis, the flour was cold macerated in ethanol-water (70:30 v/v) in the dark and at room temperature for 7 days with daily agitation. After filtration, the extract was evaporated under reduced pressure to remove the ethanol in order to obtain the "flour extract". The same procedure was made for the grain, except that this was triturated in a blender before maceration. At the end of this procedure, we obtained the "grain extract".

#### *2.3.1 Determination of total polyphenolic contents*

The extracts were prepared following a standardized procedure: 0.5 g of the extracts was dissolved in 10 mL of ethanol and the volume adjusted to 100mL with water. An aliquot of 3 mL was dissolved in 100 mL of water. Final concentration was 0.15 mg/mL. The total polyphenol concentrations in the extracts were measured spectrophotometrically as described by modified Folin-Ciocalteau method.[6] Briefly, 0.5 mL of 2N Folin-Ciocalteau reagent was added to a 1 mL of each sample (0.15 mg/mL), and this mixture was allowed to stand for 5 min before the addition of 2 mL of 20% $Na_2CO_3$. The solution was then allowed to stand for 10 minutes before reading at 730 nm in a Shimadzu-UV-1201 (Shimadzu, Kyoto, Japan) spectrophotometer. The estimation of phenolic compounds in the extracts was carried out in triplicate. It is very

difficult to choose suitable standards for total phenolic determination in plant extracts due to the chemical heterogeneity of plants products and the specificity of phenolic reagents. Thus, it is only possible to get relative equivalents with the standard used. The total polyphenol content was expressed as microgram equivalents of gallic acid per gram of flaxseed. The equation obtained for standard curve of gallic acid in the range of 0.001 – 0.020 mg/mL was $y = 40.112x + 0.0581$ ($R^2 = 0.9994$).

*2.3.2 Determination of flavonoids*

The analysis was made according to Woisky and Salatino.[7] To 2 mL of each reference solution, 20 mL of methanol and 1mL of 5% $AlCl_3$ (w/v) were added and the volume made up to 50 mL with methanol at 20ºC. After 30 minutes, the absorbances were measured at 425 nm in a Shimadzu-UV-1201 spectrophotometer. The same procedure was made to analyse of the extracts. The blank was the 5% $AlCl_3$ (w/v) alone. The estimation of flavonoids was carried out in triplicate. The contents were expressed as microgram equivalents of quercetin per gram of flaxseed. The equation obtained for standard curve of quercetin in the range of 0.02 – 0.60 mg/mL was $y = 0.891x + 0.0149$ ($R^2 = 0.9723$).

*2.3.3 Determination of ascorbic acid content*

Sulphuric acid solution 1M was used to solubilize and prepare the curve of ascorbic acid used as reference. The references samples were mixed with trichloroacetic acid 13,3% and 2,4-dinitrophenylhydrazine. The procedure followed the description of Jacques-Silva et al.[8] After an incubation period, the samples were measured at 520 nm in a Shimadzu-UV-1201 spectrophotometer. The equation obtained for standard curve of ascorbic acid in the range of 1.5 – 4.5 mg/mL was $y = 0.0534x + 0.0713$ ($R^2 = 0.9790$). The extracts were measured using the same procedure. The estimation of ascorbic acid concentration was carried out in triplicate.

*2.3.4 HPLC analysis*

High performance liquid chromatography (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software. Reverse phase chromatographic analyses were carried out

under gradient conditions using C18 column (4.6 mm x 150 mm) packed with 5 μm diameter particles; the mobile phase was water containing 2% acetic acid (A) and methanol (B), and the composition gradient was: 5% of B until 2 minutes and changed to obtain 25%, 40%, 50%, 60%, 70% and 100% B at 10, 20, 30, 40, 50 and 80 minutes, respectively, following the method described by Laghari et al.[9] with slight modifications. The presence of six antioxidants compounds was investigated, namely, gallic acid, chlorogenic acid, caffeic acid, quercetin, rutin and kaempferol. Identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. The flow rate was 0.7 mL/min, injection volume 40 μL and the wavelength were 254 nm for gallic acid, 327 nm for caffeic and chlorogenic acids, and 365 nm for quercetin, rutin and kaempferol. The samples and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.020 – 0.200 mg/mL for quercetin, rutin and kaempferol and 0.050 – 0.250 mg/mL for gallic, caffeic and chlorogenic acids. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to 500 nm). Calibration curve for gallic acid: Y = 13260x + 1276.4 (r = 0.9998); chlorogenic acid: Y = 12158x + 1174.9 (r = 0.9996); caffeic acid: Y = 14034x + 1527.5 (r = 0.9995); rutin: Y = 13721x + 1068.4 (r = 0.9997); quercetin: Y = 13794x + 1392.1 (r =0.9999) and kaempferol: Y = 12647x + 1178.3 (r =0.9995). All chromatography operations were carried out at ambient temperature and in triplicate.

*2.3.5 Radical - scavenging capacity - DPPH assay*

The antioxidant capacity of the extracts were evaluated by monitoring its ability in quenching the stable free radical DPPH, according to a slightly modified method previously described by Choi et al.[10] Spectrophotometric analysis (SHIMADZU-UV-1201) was used in order to determine the inhibition concentration (IC) and the inhibition percentage (IP %) of the crude extract. The DPPH quenching ability was expressed as $IC_{50}$ (concentration which gives 50% inhibition). Six different ethanol dilutions of the extracts (2.5 mL), at 250; 125; 62.5; 31.25; 15.62; and 7.81 μg/mL were mixed with 1.0 mL of a 0.3 mM DPPH ethanol solution. The absorbance was measured at 518 nm by spectrophotometer against a blank after 30 min of reaction at room temperature in the dark. DPPH solution (1.0 mL, 0.3mM) plus ethanol (2.5 mL)

was used as a control. Relative activities were calculated from the calibration curve of *L*-ascorbic acid standard solutions working in the same experimental conditions. Inhibition of free radical by DPPH in percent (IP %) was calculated in following way, according to the **Equation 1**:

**Equation 1:** $IP\% = 100 - [(ABS_{SAMPLE} - ABS_{BLANK}) / ABS_{CONTROL}) \text{ X } 100]$

Where, $ABS_{SAMPLE}$ is the absorbance of the test compound, $ABS_{BLANK}$ is the absorbance of the blank (containing 1.0 mL of ethanol plus 2.5 mL of the plant extract solution) and $ABS_{CONTROL}$ is the absorbance of the control reaction (containing all reagents except the test compound). IP% was plotted against sample concentration, and a linear regression curve was established in order to calculate the $IC_{50}$. Tests were carried out in triplicate.

*2.4 Selection of volunteers*

Twenty four individuals between the ages of 17 and 33 years, of both sexes, non-smokers, without hypertension, not on regular medication, using no antioxidant and mineral supplementation and with no previous history of stroke, myocardial infarction, angina, diabetes mellitus, cancer or any illness that had required hospitalization during the previous 12 months were recruited at Uruguaiana and enrolled in the study between March 2012 and April 2012. Female volunteers were excluded if pregnant, lactating or on hormone replacement therapy.

*2.5 Ethical issues*

This study was approved by the Ethics and Research of the Federal University of Pampa (Protocol No. 069/2011). All participants signed a consent form.

*2.6 Experimental design*

Twenty four participants were randomly assigned into one of two groups: flour flaxseed or grain flaxseed supplementation group (n = 12). They received 40 grams [11] aliquots of flour or grain brown flaxseed for daily use for a period of 14 days. [12] Subjects were instructed to consume the flaxseed *in natura* mixed with water in the morning for 2 weeks.

2.7 *Anthropometric and pressure measure*

Each participant's height was measured at the beginning of the study. Using a balance beam scale, each participant was weighed at the beginning and end of supplementation period. BMI was calculated using the equation BMI = weight (kg) / height$^2$ (m$^2$). Blood pressure was taken from the right arm with the participant seated, after a 4–5 minute rest and by person trained to carry out the process repeatedly in a similar manner. A digital manometer was used to perform these measures.

*2.8 Blood sample collection*

Fasting blood samples were taken at the beginning of the study and at the end of 14 days of supplementation in the morning between 7 and 9 h. Blood was drowned from the antecubital vein into prechilled tubes containing EDTA, Li-heparin and tubes without anticoagulant (Vacutainer - Becton, Dickinson and Company – New Jersey – USA). Blood in tubes without anticoagulant were allowed to clot at room temperature for 30 min. The plasma and serum were collected after centrifugation at 1500 x g for 10 min and analyzed immediately.

*2.9 Sample Analysis*

*2.9.1 Oxidative parameters*

The oxidative parameters, vitamin C,[8] lipid peroxidation[13] and protein carbonyls[14] in plasma were measured by the spectrophotometric methods; the assessment of DNA damage was made by comet assay[15] and frequency of micronucleus[16] in leukocytes; the activities of superoxide dismutase, catalase and glutathione peroxidase in erythrocyte were achieved using commercial kits (Randox Brazil LTDA).

*2.9.2 Biochemical and hematological analysis*

The hemograms (complete blood count) were performed in an automatic counter Cell-Dyn 3200 Hematology Analyzer (Abbott Diagnostic, St Clara, CA, USA) and total cholesterol, HDL cholesterol, triglycerides total, protein and glucose levels using automatic analyzers A25 Biosystems (Biosystems SA, Barcelona, Spain) for *in vitro* diagnostics. LDL cholesterol values were computed according to the Friedewald formula. All biochemical assays were carried out in triplicate.

*2.10 Statistical analysis*

Data were expressed as mean ± standard deviation (SD). Comparisons between groups were performed using a one-way analysis of variance (ANOVA), followed by post hoc of Bonferroni for multiple comparison tests. Results were considered statistically significant when $p<0.05$.

## 3. Results and discussion

*3.1 Phytochemical analysis*

Phenolics are largely distributed in the natural products and are the most abundant secondary metabolites found in plants.[17] The key role of phenolic compounds as antioxidant is emphasized in others reports.[18] Flavonoids occur naturally in plant foods and are a common component of our diet, and demonstrated a wide range of biochemical and pharmacological effects, including antioxidant activity.[17] The concentrations of these groups of secondary metabolites and some of the compounds present in this species, all with reported biological activities, are shown in **Table 01.** The DPPH assay is used as a tool for the *in vitro* evaluation of extracts, and its results can be related with the presence of phenolic and flavonoid compounds in plant extracts.[19] Our plant extract showed an $IC_{50}$ of 0.275 mg/mL for the brown grain and 98.64 mg/mL for brown flour flaxseed. This difference could be associated with the content of flavonoids in the extracts.

**Table 01: Phytochemical analysis of Brown Flaxseed used in this protocol.**

| Group / Compound | Brown Flaxseed – Grain (in µg/g of flaxseed) | Brown Flaxseed – Flour (in µg/ g of flaxseed) |
|---|---|---|
| Polyphenol compounds | 15940 ± 863 | 15970 ± 912 |
| Total flavonoids * | 13360 ± 748 | 5050 ± 434 |
| Ascorbic acid | 89.79 ± 14 | 70.39 ± 16 |
| Gallic acid * | 1737.26 ± 89 | 3483.67 ± 134 |
| Chlorogenic acid * | 915.82 ± 102 | 1994.37 ± 127 |
| Caffeic acid * | 811.13 ± 76 | 65.14 ± 12 |
| Rutin * | 567.47 ± 73 | 0.00 ± 0.00 |
| Quercetin * | 476.99 ± 47 | 1602.02 ± 203 |
| Kampferol * | 668.56 ± 84 | 581.11 ± 71 |

Data from the preparations were expressed as means ± S.D. Results were confirmed by an experiment that was repeated three times in triplicate. * Represents a difference between the presentation forms.

*3.2 Anthropometric and pressure parameters*

In **Table 2** is showed the anthropometric parameters of the volunteers in the two moments of this protocol (before treatment and after treatment).

**Table 02: Anthropometric and pressure parameters of the volunteers before and after ingestions of flaxseed.**

| Parameters | Brown Flaxseed – Grain | | Brown Flaxseed – Flour | |
|---|---|---|---|---|
| | Day 0 | Day 14 | Day 0 | Day 14 |
| **Total Volunteers** | 12 volunteers | | 8 volunteers | |
| **Medium Age (in Years)** | 23 years (18 – 32 years) | | 24 years (17 – 33 years) | |
| **Body Mass Index (BMI, in Kg/m²)** | 24.39 ± 5.67 | 24.27 ± 5.46 | 23.09 ± 2.33 | 23.16 ± 2.21 |
| **Systolic Pressure (in mmHg)** | 126.7 ± 14.35 | 115.8 ± 10.84 | 131.3 ± 17.27 | 121.3 ± 15.53 |
| **Diastolic Pressure (in mmHg)** | 83.33 ± 9.84 | 74.17 ± 7.93 | 85.00 ± 13.78 | 81.26 ± 11.26 |

Data are expressed as means ± S.D. In all parameter evaluated, there were no statistically different results in the column.

The study began with 24 volunteers, 12 in each group. **Table 02** shows the number of subjects in each group who remained in the study until day 14. Also in **Table 02** are shown the mean body mass index (BMI), systolic and diastolic pressure. It is observed that the results were within the normal range for healthy people according to Stahl et al.[20] There were no significant changes in these questions during the analysis period. This is in agreement with Stuglin and Prasad [21] that not found changes in blood pressure in a 4-week intervention using 32.7g/day of flaxseed.

*3.3 Oxidative parameters*

The **Figure 01** shows the results of biomarkers for oxidative damage. Lipid peroxidation is a complex process involving the interaction of oxygen-derived free radicals with polyunsaturated fatty acids, resulting in a variety of highly reactive electrophilic aldehydes. Lipid peroxidation is highly evident in several diseases.[22] Protein carbonylation is a type of protein oxidation that can be promoted by reactive oxygen species. It usually refers to a process that forms reactive ketones or aldehydes that can be reacted by 2,4-dinitrophenylhydrazine (DNPH) to form hydrazones. Direct oxidation of side chains of lysine, arginine, proline, and threonine residues, among other amino acids, in the "primary protein carbonylation" reaction produces DNPH detectable protein products.[23] The comet assay is not the only way to measure oxidative DNA damage, but it is one of the most sensitive and accurate, being relatively free of artefacts. It is a valuable tool in population monitoring, for example in assessing the role of oxidative stress in human disease, and in monitoring the effects of dietary antioxidants.[24] For assessing the clastogenic or aneugenic potential of a test compound, micronucleus induction in cells has been shown repeatedly to be a sensitive and a specific parameter.[25]

In "**A**" we have the results of lipid peroxidation. We can see that the ingestion of brown flaxseed grain during 14 days cause a high reduction in this parameter in order of 44.37 ± 16.6%. This result confirmed those found by Kaithwas and Majundar.[26] In their paper, a similar reduction was finding in liver, hearth and kidney in diabetic rats after an administration of diets with flaxseed oil for 3 weeks. Similar result wasn't observed in the ingestion of brown flaxseed flour. This lack of effect could be related with the fact that the flour has a lower level of flavonoids (**Table 1**). Although the concentration of polyphenols is similar, the concentration of total flavonoids in the flour is almost 3 times lower than in the grain. Flavonoids acts by the delocalization of electrons over aromatic ring, directly neutralizing the free radicals.[27]

**Figure 01B** shows the results of protein carbonylation. We observed a significant effect of the dietary intervention with brown flaxseed grain in protect the proteins against the reactive species. This result corroborated those showed by Chang et al., [28] which tested a protocol of one week in health volunteer (male) with consume of a high-polyphenol diet and obtained lower contents of carbonyl protein. As happen in lipid peroxidation this positive effect wasn't achieved with brown flaxseed flour. This

absence of result is also related with the flavonoids contents who are higher in the grain and shows a remarkable capacity of neutralize reactive species.[27]

In **Figure 01C** and **Figure 01D** are showed the results of DNA damage and micronucleus frequency, respectively. Our data shows no alteration in these two parameters. These results are also in agreement with a previous study of Bub et al.[29] In the cited study, healthy men received a beverage rich in polyphenols for 14 days, and no alterations in genetic parameters was observed.

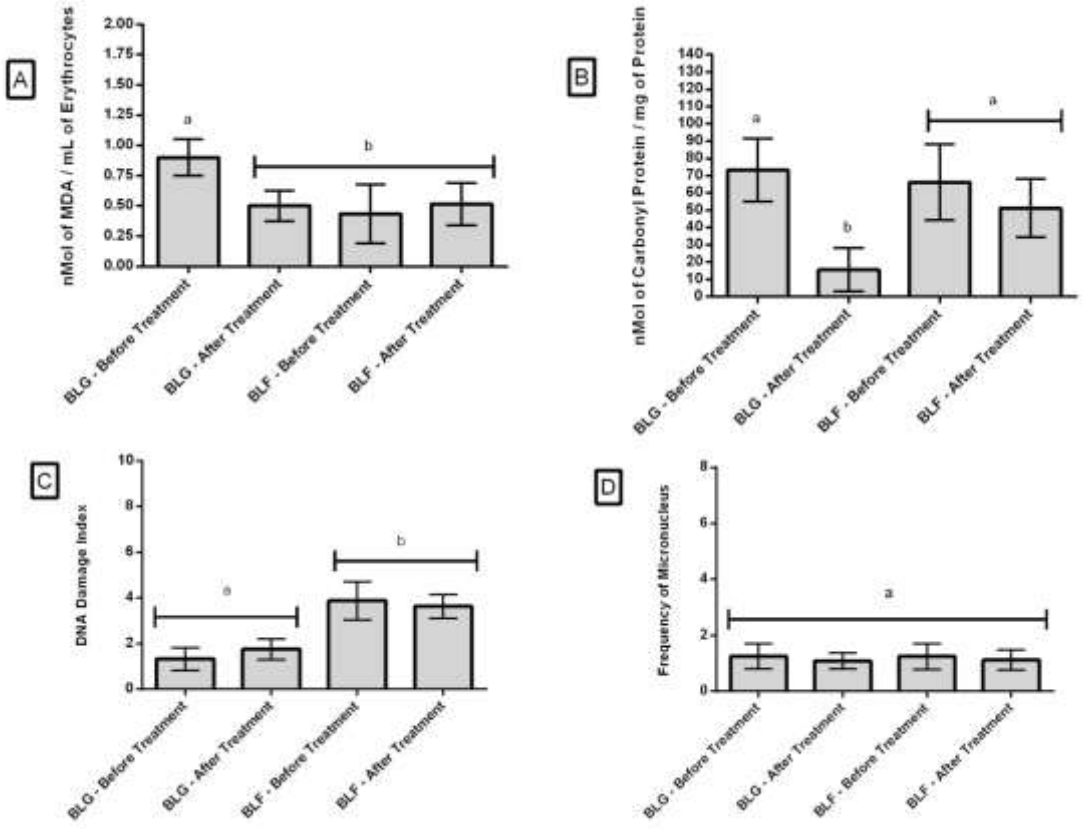


**Figure 01:** Oxidative damage markers in healthy volunteers before and after ingestions of flaxseed. In A: lipid peroxidation levels; B: carbonyl protein contents; C: DNA damage index; D: frequency of micronucleus. Data are expressed as means±S.D. Different letters means statistically different results ($p<0.05$). Legend: BLG: Brown Flaxseed Grain; BLF: Brown Flaxseed Flour.

In **Figure 02**, we show the results of oxidative defenses biomarkers in plasma. In "**A**" we show the variation in plasmatic polyphenols.

Polyphenols are secondary plant metabolites, widely present in commonly consumed foods of plant origin, and they are accruing a body of evidence as bioactive components in a wide range of biological systems.[17] The three main types of polyphenols are flavonoids, phenolic acids and tannins, which are powerful antioxidants *in vitro*. These compounds are considered to carry many potential beneficial health effects. As can be seen in **Figure 02A**, the polyphenol concentration decreased significantly after 14 days of supplementation. These findings meet the data of Machado et al., [30] which demonstrated that the polyphenol concentrations in feed intake are available in the circulation for up to 6 to 8 hours. Moreover, it is well established that *Linum usitatissimum* presents high levels of linoleic and linolenic acids. [31] These two compounds in high concentrations can cause a reduction of the polyphenols to cause increased levels of reactive oxygen species when they are metabolized.[32]

Acid ascorbic (vitamin C) has been implicated in many biological processes. It is a cofactor for several enzymatic steps in the synthesis of collagen, monoamines, amino acids, peptide hormones and carnitine and plays an important role in antioxidant defense at a number of levels.[33] **Figure 02B** shows a significantly increased the vitamin C after 14 day ingestion of brown flaxseed meal. These findings are a direct consequence of the concentrations of vitamin C found in the seed (**Table 01**) and consumed during the test period (2 weeks).

In **Figure 02**, "**C**", "**D**" and "**E**" is showed the results of s*uperoxide dismutase* (SOD), c*atalase (*CAT*)* e *glutathione peroxidase* (GPx). Mammalian cells have elaborate antioxidant defense mechanisms to control the damaging effects of ROS. SOD blunts the cascade of oxidations initiated by anion superoxide. MnSOD appears to be a central player in the redox biology of cells and tissues.[34] Catalase is well studied enzymes that play critical roles in protecting cells against the toxic effects of hydrogen peroxide.[35] Glutathione peroxidase is a selenoenzyme that plays a key role in protecting the organism from oxidative damage by catalyzing the reduction of harmful hydroperoxides with thiol cofactors.[36] All these enzymatic parameters didn't show significantly alterations in their rates. This kind of profile can be explained by the time of our protocol. Other studies involving polyphenols and antioxidants enzymes usually observe a positive correlation with diets within 30 days or more.[37]

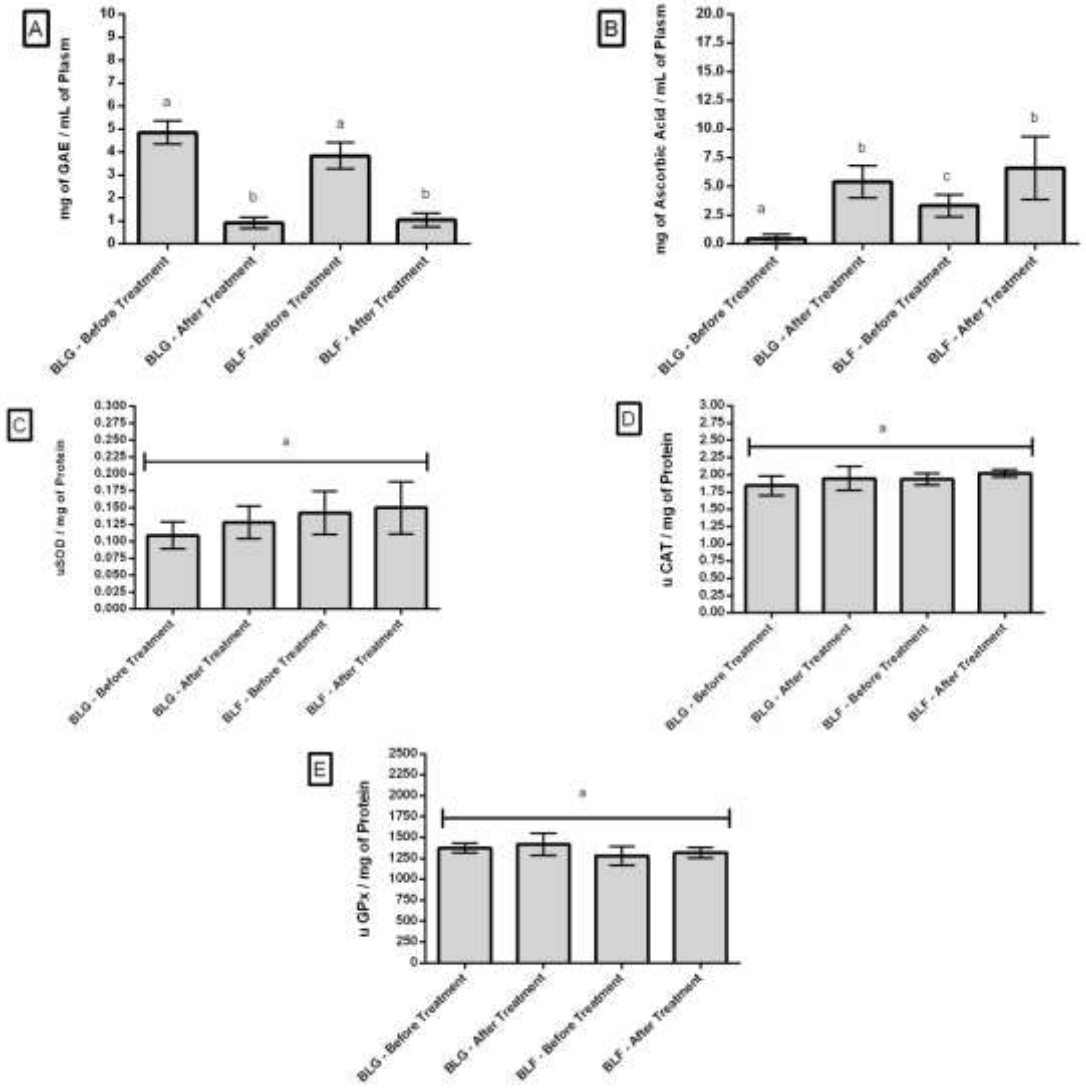


**Figure 02:** Oxidative defenses markers in healthy volunteers before and after ingestions of flaxseed. In A: polyphenols contents; B: ascorbid acid contents; C: superoxide dismutase activity; D: catalase activity; E: glutathione peroxidase activity. Data are expressed as means±S.D. Different letters means statistically different results ($p<0.05$). Legend: BLG: Brown Flaxseed Grain; BLF: Brown Flaxseed Flour.

*3.3 Biochemical and hematological parameters*

All clinical parameter evaluated in the protocol are showed in **Table 03**. All tests showed no significantly alteration at this time of supplementation. This is in agreement with Zhang et al.[38] In their paper, the lignans present in *Linum usitatissimum*

were tested in health volunteers for a period of 8 weeks. Alterations in clinical parameters were only observed after 4 weeks of treatment, therefore, in our study of 14 days, there wasn't enough time for an adaptation in organism and a significantly alteration in these parameters.

**Table 03: Hematological and biochemical parameters of the volunteers before and after ingestions of flaxseed.**

| Parameters | Brown Flaxseed – Grain | | Brown Flaxseed – Flour | |
|---|---|---|---|---|
| | Day 0 | Day 14 | Day 0 | Day 14 |
| **Glucose (mg/dL)** | 100.32 ± 9.70 | 89.10 ± 15.83 | 96.65 ± 5.30 | 102.10 ± 14.21 |
| **Total Cholesterol (mg/dL)** | 174.80 ± 28.13 | 185.60 ± 16.75 | 172.40 ± 39.20 | 177.25 ± 38.11 |
| **HDL Cholesterol (mg/dL)** | 58.10 ± 12.40 | 56.54 ± 7.90 | 39.25 ± 4.20 | 50.54 ± 11.32 |
| **LDL Cholesterol (mg/dL)** | 98.22 ± 32.60 | 90.90 ± 18.21 | 90.35 ± 29.70 | 104.70 ± 34.92 |
| **Triglycerides (mg/dL)** | 126.30 ± 68.70 | 134.80 ± 83.50 | 213.96 ± 134.50 | 110.27 ± 68.70 |
| **Total Protein (g/dL)** | 7.41 ± 0.79 | 6.83 ± 0.39 | 7.12 ± 0.35 | 6.90 ± 0.31 |
| **Hemoglobin (g/dL)** | 11.88 ± 0.94 | 11.98 ± 1.05 | 11.23 ± 1.07 | 10.69 ± 1.09 |
| **Hematocrit (%)** | 35.46 ± 2.72 | 35.40 ± 3.18 | 33.65 ± 2.73 | 31.50 ± 3.49 |
| **Erythrocytes ($10^6$/mm$^3$)** | 4.28 ± 0.37 | 4.23 ± 0.43 | 4.15 ± 0.29 | 3.92 ± 0.52 |
| **Leukocytes ($10^3$/mm$^3$)** | 8.43 ± 2.05 | 8.41 ± 2.90 | 6.96 ± 1.90 | 9.14 ± 1.89 |
| **Platelets ($10^3$/mm$^3$)** | 322.14 ± 55.12 | 281.80 ± 60.22 | 206.90 ± 66.31 | 186.30 ± 50.30 |

Data are expressed as means ± S.D. In all parameter evaluated, there were no statistically different results in the column.

## 4. Conclusion

In conclusion, the results show that supplementation with brown flaxseed for 14 days did not significantly change biochemical parameters. However, the use of brown flaxseed grain improved significantly oxidative stress markers, lowering the lipid peroxidation and the protein carbonilation. Thus, it is possible to suggest that the flaxseed could be a valuable adjunct to treating diseases which are based on the production of free radicals.

## Conflict of interest

The authors declare that there are no conflicts of interest.

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## MANUSCRITO II

# Golden flaxseed (*Linum usitatissimum* L) supplementation in healthy subjects does not change biochemical and hematological parameters.

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# Golden flaxseed (*Linum usitatissimum* L.) supplementation in healthy subjects does not change biochemical and hematological parameters.

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**Abstract**

Plants are considered as the best source of natural antioxidants. Among them, a functional food largely used is the flaxseed. Flaxseed (*Linum usitatissimum* L.) is composed by minerals, vitamins, proteins, lipids, moreover, contain significant amounts of other bioactive components such as polyphenols, polyunsaturated fatty acids, fibers and lignans. The objective of this study was to evaluate the presentation form more effective of golden flaxseed (grain or flour) and the real benefits under the oxidative status, glycemic level and lipid profile in healthy human volunteers. In order to, 24 volunteers were selected and instructed to ingest daily 40g of flaxseed *in natura* for 14-days. Collection of fasting venous blood, anthropometric and blood pressure measurements were performed before and after the supplementation period. The results demonstrated that clinical parameters do not change significantly in 14-days supplementation, but significant changes in markers of oxidative stress were observed. This research indicates that the flaxseed could be a valuable adjunct in treating diseases that are based on the production of free radicals.

**Keywords:** *Linum usitatissimum*, flaxseed, antioxidant, biochemical parameters, hematological parameters.

## 1. Introduction

Nowadays, the use of antioxidants compounds such as plant polyphenols, vitamin C, phenolic acids and flavonoids in foods are believed to have potential health benefits including the prevention and lowering incidence of chronic pathologies, in which oxidative stress is involved, by quenching reactive species in biological systems (Anwar and Przybylski, 2012).

Plants are considered as the best source of natural antioxidants (Anwar and Przybylski, 2012). Among the plant sources, a functional food largely used is the flaxseed. Flaxseed (*Linum usitatissimum* L.), belongs to the family Linaceae, is composed basically by minerals, vitamins, proteins, lipids, moreover, contain significant amounts of other bioactive components such as polyphenols, polyunsaturated fatty acids, fibers, and lignans (Anwar and Przybylski, 2012; Epaminondas et al., 2011).

Epidemiological evidences show that consumption of flaxseed and its derivate is correlated with a lower incidence of chronic pathologies such as cancer and cardiovascular diseases risk factors (Ayella et al., 2010; Fukumitsu et al., 2010). Intervention studies in humans have shown positive effects of flaxseed on lipid profile and insulin resistance (Bloedon et al., 2008).

Scientific studies of natural products have social objectives aimed at validating the use of them so that they can ensure efficacy when used as functional foods. The objective of this study was to evaluate the presentation of golden flaxseed more effective (grain or flour) and what is the real benefits under the oxidative status, glycemic level and lipid profiles in healthy human volunteers.

## 2. Material and Methods

### *2.1 Chemicals*

All the chemicals were of analytical grade. Solvents for HPLC analysis were purchased from Merck (Darmstadt, Germany). All the others reagents were acquired from Sigma Chemical Co. (St. Louis, MO, USA).

*2.2 Plant material*

Flaxseed was purchased at local supermarket in Uruguaiana (State Rio Grande do Sul, Brazil) on February of 2012.

*2.3 Phytochemical analysis*

For the phytochemical analysis, the flour was cold macerated in ethanol-water (70:30 v/v) in the dark and at room temperature for 7 days with daily agitation. After filtration, the extract was evaporated under reduced pressure to remove the ethanol in order to obtain the "flour extract". The same procedure was made for the grain, except that this was triturated in a blender before maceration. At the end of this procedure, we obtained the "grain extract".

*2.3.1 Determination of total polyphenolic contents*

The extracts were prepared following a standardized procedure: 0.5 g of the extracts was dissolved in 10 mL of ethanol and the volume adjusted to 100mL with water. An aliquot of 3 mL was dissolved in 100 mL of water. Final concentration was 0.15 mg/mL. The total polyphenol concentrations in the extracts were measured spectrophotometrically as described by modified Folin-Ciocalteau method (Chandra and de Mejia Gonzalez, 2004). Briefly, 0.5 mL of 2N Folin-Ciocalteau reagent was added to a 1 mL of each sample (0.15 mg/mL), and this mixture was allowed to stand for 5 min before the addition of 2 mL of 20% $Na_2CO_3$. The solution was then allowed to stand for 10 minutes before reading at 730 nm in a Shimadzu-UV-1201 (Shimadzu, Kyoto, Japan) spectrophotometer. The estimation of phenolic compounds in the extracts was carried out in triplicate. It is very difficult to choose suitable standards for total phenolic determination in plant extracts due to the chemical heterogeneity of plants products and the specificity of phenolic reagents. Thus, it is only possible to get relative equivalents with the standard used. The total polyphenol content was expressed as microgram equivalents of gallic acid per gram of flaxseed. The equation obtained for standard curve of gallic acid in the range of 0.001 – 0.020 mg/mL was $y = 40.112x + 0.0581$ ($R^2 = 0.9994$).

*2.3.2 Determination of flavonoids*

The analysis was made according to Woisky and Salatino (1998). To 2 mL of each reference solution, 20 mL of methanol and 1mL of 5% $AlCl_3$ (w/v) were added and

the volume made up to 50 mL with methanol at 20ºC. After 30 minutes, the absorbances were measured at 425 nm in a Shimadzu-UV-1201 spectrophotometer. The same procedure was made to analyse of the extracts. The blank was the 5% $AlCl_3$ (w/v) alone. The estimation of flavonoids was carried out in triplicate. The contents were expressed as microgram equivalents of quercetin per gram of flaxseed. The equation obtained for standard curve of quercetin in the range of 0.02 – 0.60 mg/mL was $y = 0.891x + 0.0149$ ($R^2 = 0.9723$).

*2.3.3 Determination of ascorbic acid content*

Sulphuric acid solution 1M was used to solubilize and prepare the curve of ascorbic acid used as reference. The references samples were mixed with trichloroacetic acid 13,3% and 2,4-dinitrophenylhydrazine. The procedure followed the description of Jacques-Silva et al. (2001). After an incubation period, the samples were measured at 520 nm in a Shimadzu-UV-1201 spectrophotometer. The equation obtained for standard curve of ascorbic acid in the range of 1.5 – 4.5 mg/mL was $y = 0.0534x + 0.0713$ ($R^2 = 0.9790$). The extracts were measured using the same procedure. The estimation of ascorbic acid concentration was carried out in triplicate.

*2.3.4 HPLC analysis*

High performance liquid chromatography (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software. Reverse phase chromatographic analyses were carried out under gradient conditions using C18 column (4.6 mm x 150 mm) packed with 5 μm diameter particles; the mobile phase was water containing 2% acetic acid (A) and methanol (B), and the composition gradient was: 5% of B until 2 minutes and changed to obtain 25%, 40%, 50%, 60%, 70% and 100% B at 10, 20, 30, 40, 50 and 80 minutes, respectively, following the method described by Laghari et al. (2011) with slight modifications. The presence of six antioxidants compounds was investigated, namely, gallic acid, chlorogenic acid, caffeic acid, quercetin, rutin and kaempferol. Identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. The flow rate was 0.7

mL/min, injection volume 40 μL and the wavelength were 254 nm for gallic acid, 327 nm for caffeic and chlorogenic acids, and 365 nm for quercetin, rutin and kaempferol. The samples and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.020 – 0.200 mg/mL for quercetin, rutin and kaempferol and 0.050 – 0.250 mg/mL for gallic, caffeic and chlorogenic acids. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to 500 nm). Calibration curve for gallic acid: Y = 13260x + 1276.4 (r = 0.9998); chlorogenic acid: Y = 12158x + 1174.9 (r = 0.9996); caffeic acid: Y = 14034x + 1527.5 (r = 0.9995); rutin: Y = 13721x + 1068.4 (r = 0.9997); quercetin: Y = 13794x + 1392.1 (r =0.9999) and kaempferol: Y = 12647x + 1178.3 (r =0.9995). All chromatography operations were carried out at ambient temperature and in triplicate.

*2.3.5 Radical - scavenging capacity - DPPH assay*

The antioxidant capacity of the extracts were evaluated by monitoring its ability in quenching the stable free radical DPPH, according to a slightly modified method previously described by Choi et al. (2002). Spectrophotometric analysis (SHIMADZU-UV-1201) was used in order to determine the inhibition concentration (IC) and the inhibition percentage (IP %) of the crude extract. The DPPH quenching ability was expressed as $IC_{50}$ (concentration which gives 50% inhibition). Six different ethanol dilutions of the extracts (2.5 mL), at 250; 125; 62.5; 31.25; 15.62; and 7.81 μg/mL were mixed with 1.0 mL of a 0.3 mM DPPH ethanol solution. The absorbance was measured at 518 nm by spectrophotometer against a blank after 30 min of reaction at room temperature in the dark. DPPH solution (1.0 mL, 0.3mM) plus ethanol (2.5 mL) was used as a control. Relative activities were calculated from the calibration curve of *L*-ascorbic acid standard solutions working in the same experimental conditions. Inhibition of free radical by DPPH in percent (IP %) was calculated in following way, according to the **Equation 1**:

**Equation 1:** $IP\% = 100 - [(ABS_{SAMPLE} - ABS_{BLANK}) / ABS_{CONTROL}) \text{ X } 100]$

Where, $ABS_{SAMPLE}$ is the absorbance of the test compound, $ABS_{BLANK}$ is the absorbance of the blank (containing 1.0 mL of ethanol plus 2.5 mL of the plant extract solution) and

$ABS_{CONTROL}$ is the absorbance of the control reaction (containing all reagents except the test compound). IP% was plotted against sample concentration, and a linear regression curve was established in order to calculate the $IC_{50}$. Tests were carried out in triplicate.

*2.4 Selection of volunteers*

Twenty four individuals between the ages of 17 and 34 years, of both sexes, non-smokers, without hypertension, not on regular medication, using no antioxidant and mineral supplementation and with no previous history of stroke, myocardial infarction, angina, diabetes mellitus, cancer or any illness that had required hospitalization during the previous 12 months were recruited at Uruguaiana and enrolled in the study between March 2012 and April 2012. Female volunteers were excluded if pregnant, lactating or on hormone replacement therapy.

*2.5 Ethical issues*

This study was approved by the Ethics and Research of the Federal University of Pampa (Protocol No. 069/2011). All participants signed a consent form.

*2.6 Experimental design*

Twenty four participants were randomly assigned into one of two groups: flour flaxseed or grain flaxseed supplementation group (n = 12). They received 40 grams (Bloedon et al., 2008) aliquots of flour or grain of golden flaxseed for daily use for a period of 14 days (Rajesha et al., 2006). Subjects were instructed to consume the flaxseed *in natura* mixed with water in the morning for 2 weeks.

2.7 *Anthropometric and pressure measure*

Each participant's height was measured at the beginning of the study. Using a balance beam scale, each participant was weighed at the beginning and end of supplementation period. BMI was calculated using the equation BMI = weight (kg) / height$^2$ ($m^2$). Blood pressure was taken from the right arm with the participant seated,

after a 4–5 minutes rest and by person trained to carry out the process repeatedly in a similar manner. A digital manometer was used to perform these measures.

*2.8 Blood sample collection*

Fasting blood samples were taken at the beginning of the study and at the end of 14 days of supplementation in the morning between 7 and 9 h. Blood was drowned from the antecubital vein into prechilled tubes containing EDTA, Li-heparin and tubes without anticoagulant (Vacutainer - Becton, Dickinson and Company – New Jersey – USA). Blood in tubes without anticoagulant were allowed to clot at room temperature for 30 min. The plasma and serum were collected after centrifugation at 1500 x g for 10 min and analyzed immediately.

*2.9 Sample Analysis*

*2.9.1 Oxidative parameters*

The oxidative parameters, vitamin C (Jacques-Silva et al., 2001), lipid peroxidation (Ohkawa et al., 1979) and protein carbonyls (Levine, 2002) in plasma were measured by the spectrophotometric methods; the assessment of DNA damage was made by comet assay (Singh et al., 1988) and frequency of micronucleus (Schmid, 1975) in leukocytes; the activities of superoxide dismutase, catalase and glutathione peroxidase in erythrocytes were achieved with commercial kits (Randox Brazil LTDA).

*2.9.2 Biochemical and hematological analysis*

The hemograms (complete blood count) were performed in an automatic counter Cell-Dyn 3200 Hematology Analyzer (Abbott Diagnostic, St Clara, CA, USA) and total cholesterol, HDL cholesterol, triglycerides total, protein and glucose levels using automatic analyzers A25 Biosystems (Biosystems SA, Barcelona, Spain) for *in vitro* diagnostics. LDL cholesterol values were computed according to the Friedewald formula. All biochemical assays were carried out in triplicate.

*2.10 Statistical analysis*

Data were expressed as mean ± standard deviation (SD). Comparisons between groups were performed using a one-way analysis of variance (ANOVA), followed by post hoc of Bonferroni for multiple comparison tests. Results were considered statistically significant when $p<0.05$.

## 3. Results and discussion

*3.1 Phytochemical analysis*

Polyphenols are secondary metabolites of plants and are largely distributed in vegetal kingdom (Hu et al., 2011). Dietary polyphenols exhibit many biologically significant functions, specially decreased risk of diseases associated with oxidative stress, such as cancer, degenerative disorders and cardiovascular disease. From amongst these, flavonoids compounds have been researched most extensively for the identification of structures responsible for enhancing direct antioxidant properties (Ciesla et al., 2013). The concentrations of these groups of secondary metabolites and some of the compounds present in this species, all with reported biological activities, are shown in **Table 01.**

The DPPH assay is used as a tool for the *in vitro* evaluation of extracts, and its results can be related with the presence of phenolic compounds in plant extracts (Ciesla et al., 2013). Our plant sample showed an $IC_{50}$ of 0.226 mg/mL for the golden grain and 4.040 mg/mL for golden flour flaxseed. This difference could be associated with the content of flavonoids in the extracts.

**Table 01: Phytochemical analysis of Golden Flaxseed used in this protocol.**

| Group / Compound | Golden Flaxseed – Grain (in µg/g of flaxseed) | Golden Flaxseed – Flour (in µg/ g of flaxseed) |
|---|---|---|
| Polyphenol compounds | 10560 ± 839 | 10550 ± 749 |
| Total flavonoids * | 9740 ± 642 | 6234 ± 673 |
| Ascorbic acid | 155.04 ± 28.14 | 161.74 ± 11.26 |
| Gallic acid | 416.08 ± 35.64 | 476.12 ± 54.18 |
| Chlorogenic acid * | 419.60 ± 19.39 | 2500.09 ± 127.32 |
| Caffeic acid * | 371.18 ± 56.32 | 2705.46 ± 98.04 |
| Rutin * | 715.76 ± 64.19 | 272.71 ± 41.56 |
| Quercetin * | 574.22 ± 37.86 | 1037.56 ± 104.38 |
| Kampferol * | 695.80 ± 46.32 | 174.88 ± 19.47 |

Data from the preparations were expressed as means ± S.D. Results were confirmed by an experiment that was repeated three times in triplicate. * Represents differences between the presentation forms.

*3.2 Anthropometric and pressure parameters*

In **Table 02** is showed the anthropometric parameters of the volunteers in the two moments of this protocol (before treatment, indicate as Day Zero and after a 14 days treatment, indicate as Day 14).

**Table 02: Anthropometric and pressure parameters of the volunteers before and after ingestions of flaxseed.**

| Parameters | Golden Flaxseed – Grain | | Golden Flaxseed – Flour | |
|---|---|---|---|---|
| | Day 0 | Day 14 | Day 0 | Day 14 |
| **Total Volunteers** | 12 volunteers | | 10 volunteers | |
| **Medium Age (in Years)** | 26 years (18 – 33 years) | | 23 years (17– 34 years) | |
| **Body Mass Index (BMI, in Kg/m²)** | 23.62 ± 4.15 | 23.58 ± 4.07 | 22.89 ± 4.54 | 23.06 ± 4.49 |
| **Systolic Pressure (in mmHg)** | 123.3 ± 11.55 | 114.2 ± 15.64 | 128.8 ± 9.91 | 126.0 ± 11.74 |
| **Diastolic Pressure (in mmHg)** | 77.50 ± 8.66 | 69.17 ± 7.93 | 84.44 ± 8.81 | 83.33 ± 10.00 |

Data are expressed as means ± S.D. In all parameter evaluated, there were no statistically different results in the column.

The study began with 24 volunteers, 12 in each group. **Table 02** shows the number of subjects in each group who remained in the study until day 14. Also in **Table 02** are shown the mean body mass index (BMI), systolic and diastolic pressure. It is observed that the results were within the normal range for healthy people, according to Stahl et al. (2012). There were no significant changes in these questions during the analysis period. This is in agreement with Stuglin and Prasad (2005) that not found changes in blood pressure in 4 week intervention using 32.7g/day of flaxseed. In other hand, Paschos et al. (2007) found that supplementation with 15 mL/day flaxseed oil for 12 weeks significantly decreased both systolic and diastolic pressure in middle-aged dyslipidaemic men.

*3.3 Oxidative parameters*

The **Figure 01** shows the results of biomarkers for oxidative damage. Lipid peroxidation is a complex process involving the interaction of oxygen-derived free radicals with polyunsaturated fatty acids, resulting in a variety of highly reactive electrophilic aldehydes. Lipid peroxidation is highly evident in several diseases (Tsang and Chung, 2009). Oxidative alterations of proteins by reactives species mainly affect amino acid side chains. Detection and quantitative analysis of carbonyl groups have been based on the formation hidrazones after derivatization with 2,4-dinitrophenylhydrazine (DNPH) (Tamarit et al., 2012).The comet assay is not the only way to measure oxidative DNA damage, but it is one of the most sensitive and reproducible (Furness et al., 2011). It is a valuable tool for measuring DNA damage in pathological and physiological conditions and dietary studies (Fikrova et al., 2011). For assessing the clastogenic or aneugenic potential of a test compound, micronucleus induction in cells has been shown repeatedly to be a sensitive and a specific parameter (Frieauff et al., 2013).

In "**A**" we have the results of Lipid Peroxidation. We can see that the ingestion of golden flaxseed grain during 14 days cause a high reduction in this parameter in order of 54.88 ± 20.8%. This result confirmed those found by Kaithwas and Majundar (2012). In their paper, a similar reduction was finding in liver, hearth and kidney in diabetic rats after an administration of diets with flaxseed oil for 3 weeks. Similar result wasn't observed in the ingestion of golden flaxseed flour. This lack of effect could be related with the fact that the flour has a lower level of flavonoids (**Table 01**). Although the concentration of polyphenols is similar, the concentration of total flavonoids in the flour is lower than in the grain. Flavonoids acts by the deslocalization of electrons over aromatic ring, directly neutralizing the free radicals (Katz et al., 2011).

**Figure 01B** shows the results of protein carbonylation. We observed a significant effect of the dietary intervention with golden flaxseed grain in protect the proteins against the reactive species. This result corroborated those showed by Chang et al. (2010) which tested a protocol of one week in health volunteer (male) with consuming a high-polyphenol diet and obtained lower contents of carbonyl protein. As happen in lipid peroxidation this positive effect wasn't achieved with golden flaxseed flour. This absence of result is also related with the flavonoids content who are higher in

the grain and shows a remarkable capacity of neutralize reactive species (Katz et al., 2011).

In **Figure 01C** and **Figure 01D** are showed the results of DNA damage and micronucleus frequency, respectively. Our data shows no alteration in these two parameters. These results are also in agreement with a previous study of Giovannelli et al. (2011). In the cited study, post-menopausal woman received a beverage rich in polyphenols for 30 days, and wasn't observed protective activity towards oxidative DNA damage.

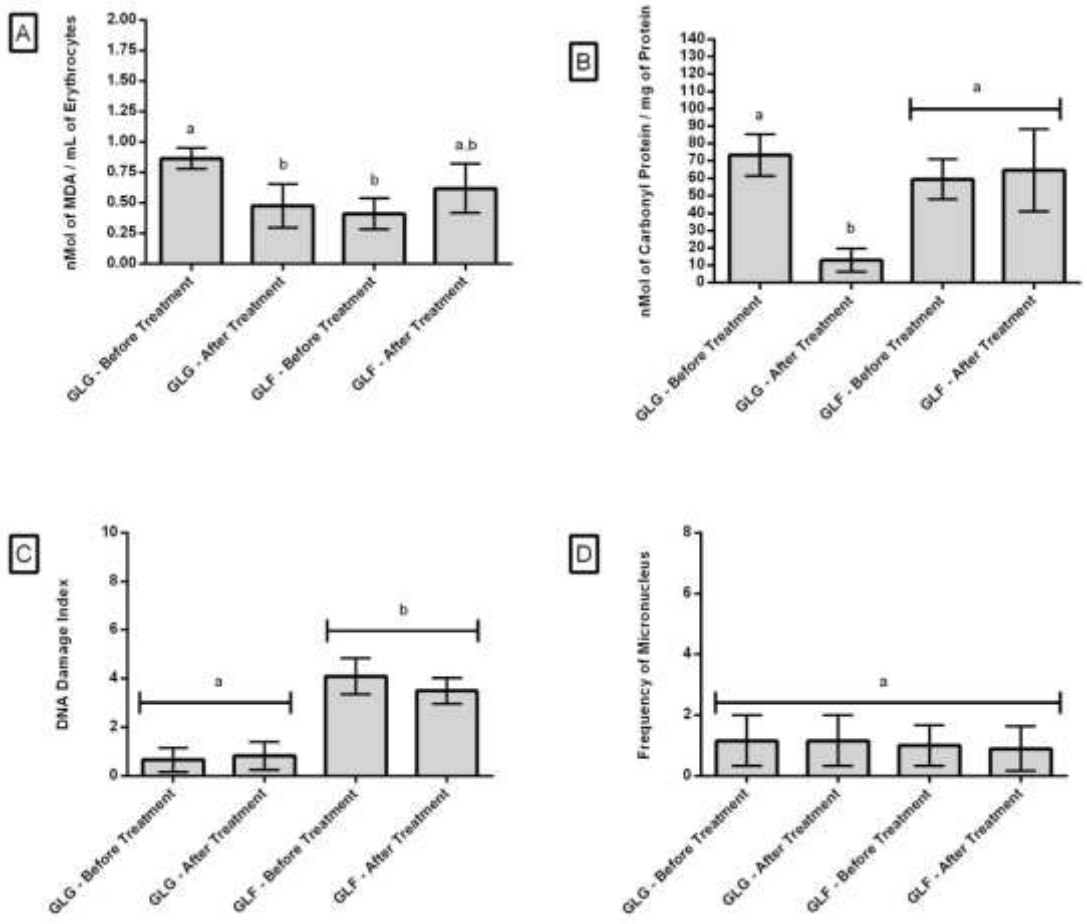


**Figure 01:** Oxidative damage markers in healthy volunteers before and after ingestions of flaxseed. In A: lipid peroxidation levels; B: carbonyl protein contents; C: DNA damage index; D: frequency of micronucleus. Data are expressed as means±S.D. Different letters means statistically different results ($p<0.05$). Legend: GLG: Golden Flaxseed Grain; GLF: Golden Flaxseed Flour.

In **Figure 02**, we show the results of oxidative defenses biomarkers in plasma. In "**A**" we show the variation in plasmatic Polyphenols. Polyphenols are secondary plant metabolites, widely present in commonly consumed foods of plant origin, and they are accruing a body of evidence as bioactive components in a wide range of biological systems (Perez-Jimenez et al., 2010). The three main types of polyphenols are flavonoids, phenolic acids and tannins, which are powerful antioxidants *in vitro*. These compounds are considered to carry many potential beneficial health effects. As can be seen in **Figure 02A**, the polyphenol concentration decreased significantly after 14 days of supplementation. These findings meet the data of Mitjavila and Moreno (2012), which demonstrated that the bioavailability for most of polyphenol is lower 8 hours and there is no evidence that polyphenols are stored in the body. Moreover, it is well established that *Linun usitatissimum* presents high levels of linoleic and linolenic acids (Chetana et al., 2010). These two compounds in high concentrations can cause a reduction of the polyphenols to cause increased levels of reactive oxygen species when they are metabolized (Furukawa et al., 2004). Acid ascorbic (vitamin C) has been implicated in many biological processes. It is a cofactor for several enzymatic steps in the synthesis of collagen, monoamines, amino acids, peptide hormones, and carnitine and plays an important role in antioxidant defense at a number of levels (Wilkinson et al., 2005). **Figure 02B** shows a significantly increased the vitamin C after 14 days ingestion of golden flaxseed grain. These findings are a direct consequence of the concentrations of vitamin C found in the seed and consumed during the test period (2 weeks). Also in **Figure 02**, "**C**", "**D**" and "**E**", is showed the results of *superoxide dismutases* (SOD), *catalase* (CAT) e *glutathione peroxidase* (GPx). Mammalian cells have elaborate antioxidant enzymatic defense mechanisms to control the damaging effects of ROS. SOD blunts the cascade of oxidations initiated by anion superoxide. MnSOD appears to be a central player in the redox biology of cells and tissues (Buettner, 2011). Catalase is well studied enzymes that play critical roles in protecting cells against the toxic effects of hydrogen peroxide (Goyal and Basak, 2010). Glutathione Peroxidase is a selenoenzyme, which plays a key role in protecting the organism from oxidative damage by catalyzing the reduction of harmful hydroperoxides with thiol cofactors (Bhabak and Mugesh, 2010). All these enzymatic parameter don´t showed significantly alterations in their rates. This kind of profile can be explained by the time of our protocol. Valentová et al. (2013) demonstrated that enzymatic oxidative defense status was not influenced by the intake beverage rich in polyphenols for a

period of 10 days. Also, other studies involving polyphenols and antioxidants enzymes usually observe a positive correlation with diets within 30 days or more (Nelson et al., 2006).

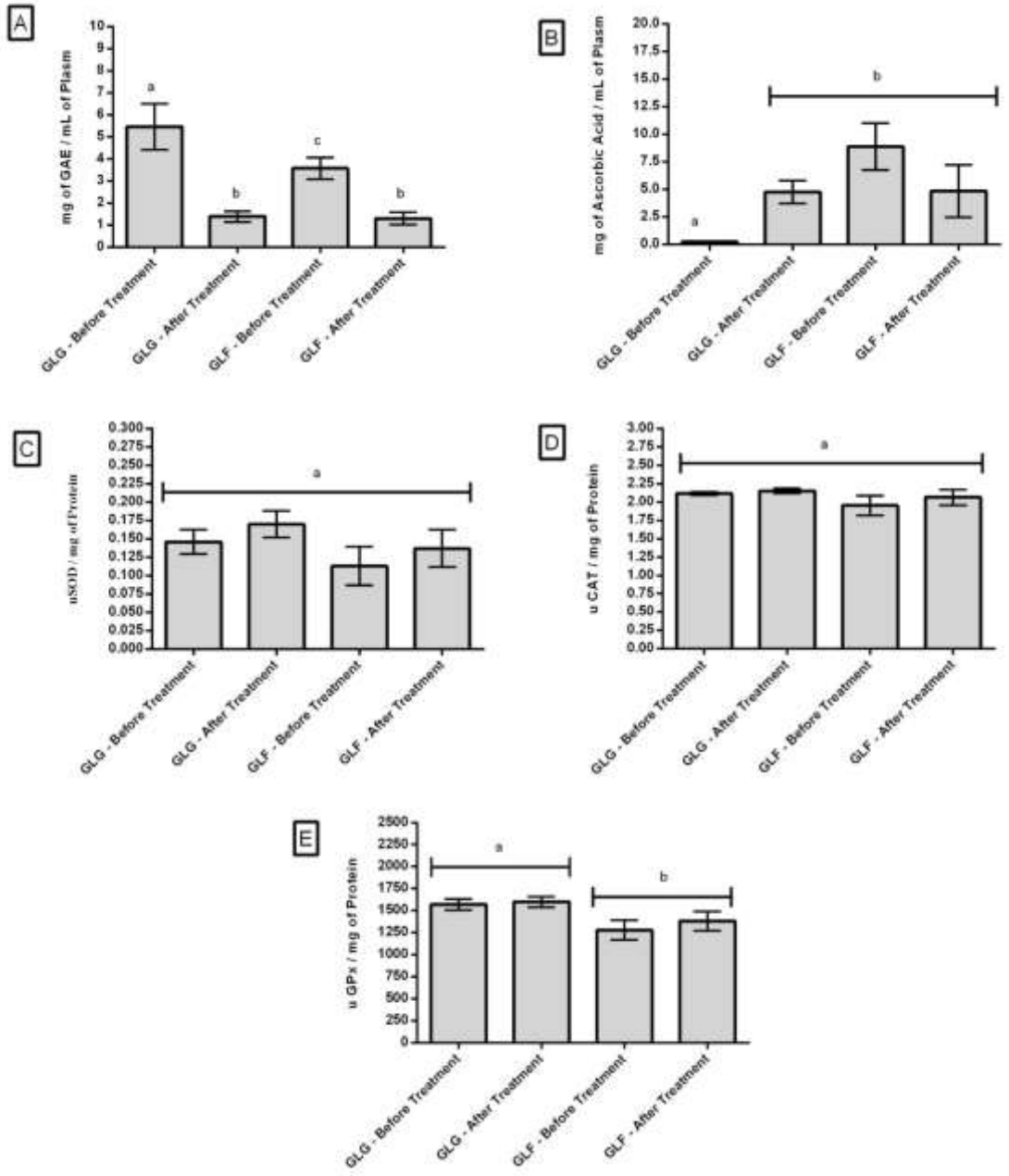


**Figure 02:** Oxidative defenses markers in healthy volunteers before and after ingestions of flaxseed. In A: polyphenols contents; B: ascorbid acid contents; C: superoxide dismutase activity; D: catalase activity; E: glutathione peroxidase activity. Data are expressed as means±S.D. Different letters means statistically different results ($p<0.05$). Legend: GLG: Golden Flaxseed Grain; GLF: Golden Flaxseed Flour.

*3.4 Biochemical and hematological parameters*

All clinical parameter evaluated in the protocol are showed in **Table 03**. All tests showed no significantly alteration at this time of supplementation. This is in agreement with Zhang et al. (2008). In their paper, the lignans present in *Linum usitatissimum* were tested in health volunteers for a period of 8 weeks. Alterations in clinical parameters were only observed after 4 weeks of treatment, therefore, in our study of 14 days, there wasn´t enough time for an adaptation in organism and a significantly alteration in these parameters.

**Table 03: Hematological and biochemical parameters of the volunteers before and after ingestions of flaxseed.**

| Parameters | Golden Flaxseed – Grain | | Golden Flaxseed – Flour | |
|---|---|---|---|---|
| | Day 0 | Day 14 | Day 0 | Day 14 |
| **Glucose (mg/dL)** | 82.56 ± 8.71 | 95.38 ± 7.68 | 101.30 ± 8.39 | 103.40 ± 14.03 |
| **Total Cholesterol (mg/dL)** | 188.10 ± 23.64 | 194.50 ± 32.22 | 177.90 ± 36.85 | 184.00 ± 42.13 |
| **HDL Cholesterol (mg/dL)** | 60.92 ± 9.82 | 59.67 ± 11.30 | 53.00 ± 10.30 | 44.40 ± 5.46 |
| **LDL Cholesterol (mg/dL)** | 95.25 ± 43.54 | 96.47 ± 33.92 | 100.00 ± 40.36 | 108.00 ± 41.44 |
| **Triglycerides (mg/dL)** | 149.60 ± 87.43 | 156.80 ± 92.57 | 105.00 ± 69.16 | 149.00 ± 72.44 |
| **Total Protein (g/dL)** | 7.41 ± 0.66 | 7.16 ± 0.57 | 7.40 ± 0.51 | 7.10 ± 0.73 |
| **Hemoglobin (g/dL)** | 11.93 ± 1.19 | 11.77 ± 1.06 | 10.51 ± 0.65 | 10.99 ± 0.89 |
| **Hematocrit (%)** | 35.12 ± 3.76 | 34.96 ± 3.24 | 32.44 ± 1.93 | 32.88 ± 2.75 |
| **Erythrocytes ($10^6/mm^3$)** | 4.23 ± 0.49 | 4.18 ± 0.41 | 4.19 ± 0.43 | 4.11 ± 0.46 |
| **Leukocytes ($10^3/mm^3$)** | 9.23 ± 2.47 | 8.31 ± 1.48 | 8.01 ± 1.36 | 7.21 ± 1.81 |
| **Platelets ($10^3/mm^3$)** | 291.16 ± 44.58 | 300.58 ± 48.64 | 249.90 ± 51.51 | 214.00 ± 52.79 |

Data are expressed as means ± S.D. In all parameter evaluated, there were no statistically differences within the columns.

## 4. Conclusion

In conclusion, these results demonstrate that clinical parameters do not change significantly with the use of golden flaxseed for periods up to14 days, but significant changes in markers of oxidative stress were observed.

## 5. Conflict of interest statement

All authors report no conflict of interest.

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# PARTE III

## 5. DISCUSSÃO GERAL

Vários estudos têm demonstrado a importância da dieta no desenvolvimento de doenças crônicas, como câncer, desordens cardiovasculares e neurodegenerativas. O consumo de frutas, hortaliças e cereais está associado à redução no risco de desenvolvimento dessas doenças e esta relação pode ser atribuída à presença de antioxidantes naturais nos alimentos, como vitamina C, tocoferóis, carotenóides e polifenóis, além de outros compostos bioativos (ALVAREZ-JUBETE et al., 2010).

Embora os antioxidantes sejam encontrados em diversos alimentos, há variação na concentração e no tipo desses compostos devido a fatores genéticos e ambientais, bem como, condições de processamento. Portanto, a concentração de compostos fenólicos varia entre dietas, dependendo do tipo e quantidade de alimento consumido (ALVAREZ-JUBETE et al., 2010).

Os polifenóis destacam-se por demonstrarem maior capacidade antioxidante *in vitro* que outros tradicionalmente utilizados, como vitamina C e α-tocoferol, enfatizando sua importância como antioxidantes na dieta. Entre os compostos fenólicos, os flavonoides têm sido extensivamente pesquisados, visto serem estes os polifenóis com maior atividade antioxidante relatada (IGNAT et al., 2011).

As concentrações de polifenóis e alguns dos seus principais representantes, presente na dieta utilizada neste estudo estão demonstradas nas **Tabelas 01 dos manuscritos.** Observa-se que embora as concentrações dos polifenóis presentes na linhaça de ambas as variedades e formas de apresentação sejam semelhantes, a quantidade de flavonoides apresenta-se maior nos grãos de linhaça.

O teste do DPPH tem sido amplamente utilizado como uma ferramenta *in vitro* para a identificação de fontes potenciais de antioxidantes (ALVAREZ-JUBETE et al., 2010). Neste estudo, o teste do DPPH determinou a concentração em que 50% da autooxidação do radical 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) está inibida ($IC_{50}$). As amostras de grão de linhaça dourada e marrom, e farinha de linhaça dourada e marrom exibiram $IC_{50}$ de 0,226 mg/mL e 0,275 mg/mL, e 4,040 mg/mL e 98,64 mg/mL respectivamente. Estes resultados indicam que os grãos de linhaça são fontes ricas de polifenóis, entretanto as farinhas de linhaça apresentam uma capacidade menor em

inibir a autooxidação do radical DPPH$^{\bullet}$, a qual pode ser decorrente do conteúdo de flavonoides que é inferior nas farinhas se comparado aos grãos de linhaça de ambas as variedades (**Tabelas 01 dos manuscritos)**.

As **Tabelas 02 dos manuscritos** mostram as médias dos parâmetros antropométricos e as medidas pressóricas nos voluntários antes e após a suplementação com linhaça. Observa-se que os resultados de pressão sistólica, diastólica e IMC encontram-se dentro dos limites considerados normais para pessoas saudáveis de acordo com Stahl e colaboradores (2012). Não houve mudanças significativas nestes parâmetros durante o período analisado, corroborando os achados de Stuglin e Prasad (2005) que também não encontraram alterações na pressão arterial na intervenção dietética com 3 *muffins* contendo 32,7 g/dia de linhaça durante 4 semanas. Por outro lado, Paschos e colaboradores (2007) relataram que a suplementação com 15 mL/dia de óleo de linhaça por 12 semanas, diminui significativamente a pressão sistólica e diastólica em homens de meia idade dislipidêmicos.

Os parâmetros bioquímicos e hematológicos avaliados neste protocolo são mostrados na **Tabela 03 dos manuscritos**. Estes parâmetros não sofreram alteração significativa no período de suplementação. Zhang et al. (2008) testaram as lignanas presentes no *Linum usitatissimum* em voluntários saudáveis por períodos de 8 semanas. Alterações em parâmetros clínicos somente foram observadas após 4 semanas de tratamento, portanto, neste estudo de 14 dias, não houve tempo suficiente para uma adaptação do organismo e alterações significativas nestes parâmetros.

A peroxidação lipídica é um processo complexo que envolve a interação de espécies reativas com os ácidos graxos poliinsaturados das membranas celulares, resultando na formação de hidro ou lipoperóxidos, que são altamente reativos e podem dar início a uma cascata oxidativa, com severos danos a integridade da membrana (TSANG e CHUNG, 2009). Podemos observar nas **Figuras 01A dos manuscritos** que a ingestão de grãos de linhaça marrom ou dourada durante 14 dias causou redução de 44,37± 16.6% e 54,88 ± 20.8% respectivamente, nos níveis de lipoperoxidação. Este resultado está de acordo com os encontrados por Kaithwas e Majundar (2012) em fígado, coração e rim de ratos diabéticos após a administração de dietas com óleo de linhaça nas concentrações de 1, 2 e 3 mg/kg durante 3 semanas. Resultados similares não foram encontrados, neste estudo, com a ingestão das farinhas de linhaça dourada ou marrom. Esta ausência de efeitos pode estar relacionada com o menor teor de flavonoides presente nas farinhas de linhaça dourada e marrom, embora a concentração

de polifenóis seja similar a dos respectivos grãos. Os flavonoides atuam pela deslocalização do elétron no anel aromático, neutralizando diretamente os radicais livres e consequentemente o dano oxidativo (KATZ et al., 2011).

Alterações oxidativas de proteínas por espécies reativas afetam principalmente as cadeias laterais de aminoácidos e a detecção e quantificação dos grupos carbonil é baseada na formação de hidrazonas após derivatização com 2,4-dinitrofenilhidrazina (DNPH) (STADTMAN e LEVINE, 2000). Nas **Figuras 01B dos manuscritos** observamos efeito positivo da intervenção dietética com grãos de linhaça dourada ou marrom em proteger as proteínas citoplasmáticas do dano oxidativo causado pelas espécies reativas. Este resultado confirma os achados de Chang et al. (2010) que testaram o efeito de uma dieta rica em polifenóis em voluntários do sexo masculino, sadios, durante 1 semana. Assim, como na peroxidação lipídica, este efeito positivo não foi observado com o uso das farinhas de linhaça dourada ou marrom, sendo esta ausência de efeitos provavelmente relacionada ao conteúdo de flavonoides que é maior nos grãos e mostram uma alta capacidade de neutralizar espécies reativas (KATZ et al., 2011).

O teste cometa não é a única forma de mensurar o dano oxidativo ao DNA, porém é um dos mais sensíveis e acurados, sendo relativamente livre de artefatos. É uma ferramenta importante no monitoramento de populações, como por exemplo, na avaliação do estresse oxidativo nas doenças humanas e monitoramento de antioxidantes da dieta (COLLINS, 2009). Por outro lado, para avaliar o potencial clastogênico e aneugênico de um determinado composto, o teste da indução de micronúcleos tem se mostrado um parâmetro reprodutível, específico e sensível (FRIEAUFF et al., 2013). As **Figuras 01C e 01D dos manuscritos** mostram os resultados do dano ao DNA e da frequência de micronúcleos, respectivamente. Os resultados não mostram alterações nestes dois parâmetros, confirmando os estudos prévios de Giovanelli et al. (2011) e de Bub et al. (2003). Nestes estudos, foram utilizadas bebidas ricas em polifenóis por 30 dias e 14 dias, respectivamente, não sendo observado efeito protetor ao material genético durante o período experimental.

Como pode ser visto na **Figura 02A dos manuscritos** a concentração de polifenóis plasmáticos decresceu significativamente após os 14 dias de suplementação com grãos e farinha de linhaça de ambas as variedades. Estes achados confirmam os encontrados por Machado et al. (2012) e Mitjavila e Moreno (2012) que avaliaram a biodisponibilidade dos polifenóis na circulação e observaram que os compostos

fenólicos ingeridos permanecem na corrente sanguínea por até 8 horas. Além disso, está bem estabelecido que o *Linum usitatissimum* apresenta altos níveis de ácidos graxos poliinsaturados, Linoleico e Linolênico (CHETANA et al., 2010) e que estes compostos quando metabolizados geram espécies reativas. Baseado nesta hipótese, possivelmente a redução nos níveis plasmáticos de polifenóis é decorrente do consumo dos mesmos na neutralização das espécies reativas geradas na metabolização dos ácidos graxos poliinsaturados presentes na linhaça (FURUKAWA et al., 2004).

O ácido ascórbico (vitamina C) atua como cofator em várias reações enzimáticas, como síntese de colágeno, aminoácidos, hormônios peptídicos e carnitina, e desempenha um papel importante na defesa antioxidante (WILKINSON et al., 2005). As **Figuras 02B dos manuscritos** mostram um aumento dos níveis de vitamina C após 14 dias de ingestão de linhaça, indicando que a linhaça é uma fonte de vitamina C.

O organismo possui vários sistemas de defesa antioxidante que atuam na detoxificação das espécies reativas de forma diferenciada. Dentre eles, o sistema enzimático antioxidante parece ser o principal meio de remoção de EROs formadas durante o metabolismo intracelular (YU, 1994). A atividade das enzimas antioxidantes, SOD, CAT e GPx são mostrados nas **Figuras 02C**, **02D** e **02E dos manuscritos**. Observa-se que a atividade destas enzimas não sofreram alterações significativas após 14 de dias de suplementação com linhaça de ambas as variedades e formas de apresentação. Este perfil enzimático provavelmente está relacionado com o período de tempo do protocolo, visto que outros estudos envolvendo enzimas antioxidantes, usualmente, observam correlação positiva com dieta a partir de 30 dias de intervenção (NELSON et al., 2006).

## 6. CONCLUSÕES

Os resultados indicam que a suplementação com linhaça durante 14 dias não provoca alterações significativas em parâmetros hematológicos, bioquímicos, antropométricos e de pressão arterial. Entretanto, os marcadores de estresse oxidativo, como peroxidação lipídica e carbonilação de proteínas, reduziram significativamente com a suplementação com grãos de linhaça dourada e marrom. Portanto, períodos de suplementação de 2 semanas com linhaça na forma de grão podem ser um importante cofator na prevenção de doenças que têm como base a produção de radicais livres.

## 7. PERSPECTIVAS

Este trabalho tem como perspectivas:

- Realizar estudo em indivíduos com Síndrome Metabólica suplementando-os diariamente com 40 g de grão de linhaça durante 30 dias, para análise de perfil lipídico, hematológico, nível glicêmico, medidas antropométricas e pressóricas, parâmetros de estresse oxidativo, lipoproteicos (Apo I) e inflamatórios (PCR, IL-1B, IL-6, IL-10 e TNF-α).

- Realizar estudo em indivíduos saudáveis suplementando-os com 40 g de farinha de linhaça triturada no momento do uso, durante 30 dias, para análise de perfil lipídico, hematológico, nível glicêmico, medidas antropométricas e pressóricas e parâmetros de estresse oxidativo.

- Realizar estudo em indivíduos com diagnóstico de diabettes, utilizando grupos suplementados com semente *in natura*, lignana SDG isolada, formulação de lignanas da linhaça e ácidos graxos poliinsaturados para posterior comparação dos efeitos biológicos e determinação do possível princípio ativo.

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## 9. ANEXOS

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DO PAMPA
Comitê de Ética em Pesquisa
Unipampa/CEP - Portaria nº 778/09/GR/Unipampa
Registrado na CONEP – Ofício nº 3210/CNS/GM/MS
Endereço eletrônico: cep@unipampa.ed.u.br

Uruguaiana, 19 de dezembro de 2011.

**CARTA DE APROVAÇÃO Nº 069 2011**

Prezada Pesquisadora Responsável

Vanusa Manfredini

Comunicamos que o protocolo de pesquisa intitulado AVALIAÇÃO DOS EFEITOS ANTIOXIDANTES E ANTI-GENOTÓXICOS IN VIVO DA SEMENTE DE Linum usitatissimum L., registro ProPesq 10.143.11, registro Unipampa/CEP 091 2011, foi avaliado por este CEP e está **aprovado** para execução a partir da presente data.

Lembramos que qualquer alteração no protocolo de pesquisa submetido a avaliação deverá ser comunicada ao Unipampa/CEP imediatamente, bem como eventos adversos, e que o relatório parcial deverá ser entregue em **junho de 2012**.

Atenciosamente.

Rosana Soibelmann Glock
Coordenadora CEP

**CARTA DE APROVAÇÃO DO COMITÊ DE ÉTICA EM PESQUISA DA UNIPAMPA.**