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AVALIAÇÃO DO POTENCIAL ANTIOXIDANTE DE HIDROLISADOS PROTEICOS DA SEMENTE DO GIRASSOL SOBRE CÉLULAS INTESTINAIS CACO-2

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A ata de defesa de dissertação de mestrado com as respectivas assinaturas dos membros da banca encontra-se arquivada junto à documentação do aluno.

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Aos meus pais: Jucileide e José Lopes,
que me incentivam a perseguir minha realização pessoal e profissional,
proporcionando-me todo o suporte necessário.
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RESUMO

O estresse oxidativo, causado pela desregulação entre a formação de substâncias pró-oxidantes e antioxidantes, está associado a um aumento da incidência de doenças crónicas não transmissíveis (diabetes tipo II, doença de Parkinson e alguns tipos de câncer). A farinha de girassol, um co-produto da produção de biodiesel, é rica em proteínas com funcionalidade desconhecida. Estudos mostram que os peptídeos, gerados após um processo de hidrólise, podem ter uma ação antioxidante benéfica para a saúde. Contudo, estas características foram pouco estudadas em hidrolisados do girassol. Frente ao exposto, o presente trabalho visou avaliar a atividade antioxidante de hidrolisados peptídicos derivados do girassol por métodos químicos in vitro, in sílico e sobre células intestinais Caco-2. A proteína da farinha desengordurada foi isolada por pH isoelétrico (4,5) e os compostos fenólicos foram removidos por extração com álcool (70%). O isolado proteico de girassol (SFPi) foi hidrolisado pela enzima alcalase (pH 9, a 50ºC/90 min) e ultrafiltrado (<5kDa), gerando a fração filtrada do hidrolisado de girassol (SPHf). A hidrólise com alcalase gerou peptídeos de baixo peso molecular (<1 kDa) e este resultado foi intensificado pela digestão simulada. Apesar da alta concentração de aminoácidos ácidos e hidrofóbicos, o perfil de hidrofobicidade foi reduzido após a hidrólise. A SPHf aumentou significativamente a atividade antioxidante em comparação com a SFPi digerido nos ensaios ABTS e DPPH. Após a digestão, a atividade antioxidante por ORAC foi mantida na fração SPHf. Dos 196 peptídeos identificados, 13 apresentaram maior probabilidade de atividade antioxidante utilizando uma ferramenta de análise de bioinformática de aprendizagem profunda. Além disso, vários peptídeos continham di- e tri-peptídeos com atividade antioxidante na sua estrutura, com referências cruzadas em bases de dados de peptídeos bioativos alimentares. O SPHf não apresentou atividade antimicrobiana, contudo atenuou a produção de espécies reativas de oxigênio induzido por peróxido de hidrogênio em células gastrointestinais Caco-2. O hidrolisado de girassol foi capaz de reverter o aumento do teor de GSH e da atividade da CAT induzido pelo estímulo de H_2O_2. Embora não significativo, o mesmo perfil foi observado com a atividade da SOD. Através de métodos químicos, biológicos e de bioinformática, este estudo mostra o potencial efeito antioxidante dos peptídeos do girassol com baixo teor de fenólicos. A identificação das

sequências peptídicas possibilitou a análise química e estrutural relacionado com a funcionalidade bioativa dos peptídeos do girassol, abrindo novas perspectivas para o estudo de biomoléculas alimentares com efeito positivo sobre a saúde e o surgimento de novos ingredientes para produção de suplementos ou medicamentos.

Palavras-chave: composto bioativo; peptídeos do girassol; hidrólise de proteínas; digestão simulada; atividade antioxidante

ABSTRACT

Oxidative stress, caused by deregulation between the formation of pro-oxidant and antioxidant substances, is associated with an increased incidence of chronic non-communicable diseases (type II diabetes, Parkinson's disease, and some types of cancer). Sunflower meal, a co-product of biodiesel production, is rich in proteins with unknown functionality. Studies show that peptides, generated after a process of hydrolysis, may have an antioxidant action beneficial to health. However, these characteristics have been little studied in sunflower hydrolysates. In view of the above, this study aimed to evaluate the antioxidant activity of peptide hydrolysates derived from sunflower using chemical methods in vitro, in silico and on Caco-2 intestinal cells. The protein from the defatted flour was isolated by isoelectric pH (4.5) and the phenolic compounds were removed by alcohol extraction (70%). The sunflower protein isolate (SFPi) was hydrolyzed by the enzyme alcalase (pH 9, at 50ºC/90 min) and ultrafiltered <5kDa, generating the filtered fraction of sunflower hydrolysate (SPHf). Hydrolysis with alcalase generated low molecular weight peptides (<1 kDa) and this result was intensified by simulated digestion. Despite the high concentration of acidic and hydrophobic amino acids, the hydrophobicity profile was reduced after hydrolysis. SPHf significantly increased antioxidant activity compared to digested SFPi in the ABTS and DPPH assays. After digestion, antioxidant activity by ORAC was maintained in the SPHf fractions. Of the 196 peptides identified, 13 showed a higher probability of antioxidant activity using the deep learning bioinformatics analysis tool. In addition, several peptides contained di- and tri-peptides with antioxidant activity in their structure, cross-referenced in food bioactive peptide databases. SPHf did not show antimicrobial activity, but it did prevent hydrogen peroxide-induced oxidative stress in Caco-2 gastrointestinal cells. Sunflower hydrolysate was able to reverse the increase in GSH content and CAT activity induced by H_2O_2 stimulation. Although not significant, the same profile was observed with SOD activity. Using chemical and biological methods, this study shows the potential antioxidant effect of sunflower peptides with a low phenolic content. The identification of peptide sequences enabled chemical and structural analysis related to the bioactive functionality of sunflower peptides, opening new perspectives for the study of food biomolecules with a positive effect

on health and the emergence of new ingredients to produce supplements or medicines.

Keywords: bioactive compound; sunflower peptides; protein hydrolysis; simulated digestion; antioxidant activity

SUMÁRIO

LISTA DE ABREVIATURAS E SIGLAS

AA – aminoácido

AAAB: α-aminobutyric acid

AAPH: 2,2'-azobis(2-amidino-propano) dihidroclorado

AAT: Antioxidant activity

ABTS: 2,2-azino-bis (ethylbenzo-thiazoline6-sulfonic acid

AC: Ácido clorogênico/ Chlorogenic acid

AG: Ácido gálico

AOAC - Association of Official Analytical Chemists

ATCC: American Type Culture Collection

B: volume of consumed base

BHI: Brain heart infusion

$CaCl_2$: Cloreto de cálcio

Caco-2: Adenocarcinoma de cólon humano

CAT: Catalase

CO_2: Dióxido de carbono

CONAB: Companhia Nacional de Abastecimento

Da: Dalton

DAD: Diode array detector

DCF: Dichlorofluorescein

DH: Degree of hydrolysis

DNA: Ácido desoxirribonucleico

DPPH: 2,2-difenil-1-picril-hidrazil

DS: Deffated sunflower

EDTA: Ethylenediaminetetraacetic acid

EO: Estresse oxidativo

ERN: Espécies reativas de nitrogênio

EROs: Espécies reativas de oxigênio

FAO: Food and Agriculture Organization

SE-FPLC: Molecular exclusion chromatography

g: Grama

GAE: Gallic acid equivalent

GSH: Glutationa peroxidase

H_2O_2: Peróxido de hidrogênio

HCL: Ácido clorídrico

htotal: total peptide bond content

INFOGEST: Static in vitro simulation of gastrointestinal food digestion

KDa: Kilodalton

kHz: Kilohertz

M: Mol

mg: Miligrama

MIC: Inhibitory concentration

mL: Mililitro

mmol: Milimol

Mp: mass of protein

NaCl: Cloreto de sódio

$NaNO_2$: Nitrato de sódio

NaOH: Hidróxido de sódio

Nb: normality of the base

nM: Nanômetro

NO/NO^-: Óxido nítrico

O_2^-: Radical superóxidos

OH: Hidroxila

ORAC: Capacidade de Absorção de Radicais de Oxigênio

pH: Potencial hidrogeniônico

Predicted chelation score (CHEL)

Predicted free radical scavenger score (FRS)

PTFE: Hydrophilic polytetrafluoroethylene

RP-HPLC: Reversed-phase high-performance liquid chromatography

SDS-PAGE/SDS: Sodium dodecyl sulfate-polyacrilamide gel

SPHf: Filtered sunflower hydrolysates

SFPi: Sunflower protein isolate

SPHr: Retained sunflower hydrolysates

SOD: Superóxido dismutase

TFA: Trifluoroacetic acid

U/g: Unidade enzimática

UV: Ultra violeta

α: average degree of dissociation

INTRODUÇÃO

O mercado consumidor de alimentos tem se tornado cada vez mais exigente quanto a qualidade, origem e contribuições nutricionais e funcionais dos alimentos. Acredita-se que essa modificação tenha ocorrido após a grande difusão provocada pelos meios de comunicação em tempo real, o que facilitou o acesso a informações relacionadas ao impacto dos alimentos à saúde. Associado a isso, a indústria alimentícia tem buscado por medidas que atendam a esses novos padrões.

Tem se estudado que o girassol, uma cultura oleaginosa destinada principalmente a produção de óleos para consumo humano e biocombustível, apresenta em sua constituição compostos bioativos que podem trazer benefícios a saúde do consumidor. Entre esses compostos destaca-se o alto conteúdo proteico da farinha desengordurada do girassol, que vem sendo aplicado na fortificação de produtos de panificação a base de cereais (LAI *et al.,* 2017). O isolamento e descoberta de novas propriedades das proteínas vegetais são de grande interesse na indústria alimentícia, assim como apresenta benefícios ecológicos e sustentáveis, uma vez que encontra aplicação à subprodutos da extração do óleo das culturas oleaginosas.

Uma das propriedades fisiológicas dos compostos bioativos presentes nos alimentares é a atividade antioxidante, uma vez que o estresse oxidativo é um dos principais fatores envolvidos no surgimento/desenvolvimento de doenças crônico degenerativas não transmissíveis, como a doença inflamatória intestinal (VELLOSA *et al.,* 2020). A hidrólise das proteínas do isolado do girassol pode expor sítios específicos da proteína com propriedades antioxidantes ausentes na proteína íntegra e representar uma alternativa promissora a prevenção/tratamento dessa doença.

Este trabalho propôs a caracterização físico-química de hidrolisados proteicos do girassol, o estudo da bioatividade através de ensaios antioxidantes químicos *in vitro*, cultura celular e métodos *in sílico* de bioinformática. A atividade biológica dos peptídeos para ter impacto fisiológico, deve permanecer após o processo digestivo. Dessa forma foi avaliado a resistência da bioatividade dos peptídeos após digestão simulada. O estudo com cultura de células adiciona fatores biológicos intrínsecos de uma célula e interação com o composto

alimentar. A célula Caco-2, escolhidas para este estudo, é um modelo biológico de linhagem celular de enterócito e corresponde ao primeiro tipo celular a entrar em contado com os metabólitos oriundos do processo digestivo. Os enterócitos são susceptíveis ao estresse oxidativo frequentemente ocasionado no ambiente gástrico-intestinal, representando um bom modelo de estudo para avaliação de propriedades antioxidantes. Diante disso, na literatura, existem poucas informações a respeito dos potenciais efeitos antioxidantes que os peptídeos bioativos do girassol com baixo teor de compostos fenólicos provocam em células Caco-2 e sua relação com estrutura e função.

OBJETIVOS

Objetivo principal

- ✓ Avaliar a atividade antioxidante de hidrolisados peptídicos derivados do girassol por métodos químicos *in vitro, in sílico* e sobre células intestinais Caco-2.

Objetivos específicos

- Obtenção de isolado proteico a partir da farinha desengordurada do girassol e processo de extração dos fenólicos

- Obtenção de potenciais peptídeos bioativos do girassol a partir da hidrólise empregando a enzima Alcalase®

- Caracterização do girassol e hidrolisados peptídicos quanto ao teor de proteína, umidade, grau de hidrólise, tamanho molecular e hidrofobicidade

- Avaliação da atividade antioxidante dos hidrolisados peptídicos utilizando técnicas *in vitro* tais como DPPH, ORAC e ABTS, antes e após digestão simulada (Infogest)

- Identificação de sequências peptídicas com bioatividade antioxidante por espectrometria de massas

- Avaliação do potencial efeito antimicrobiano de peptídeos bioativos do girassol obtidos por hidrólise enzimática (Alcalase®)

- Avaliação da atividade antioxidante dos isolados peptídicos em células Caco-2 estimuladas com peróxido: avaliação da viabilidade celular, produção de espécies reativas de oxigênio, produção de óxido nítrico, conteúdo de glutationa e atividade da super peroxido dismutase e catalase

- Avaliação *in sílico* da atividade antioxidante por meio de ferramentas de bioinformática, cruzamento com banco de dados (BIOPEP-UWM) e predição por algoritmo de aprendizagem (*deep learn*ing)

CAPÍTULO 1

REVISÃO BIBLIOGRÁFICA

AVANÇOS DA APLICAÇÃO DE COMPOSTOS BIOATIVOS DO GIRASSOL (*Helianthus annuus L.)* FRENTE AO ESTRESSE OXIDATIVO

Daniel S. Lopes e Fabiana A. B. Galland.

O artigo será submetido à revista *Brazilian Journal of Food Technology*.

RESUMO

A farinha desengordurada do girassol, co-produto da produção de biodiesel, é rica em compostos bioativos como compostos fenólicos, proteínas e peptídeos, com potencialidade para serem usados como ingredientes funcionais em alimentos. Apesar disso, existem ainda poucas aplicações comerciais deste subproduto em alimentos funcionais. Dentre as diversas funcionalidades a potencialidade antioxidante de compostos alimentares tem sido alvo de estudo, de forma a amenizar os impactos à saúde induzidas pelo estresse oxidativo observado em diversas patologias. O objetivo desta revisão foi avaliar os principais estudos com compostos bioativos da farinha do girassol em relação a sua potencialidade antioxidante. Os principais compostos bioativos estudados na farinha do girassol foram os compostos fenólicos e peptídeos derivados da hidrólise. O principal método de extração dos fenólicos foram o metanólico e hidroetanólico, e a principal enzima estudada para geração de peptídeos foi a alcalase. Estes compostos apresentaram potencial antioxidante, apesar de poucos estudos avaliarem a resistência a processos digestivos e a presença de compostos fenólicos em hidrolisados proteicos. Os estudos para avaliação da atividade antioxidante se baseiam principalmente em ensaios químicos, como ABTS, DPPH e ORAC e em menor escala em ensaios biológicos, com o uso de cultura de células. Neste contexto, a falta de estudos em modelos biológicos avaliando a potencial ação antioxidante dos bioativos do girassol se fazem necessária, visando a compreensão dos mecanismos de ação na maquinaria celular. Ainda, a relação estrutura função dos compostos isolados é pouco compreendida. Este estudo traz uma perspectiva de novas áreas de pesquisa para melhor exploração desta matriz e entendimento do potencial bioativo da torta do girassol. Também, novas aplicações pela indústria de alimentos e farmacêutica.

Palavras chaves: girassol; compostos fenólicos; peptídeos bioativos; atividade antioxidante; estresse oxidativo

1 Introdução

O girassol (*Helianthus annuus L*) é uma cultura oleaginosa de grande importância econômica mundial destinada principalmente a produção de óleo e biodiesel (UDSA, 2023). Após o processamento da extração do óleo, forma-se um resíduo/subproduto, a torta do girassol, que pode trazer impactos ambientais caso seja descartada de forma incorreta na natureza. Contudo, de forma a deixar o processo mais sustentável, os compostos presentes na torta do girassol tem sido alvo de estudo para novas aplicações em alimentos. Este subproduto é rico em compostos bioativos com potencial para serem usados como matéria prima para produção de novos alimentos funcionais (KACHRIMANIDOU *et al.,* 2015).

Os compostos bioativos derivados de alimentos podem apresentar diversas funções com impactos positivos à saúde (HAN *et al.,* 2019). Uma das propriedades fisiológicas mais visadas dos compostos alimentares é a atividade antioxidante, uma vez que o estresse oxidativo é um dos principais fatores envolvidos nas doenças crônicas degenerativas, como diabetes do tipo II, Parkinson e outras (HAN *et al.,* 2019).

Esta revisão reúne os principais estudos que avaliaram o potencial antioxidante de fenólicos e peptídeos derivados da farinha do girassol, as principais metodologias utilizadas e os mecanismos de ação sugeridos para tal bioatividade. Este estudo traz uma perspectiva de novas áreas de estudo para melhor exploração desta matriz e contribui para melhor entendimento do potencial bioativo da torta do girassol e novas aplicações pela indústria de alimentos e farmacêutica.

2 Semente do girassol

Originária do continente americano, o girassol, é uma planta dicotiledônea, de ciclo anual e adaptada a diferentes condições climáticas (SEILER *et al.,* 2012; KAYA *et al.,* 2012 e 2014). É cultivada em diversos países como Argentina, Rússia, Turquia, Ucrânia e outros (USDA, 2023). No Brasil, a cultura teve sua inserção no final da década de 1990, sendo cultivada principalmente no Centro-Oeste, com destaque para os estados do Mato Grosso, Goiás e o Distrito Federal. Para a safra de 22/23, a produção da semente no Brasil atingiu a marca de 85,2 mil toneladas, tendo como valor média de

produtividade de 1,520 kg/hectar dentro de uma área de cultivo de 56,1 mil hectares (CONAB, 2023).

O grande interesse pela cultura se dá devido a sua alta aplicabilidade na produção de óleo para consumo humano, biocombustível e em menor escala para a produção de ração de animais e alimentos derivados (RIZZELLO *et al.,* 2016; CHALAMAIAH *et al.,* 2019). Cerca de 40-50% do conteúdo da semente de girassol é composto por lipídeos (LEITE *et al.,* 2005). O óleo gerado na extração tem uma alta valorização no mercado em comparação outros óleos vegetais, devido a sua cor clara e sabor suave.

No geral, o óleo do girassol é obtido por prensagem a frio, método mais indicado ao consumo humano por se tratar de um processamento menos tóxico e por preservar os constituintes químicos presentes no óleo (GONZALEZ-PÉREZ *et al.,* 2007). Esse processamento gera um co-produto, a torta do girassol, com riqueza de compostos nutricionais passíveis de serem usados em outras aplicações na indústria de alimentos. A descoberta de novas aplicações da torta do girassol pode minimizar os impactos ambientais decorrentes de seu descarte, além de tornar o processo mais sustentável.

2. 1 Composição química da semente desengordurada

Assim como na semente, a torta do girassol é rica em diversos compostos importantes para a nutrição (ALAGAWANY *et al.,* 2015; PREMNATH *et al.,* 2016). A Tabela 1 reúne informações sobre a composição nutricional da torta do girassol realizada por diversos trabalhos.

Tabela 1. Composição da farinha/torta do girassol desengordurada (g/100g).

Componente (base seca)	**Mandarino (1992)**	**González-Pérez *et al.* (2003)**	**Leite *et al* (2005)**	**Grasso *et al.* (2019)**	**Alexandrino *et al.* (2017)**	**Prado *et al.* (2020)**
Carboidratos	±28	±18-26	±20	-	±13	±57,5
Fibras	±12	-	±12	-	±16	-
Lipídeos	±3	-	±3	±1,84	±1,70	±2,32
Minerais	±3-4	±3-4	±4	±7,12	±8,63	-
Proteína	±50	±20-40	±24	±38	±61	±28
Compostos Fenólicos	-	±0,3-3	±0,3-3	-	-	

Observa-se que os estudos citados na tabela 1 apresentam variações nos resultados, como em carboidratos e proteínas. As variáveis entre os estudos podem ser justificadas pela diferença entre as metodologias adotadas para a dosagem de tais compostos, variáveis de solo e cultivo, e processamento da semente.

A torta do girassol é considerada uma boa fonte em proteínas, característica que viabiliza, por exemplo, a sua aplicabilidade para fabricação de produtos com alto valor proteico de origem vegetal, como os plant-based. O rendimento e aplicação proteica em produtos alimentícios pode ser afetado pelo método de extração do óleo. Alguns tipos de processamento, com aplicação de altas temperaturas, podem desnaturar a proteína, resultando em uma torta de girassol com maior teor de proteínas insolúveis (GONZÁLES-PERES; VEREIJKEN *et al.,* 2007).

- Carboidratos, minerais e vitaminas

A depender do tipo de variedade utilizada e processamento, a torta do girassol apresenta em sua constituição os carboidratos: glicose, rafinose, sacarose e trealose (cerca de 0,6; 3,22 e 2,29%, respectivamente) (CARRÃO PANIZZL*;* MANDARINO, 1994). Com relação a minerais, a matriz é rica em cálcio, fósforo e potássio (120; 837 e 920 mg/100g). Além disso, a torta do girassol é constituída de vitaminas do complexo B (ácido nicotínico, tiamina, ácido pantotênico, riboflavina e biotina) (LEITE *et al.,* 2005).

- Fibras

A torta do girassol gerada após a extração do óleo é rica em fibras, das quais a grande fração presente se classifica como insolúvel (lignina e celulose) (GROMPONE, 2005). Na saúde humana, o consumo de alimentos ricos em fibras insolúveis contribui beneficamente ao bom funcionamento do intestino e consequente redução de tempo da passagem do bolo alimentar pelo sistema digestivo, contribuição da circulação entero-hepética e redução dos níveis de colesterol no sangue e outros (COPPINI *et al.,* 2004; MAKKI K. *et al.* 2018). Nesse contexto, não foram encontrados trabalhos que exploram o emprego como alimento funcional. Uma oportunidade que poderia ser explorada, pois

durante o isolamento proteico da proteína do girassol, um subproduto rico em fibras é gerado (WILDERMUTH *et al.,* 2016).

- Proteína do girassol

A torta de girassol tem sido considerada atrativa pelo seu alto conteúdo proteico. Esta característica confere a potencialidade para ser utilizada como fonte de proteica a produtos fortificados ou plant-based. As proteínas vegetais, oriundas de subprodutos agroindustriais, têm tido grande interesse pelo mercado consumidor de alimentos saudáveis, uma vez que a sua obtenção gera menor impacto ambiental, menor risco de contaminação por doenças de origem animal, além de ser uma alternativa mais sustentável no processo de produção. Contudo, a variabilidade das proteinas e o conteúdo de aminoácidos essenciais são importantes para a qualidade nutricional proteíca (HAN *et al.*, 2015; RIZZELLO *et al.,* 2016).

A torta do girassol é composta maioritariamente por proteínas do tipo: globulina (55-60%) albuminas (17-60%), gluteínas (11-17%) e prolaminas (1-4% (PRADO *et al.,* 2020). Quanto ao conteúdo de aminoácidos, na Tabela 2 é mostrado a distribuição de aminoácidos essências e sua relação com os valores recomendados pela FAO (2007) para uma ingestão diária balanceada. Neste cenário, o aminoácido essencial lisina, não atende ao padrão de referência proteica estabelecido pelo órgão. Apesar disso, a sua falta pode ser complementada através do uso em paralelo com outras proteínas vegetais as quais os valores para o aminoácido em questão não seja limitante (CONDE *et al.,* 2005; SOSULSKI, 1979).

As proteínas do girassol são ricas em aminoácidos ácidos e hidrofóbicos (Valina, Isoleucina, Fenilalanina e Metionina) (Tabela 2) (PICKARDT *et al.,* 2015; ALEXANDRINO *et al.*, 2017). Esta característica pode influenciar diretamente nas propriedades bioativas que as proteínas podem apresentar no organismo. É importante salientar que o teor aminoacídico da semente pode sofrer variações a depender das condições de cultivo, variedade adotada, o processamento adotado e manejo com a cultura (GONZALEZ-PÉREZ; VEREIJKEN, 2007; CONDE *et al.,* 2005).

Tabela 2. Composição de aminoácidos essenciais na proteína isolada do girassol (mg/g).

Aminoácido	Padrão FAO	Proteína Isolada
Isoleucina	30	24-32
Leucina	59	41-86
Lisina	45	33-37
Metionina+cisteína	22	19-29
Fenilalanina+tirosina	38	72-82
Treonina	23	31-39
Triptofano	6	10-31
Valina	39	45-50

Fonte: Leite *et al.* (2005) e Food and Agriculture Organization (FAO) (2007).

Quando aplicada como ingrediente em produtos alimentício, a proteína do girassol contribui para as propriedades tecnológicas e funcionais. Essa característica pode estar relacionada a composição aminoacídica (histidina, triptofano, fenilalanina, prolina, isoleucina, valina, metionina, tirosina). Estudos indicam que estes aminoácidos contribuem para uma boa capacidade de formação de espumas e de estabilidade proteica em emulsões e géis (Tabela 3).

Como ingrediente na produção de alimentos, a farinha do girassol tem sido empregada na formulação de: pães (DELLA GATTA; PIERGIOVANNI,1996), muffins (GRASSO *et al.,* 2020a), biscoitos (SRILATHA; KRISHNAKUMARI, 2003; GRASSO *et al.,* 2019) e salsichas (GRASSO *et al.,* 2020b).

Tabela 3. Aplicações tecno/funcionais da proteína do girassol em alimentos.

Aplicação	Referência
Solubilidade	VERMEESCH *et al.* (1987); GHEYASUDDIN *et al.* (1970); SOSULSKI; FLEMING (1977); CANELLA (1978); ROSSI *et al.* (1985)
Formação de gel	BILANI *et al.* (1989); SÁNCHEZ; BURGOS, (1995; 1996 e 1997a, 1997b); PAWAR *et al.* (2001); FLEMING; SOSULSKI, (1975); BULMAGA, *et al.* (1989);
Propriedades tensioativas/de formação de emulsão	VENKTESH; PRAKASH. (1993); RAHMA; RAO. (1981); HUFFMAN *et al.* (1975); BOOMA; PRAKASH. (1990); PAWAR *et al.* (2001); CANELLA (1978); ROSSI (1982 and 1985) e KABIRULLA; WILLS. (1988)

Fonte: Adaptado de GONZALEZ-PÉREZ e VEREIJKEN, 2007.

- *Compostos fenólicos*

Os compostos fenólicos podem ser definidos como metabólitos secundários produzidos pelas plantas a partir de variados estímulos (estresse hídrico, nutricional e térmico, defesa e outros) e podem desempenhar diferentes papeis em sua fisiologia (KAMMERER *et al.,* 2007). Cerca de 4% da farinha do girassol refere-se a compostos fenólicos, dos quais o ácido clorogênico (AC) se destaca majoritariamente (aproximadamente 70%) (CARRÃO-PANIZZL*;* MANDARINO, 1994; WEISZ *et al.,* 2009). O AC é formado por um grupamento de ésteres de ácido hidroxicinamico (ácido caféico, ferúlico e cumário, ligado ao ácido quínico) (BODOIRA; MAESTRI, 2020; NACZK e SHAHIDI, 2004; NAKATANI *et al.,* 2000).

No que se refere a aplicabilidade da torta do girassol em alimentos há o grande desafio de reduzir a cor esverdeada que eventualmente é gerada durante o processamento em função do AC. Fatores como altas temperaturas (durante e após o período de maturação da semente) e variação de pH (solubilização das fibras e precipitação isoelétrica das proteínas em meio alcalino), podem favorecer o surgimento de uma coloração amarelo-esverdeada, derivada do processo oxidativo do AC e consequente escurecimento. Tal reação se dá em detrimento a presença da enzima polifenoloxidade, a qual tem como substrato o AC (MANDARINO, 1992; LEITE *et al.,* 2005). Diversos métodos para extração do AC da farinha do girassol têm sido propostos, como por exemplo, o uso de antioxidantes ou inibidores das enzimas oxidativas ou mesmo melhoramento genético. Contudo, estes procedimentos podem ser de custo elevado quando se pensa na aplicação desta farinha em escala industrial.

Portanto, a presença dos fenólicos pode ser responsável pela perda das características sensoriais e propriedades tecnológicas da proteína (GONZÁLEZ-PÉREZ; VEREIJKEN *et al.,* 2007). No estudo de Grasso *et al.* (2019), a farinha do girassol foi adicionada como ingrediente proteico complementar na formulação de salsichas. Foi observado o surgimento de uma coloração escura no produto. Entretanto, este foi aceito sensorialmente.

2. 2 Estresse oxidativo e defesa antioxidante

Nos últimos anos, doenças crônicas não transmissíveis têm aumentado na população humana, tais como diabetes do tipo II, Parkinson e alguns tipos de câncer (SANTI *et al.,* 2011). Tal decorrência pode estar associada a diversos fatores, dentre eles o desbalanço provocado entre as defesas endógenas antioxidantes das células e tecidos *versus* a produção e acúmulo de radicais livres (HUSSAIN *et al.,* 2016). Dessa forma, diversas moléculas naturais são estudadas com o intuito de amenizar ou prevenir a produção de radicais livres.

Apesar dos radicais livres serem comumente associados a distúrbios, eles também têm funções fisiológicas, atuando na sinalização celular, ativação de genes, bem como defesa contra patógenos. Contudo, em condições patológicas ocorre a produção em grande quantidade de espécies reativas de oxigênio (EROs), associada a uma baixa taxa de consumo ou eliminação dessas moléculas. Este distúrbio é chamado de estresse oxidativo (EO) (WANG *et al.,* 2020). O EO promove danos celulares e genotóxicos ao organismo. Essas reações provocam a oxidação de moléculas vitais, como proteínas, lipídeos e DNA, as quais perdem suas funções, desencadeando um desequilíbrio homeostático no organismo (AHMADINEJAD *et al.,* 2017).

Radicais livres são moléculas que apresentam um elétron desemparelhado na última camada eletrônica, oriundos de reações enzimáticas que ocorrem principalmente na mitocôndria em função da redução univalente do O_2. Os principais radicais formados são o radical superóxidos (O_2^-), hidroxila (OH), e o peróxido de hidrogênio (H_2O_2) (BARBOSA *et al.,* 2014). O peróxido de hidrogênio, apesar de não ser um radical livre, por não possuir um elétron desemparelhado, apresenta também alto potencial reativo. O excesso dessas moléculas em um organismo propicia reações de oxidação (perda de um elétron) às células sadias, prejudicando assim as suas funções fisiológicas (WANG *et al.,* 2020).

De forma a combater essas reações danosas, o organismo possui mecanismo de defesa antioxidante endógena (moléculas produzidas na própria célula) ou exógenas (compostos obtidos na alimentação) (VELLOSA *et al.,* 2020). Os antioxidantes podem atuar de forma enzimática e não enzimática (SANTI *et al.,* 2011). Enzimas endógenas tais como a superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GSH-Px) apresentam papel fundamental no combate ao estresse oxidativo primário, interrompendo a

oxidação de diversas moléculas. A SOD promove a conversão de O_2^- em H_2O_2. Este último serve de substrato para a CAT, resultando na formação de água e oxigênio. Já GSH-Px, promove a redução de H_2O_2 e hidroperóxidos orgânicos em reação dependente de glutationa reduzida (GSH), que é oxidada a glutationa oxidada (GSSG) (MUNTEANU *et al.,* 2021).

Já no contexto não enzimático, fontes exógenas ao corpo humano, advindos da alimentação, por exemplo, podem se caracterizar como uma fonte de defesa antioxidante alternativa. Neste contexto, os compostos bioativos (fenólicos e peptídeos) do girassol, podem representar uma alternativa promissora a atenuação do estresse oxidativo.

Dessa forma, diversos estudos são dedicados a avaliar a capacidade de prevenir ou reverter o estresse oxidativo e o consequente desenvolvimento de doenças a partir de moléculas antioxidantes derivadas dos alimentos. O interesse da indústria farmacológica e de alimentos por produtos naturais pode estar relacionado ao fato destas apresentarem baixa toxicidade, serem excelentes eliminadores de radicais livres e potenciais substituintes dos antioxidantes sintéticos (FABIANOWSKA-MAJEWSKA *et al.,* 2021).

2. 3 Métodos Químicos para avaliação da atividade antioxidante

Existem diversos métodos químicos que podem ser empregados na avaliação da atividade antioxidantes de forma acessível, rápida e automatizada, sendo uma alternativa a triagem e avaliação inicial de novos compostos antioxidantes. Entretanto, para a confiabilidade do ensaio, diversos fatores devem ser levados em consideração, tais como: características do analito; reprodutividade (intra e inter ensaios); possuir um único resultado avaliativo (simples e claro); equipamentos e reagentes de fácil obtenção; não apresentar limitações a polaridades do analito, bem como ser passível de controle de qualidade (MUNTEANU *et al.,* 2021).

Dois mecanismos de atuação são normalmente avaliados nos ensaios químicos: a capacidade de doação de elétrons, ou a capacidade de transferência de próton (PRIOR *et al.,* 2005). Os testes baseados na transferência do átomo de hidrogênio medem a capacidade de um antioxidante em remover os radicais livres doando um átomo de hidrogênio, como o ensaio de ABTS (2,2-azino-bis (ethylbenzo-thiazoline6-sulfonic acid) e ORAC (Capacidade de Absorção de

Radicais de Oxigênio). Os testes baseados na transferência de elétrons, detectam a capacidade de um antioxidante de transferir um elétron para reduzir íons, grupos carbonila e radicais livres, como é o caso do DPPH (sequestro do radical 2,2-difenil-1-picril-hidrazil). Devido ao baixo custo comercial dos reagentes, estabilidade dos radicais livres formados, solubilidade da molécula antioxidante e baixa interferência, tais modelos são excelentes ferramentas de avaliação dos compostos bioativos do girassol (AYOKA *et al.,* 2022).

Os métodos químicos mais utilizados para avaliação da atividade antioxidante do girassol foram o ABTS, DPPH e ORAC. De forma geral, podemos dizer que o método ABTS atua na avaliação da molécula antioxidante em sequestrar radical $ABTS^{+}$. Já o método DPPH, avalia a capacidade de redução do radical DPPH e o método ORAC, avalia a capacidade da molécula em proteger fluoresceína do dano oxidativo, provocados por radicais peroxila (SUCUPIRA *et al.,* 2012).

A atividade antioxidante de um composto não pode ser atestada em apenas um único método, pois a depender das especificidades do composto (carga, solubilidade e outros), estes podem interferir na resposta/avaliação. Além disso, um fator importante que deve ser destacado é que as respostas dos modelos químicos podem não representar a totalidade de uma fisiológica, sendo necessária à sua complementação em modelos biológicos de estudo (MUNTEANU *et al.,* 2021).

2. 3. 2 Métodos Biológicos para avaliação da atividade antioxidante

A avaliação antioxidante de compostos naturais pode ser estudada através de ensaios que utilizem organismos biológicos como modelo de estudo. Entre os organismos mais utilizados podemos citar as células isoladas em cultivo no laboratório. Este modelo permite avaliar a interação do composto em estudo com a maquinaria biológica celular, como membranas biológicas, receptores, transportadores e enzimas. Através de um estímulo oxidativo é possível criar, na célula, um desequilíbrio entre radicais livres e antioxidantes. Nesta condição patológica é possível avaliar se o composto de estudo consegue amenizar ou reverter tal condição e apresentar ação antioxidante (SHANI-LEVI *et al.,* 2017).

Através do uso das células pode-se avaliar mecanismo de ação pelo qual o composto age. É possível que este atue simplesmente como um agente

redutor, doando elétrons para os radicais livres, cortando a cadeia oxidativa, como é o caso do ácido ascórbico (vitamina C) (NJUS *et al.,* 2020). Ainda um composto antioxidante pode modular vias intracelulares de defesa antioxidante, através da interação com fatores de transcrição chave, que regulam a expressão de enzimas antioxidantes na célula, como é o caso do NRF2. A ativação deste fator de transcrição estimula a síntese de importantes enzimas antioxidantes tais como SOD, CAT e GPX, os quais combatem o estresse oxidativo e promovem citoproteção (PALL; LEVINE, 2015).

Linhagens celulares são células imortalizadas disponíveis em bancos de células, podendo ser a sua origem de diversas espécies. Seu uso tem crescido nos últimos anos, devido a fatores como: fácil manutenção em laboratório, baixo custo e por mimetizarem respostas fisiológicas similares a células sadias. Além disso, são consideradas como uma alternativa ao uso de animais em pesquisa (TYARKHT *et al.,* 2014).

A célula Caco-2, oriunda de um adenocarcinoma de cólon humano, é um dos modelos celulares mais estudados para avaliação de compostos derivados de alimentos. Este modelo mimetiza enterócitos, tipo celular que entra em contado com os metabólitos oriundos do processo digestivo (BALIMANE; CHONG, 2005). Entretanto, o modelo não permite a representação da total complexidade do processo digestivo humano, bem como a recriação de todas as suas funções. Apesar disso, partes desse processo podem ser simuladas gerando resultados fidedignos (HUR *et al.,* 2011; SHANI-LEVI *et al.,* 2017).

2. 4 Compostos bioativos do girassol e sua atuação antioxidante

2. 4. 1. 1 Compostos fenólicos

Dentre os constituintes químicos naturais presentes na torta do girassol, compostos fenólicos é uma molécula com potencial ação bioativa. Como comentado anteriormente, a presença de fenólicos, especificamente do ácido clorogênico, pode ser desfavorável para fins tecnológicos do uso da farinha como alimento. Contudo, o isolamento de extratos ricos em fenólicos podem ser utilizados para aplicação em produtos com ação funcional em suplementos. Na literatura, diversas formas são utilizadas para a extração/obtenção desses compostos: metanol, etanol, resina de adsorção, extração ácida branca e de troca iônica (WEISZ *et al.,* 2009, GONZÁLEZ-PEREZ *et al.* 2002, PICKARDT *et*

al. 2015). O método de extração pode influenciar no rendimento de obtenção e na bioatividade do produto gerado. O método com extração etanólica é um dos mais utilizado.

Os compostos fenólicos possuem bioatividade em diversas patologias. Estudos experimentais com os compostos fenólicos relatam efeitos bioativos efetivos no tratamento de doenças cardiovasculares e doenças crônicas neurodegenerativas, como agente anti-inflamatório e como agente antioxidante (SANTANA-GÁLVEZ *et al.,* 2017).

Em relação ao mecanismo de ação antioxidante, os compostos fenólicos, em específico do AC, apresentam ésteres em sua constituição (C_4, C_8 E C_{12}), os quais inibem a formação de ERO$_S$ (doação de elétrons) advindas da oxidação de lipídeos, por exemplo (NÁTHIA-NEVES; ALONSO, 2021). No ambiente celular, essa característica pode proteger os ácidos graxos presentes na membrana plasmática (ZOU *et al.,* 2016). Os estudo mais recentes (últimos cinco anos) empregando as metodologias químicas citadas anteriormente, se encontram dispostos na Tabela 4.

Na comparação dos dados entre os diferentes estudos pode-se observar que a atividade antioxidante por µmol de equivalente de trólox é bastante variável. Um dos motivos pode estar relacionado ao método de extração do fenólico, como já citado anteriormente. Ainda, a atividade antioxidante pode variar em relação a classe dos fenólicos extraídos da amostra (ácidos hidroxibenzóicos, ácidos hidroxicinâmicos, flavonóides e outros). Entretanto, o ácido clorogênico e cafeico são os compostos majoritários (ABDALLA *et al.,* 2021).

Tabela 4. Avaliação antioxidante de compostos fenólicos do girassol em modelos químicos.

Forma de obtenção	**Método**	**Resultado (µmol Trólox eq/g)**	**Referência**
Extração etanólica (75%)	DPPH	(0.005 – 0.10%) Dose dependente	Ivanova *et al.* (2021)
	DPPH	22,5	Zoumpoulakis *et al.* (2017)
Extração com hexano e metanol	DPPH	±22 - 40	Abdalla *et al.* (2021)
	ABTS	±19-31	

Extração com bissulfito de sódio	ORAC	±5,88	Alexandrino *et al.* (2021
	DPPH	±0,40	
	ABTS	±0,41	
Extração com solução etanólica (70%)	ORAC	±7,35	
	DPPH	±0,70	
	ABTS	±0,53	
Extração etanólica (80%)	ORAC	±8,50	Amakura *et al.* (2013)
Extração etanólica (70%)		203	Náthia-Neves; Alonso (2021)

2. 4. 1. 2 Hidrolisados proteicos/peptídeos bioativos

Atualmente, proteínas vegetais têm sido submetidas a processos enzimáticos com a finalidade de obtenção de hidrolisados com maior valor bioativo (TONON *et al.,* 2016). Diversos processos enzimáticos podem ser aplicados visando a obtenção de hidrolisados proteicos/peptídeos bioativos. Processos fermentativos, hidrólise enzimática e digestão podem ser citados (GARCÍA *et al.,* 2013; NAJAFIAN; BABJI, 2012; OPHEIM *et al.,* 2015; BRODKORB, *et al., 2019*; TAVANO, 2013).

A hidrólise de proteínas gera cadeias polipeptídicas menores, as quais podem expor sítios da proteína, antes escondidos dentro da estrutura terciária e quaternária da proteína. Nos peptídeos formados, propriedades funcionais diversas da proteína original podem ser geradas. No decorrer da quebra enzimática, os fragmentos da proteína se tornam mais solúveis. Tal fato se dá em virtude ao rompimento das ligações peptídicas, resultando na formação de grupos aminoacídicos menores (AGYEI *et al.,* 2016).

Os chamados peptídeos bioativos apresentam características de cadeias curtas (2-20 aminoácidos), com variedade de alvos extra e intracelulares, os quais podem agir sobre receptores e vias bioquímicas e ainda apresentar efeitos positivos sobre a saúde do consumidor (LEMES *et al.,* 2016, 2020; RIZZELLO *et al.,* 2016; CORROCHANO *et al.,* 2019).

Dados recentes (< cinco anos), referentes a atividade antioxidante de hidrolisados proteicos do girassol utilizando métodos químicos, encontram-se dispostos na tabela 5 a seguir.

Tabela 5. Avaliação antioxidante de hidrolisados proteicos do girassol em modelos químicos.

Forma de obtenção	Método	Resultado (µmol Trólox eq/g)	Referência
Hidrólise com alcalase	ABTS	15 – 25% Dose dependente	Dabbour *et al.* (2019a)
	DPPH	17,41	Dabbour, *et al.* (2019b)
Hidrólise com Neutrase® e Flavorzyme®	ABTS	±30,000	Prado *et al.* (2022)
	DPPH	±300	
Hidrólise com xilose e cisteína	DPPH	50 – 90% Dose dependente	Habinshuti *et al.* (2019)
Hidrólise com pancreatina e pepsina (Infogest)	ABTS	±175	Bisinotto *et al.* (2023)
	DPPH	±250	
	ORAC	±1000	

Como observado na tabela 5, os hidrolisados proteicos do girassol foram obtidos a partir de diferentes enzimas. O emprego de diferentes enzimas no processo enzimático resulta na formação de peptídeos com bioatividade distintas, uma vez que cada enzima possui uma região de clivagem preferencial, formando peptídeos com terminações, estruturas, composição e propriedades variadas (TONON *et al.,* 2016). As principais enzimas utilizadas foram as comerciais, provavelmente por permitirem a formação de peptídeos de forma mais econômica, padronizável e escalonável. Apenas um trabalho trouxe dados referentes a digestão dos peptídeos com simulação gastro-intestinal. Necessariamente para atingir o tecido alvo e exercer sua bioatividade, os peptídeos devem resistir ao processo digestivo e serem capazes de serem absorvidos. Dessa forma, estudos que trazem a bioacessibilidade de pepetídeos após a digestão são importantes, pois conferem propriedades fisiológicas relevantes.

Embora estes resultados sejam promissores, acredita-se que a atividade antioxidante possa ter tido influência do conteúdo de fenólico presente nos hidrolisados/peptídeos, uma vez que não foi mencionado nenhum processo de remoção/redução destes nos estudos avaliados. Sabe-se que partes dos fenólicos podem ficar aderidos na estrutura peptídica (LI *et al.,* 2021). Portanto,

avaliar a bioatividade antioxidante de hidrolisados proteicos do girassol com baixo conteúdo em fenólico se faz necessária.

2. 4. 2 Modelos biológicos

Em girassol, apenas o estudo de Megías *et al.* (2009) avaliou a estabilidade de hidrolisados proteicos de girassol após simulação de digestão humana e de células Caco-2 e a manutenção de sua bioatividade. Uma sequência peptídica FVNPQAGS foi resistente a ambos os processos e sua bioatividade (inibição da enzima conversora da angiotensina) foi preservada.

Em estudos recentes com hidrolisados proteicos do girassol obtidos por hidrólise gastrointestinal (simulado), três sequências peptídicas (DVAMPVPK; TTHTNPPPEAE e PADVTPEEKPEV), foram capazes de reduzir a produção de ROS em células Caco-2. Embora o trabalho ateste que a sequência PADVTPEEKPEV tenha sido capaz de reduzir os níveis de NF-κB (gene pró-inflamatório), nenhuma sequência foi capaz de inibir a NRF2 (molécula sinalizadora para a resposta antioxidante). Além disso, atividade antioxidante medida por moléculas endógenas à célula (GSH, SOD e CAT), não foram avaliadas (TONOLO *et al.,* 2024).

Embora existam os referidos trabalhos citados, empregando hidrolisados proteicos/peptídeos do girassol, a avaliação de sua ação como agentes antioxidantes em cultura celular ainda não foi realizada. Em se tratando dessa lacuna, trabalhos que visem essa temática tornam-se necessários para o bom reaproveitamento e uso das potencialidades bioativas da referida matriz a nível de saúde.

3 Conclusão

Em suma, a farinha desengordurada do girassol apresenta em sua constituição compostos bioativos com potencial ação antioxidante, como os fenólicos e peptídeos. A bioatividade antioxidante destes compostos podem variar em relação ao método de extração, enzima utilizada ou resistência a processos digestivos. A avaliação da atividade antioxidante foi predominantemente avaliada por métodos químicos e muito poucos com métodos biológicos, como em cultura celular. O aprofundamento destes estudos é essencial para confirmar a potencialidade antioxidante fisiológica dos compostos, bem como melhor compreensão dos mecanismos de ação. Na maior

parte dos estudos a bioatividade foi avaliada em matrizes complexas, com mistura de compostos, sem a discriminação e identificação destes. Trabalhos que isolem, identifiquem e avaliem individualmente o composto podem trazer resultados mais promissores da relação entre atividade e estrutura.

4 Referências

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CAPÍTULO 2

ANTIOXIDANT BIOACTIVITY AND SEQUENCE IDENTIFICATION OF SUNFLOWER PROTEIN HYDROLYSATES BEFORE AND AFTER *in vitro* INFOGEST PROTOCOL

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ABSTRACT

A sunflower protein hydrolysate was evaluated for its antioxidant bioactivity before and after *in vitro* simulated gastrointestinal digestion and sequence peptide identified for function-structure relationship studies. Sunflower protein isolate (SFPi) with low phenolic content was hydrolyzed by alcalase. Filtered sunflower hydrolysates (SPHf, < 5 kDa) and retained (SPHr, > 5kDa) fraction showed small molecular weight. Despite the high concentration of acidic and hydrophobic amino acids, the hydrophobicity profile was reduced after hydrolysis. SPHf significant increased antioxidant activity in comparison to SFPi in ABTS and DPPH assay. After digestion the antioxidant activity by ORAC was maintained in SPHf fractions. From 196 identified peptide, 13 presented predicted antioxidant properties using deep learning analysis tool. Furthermore, several peptides contained in its structure di- and tripeptides with antioxidant activity cross-referred in food databases. This study brings for the first-time results regarding sunflower peptide digestion stability and identified potential bioactive sequence.

Keywords: Sunflower, Antioxidant Peptide, Protein Bioactivity, Food Peptides, Oxidative Stress

1. Introduction

Sunflower, a crop of global importance, is used to produce edible oil and biofuel. As a co-product of oil extraction, the sunflower cake, presents high levels of protein content (±20-->±40g/100g) (SRIVASTAVA; VERMA, 2014; KAUR; SARAF, 2022). In parallel, there is a growing demand for protein products and the current consumer public has opted for healthier and more sustainable products. The plant-based products/meat analogs grew by about 70% in Brazil in the years 2015 to 2019, representing a big economic potential for sunflower products (BROUWER *et al.,* 2022; TVS, 2019).

Sunflower flour can be an excellent alternative for the production of protein products, with technological value and effects beyond nutrition, such as functional contributions to health (WILDERMUTH *et al.,* 2016; FILHO *et al.,* 2021). Particularly, sunflower protein hydrolysates have shown angiotensin converting enzyme (ACE) inhibitory capacity (LUO; HE, 2018), antimicrobial (HABINSHUTI *et al.,* 2019) and anti-inflammatory activity (VELLIQUETTE *et al.,* 2020). Regarding antioxidant activity, studies shown bioactivity in phenolics and protein

hydrolysates. Most studies in sunflower protein hydrolysates uses chemical antioxidant methods, such as ABTS, DPPH and FRAP (DABBOUR *et al.,* 2019a and 2019b; PRADO *et al.,* 2020; HABINSHUTI *et al.,* 2019). Very few evaluated the bioactivity and resistance of antioxidant peptide fraction in cell culture (MEGIAS *et al.,* 2009). Furthermore, little attention has been paid to the phenolic content remaining in sunflower hydrolysates, which may overestimate the bioactive effect of the peptides per se. Chlorogenic acid (CA) is the most abundant phenolic in sunflower meal and its oxidation during alkaline solubilization of the protein generates an undesirable greenish pigmentation, limiting the use of the sunflower by-product in the food industry (WILDERMUTH et al., 2016). Therefore, the isolation of sunflower protein from AC may represent a good alternative for application in food.

The enzyme alcalase has been preferentially used for sunflower protein hydrolysis to generates small molecular weight peptides with antioxidant potential (DABBOUR, *et al.,* 2019a; PRADO *et al.,* 2020; HABINSHUTI *et al.,* 2019). However, the use of commercial enzymes does not guarantee the stability of the generated peptide in the digestion tract. To promote physiological antioxidant bioactivity, peptides must resist the acid and enzymatic conditions of the gastrointestinal system to be absorbed into the circulation. Therefore, peptide integrity and bioavailability throughout digestion may be important to maintain functionality (MEGÍAS *et al.,* 2009).

The present work aimed to obtain sunflower hydrolysates with alcalase from protein isolates with low phenolic content. The sunflower hydrolysates were characterized physicochemical regarding size, amino acid composition and hydrophobicity profile. Since sunflower physiological peptide bioactivity depends on its structure and digestion resistance, it was evaluated the antioxidant bioactivity before and after simulated gastrointestinal digestion and identified the sequence of peptide cross-checking with the bioactivity database.

2. Materials and Method

2.1 Chemical reagents, characterization and phenolic compounds

- *Protein determination:* moisture content and total protein (Kjedahl method, Nx 5,75) of samples was performed as described by AOAC methods (LATIMER Jr, 2012).

- *Sunflower protein preparation:* Defatted sunflower meal (*Helianthus annuus L.*) was produced and donated by Sunbloom protein Company. Sunflower protein was isolated by sequential extractions using 70% ethanol, according to Salgado *et al.* (2010). Briefly, sunflower flour at 1:15 (w/v) ratio, pH 5 was stirred for 1 h at room temperature, centrifuged at 11000×g for 20 min at 20°C. The precipitate (fibers and proteins) was dispersed in distilled water (5 mL) and pH adjustment to 9 (NaOH 1 mol/L), stirred for 1 h, centrifuged at 11000×g for 20 min at 20ºC. Supernatants were submitted to isoelectric precipitation (pH 4.5/ HCl 1 mol/L), resting overnight, and centrifuged at 11000×g for 20 min at 4°C. This procedure was done twice to optimize phenolic extraction. Decanted sunflower isolate protein were centrifuged at 11000×g for 20 min at 4°C and lyophilized.
- *Total phenolics:* Phenolic compounds were determined by reaction with Folin-Ciocalteu according to Singleton *et al.* (1965), and the result was expressed in mg gallic acid equivalent (GAE)/g sample.
- *Amino acid profile:* was determined by RP-HPLC, coupled to a UV detector (Shimadzu Corporation, Tokyo, Japan), equipped with a Luna/Phenomenex C18 column (250 mm × 4.6 mm ×, 5 µm; Phenomenex Inc., Torrence, USA), using the wavelength of 254 nm. Identification and quantification were performed using the external standard (Pierce / PN 20088), and α-aminobutyric acid (AAAB) as internal standard (Aldrich, Milwaukee-USA), as described by White *et al.* (1986).

2. 2 Preparation of sunflower hydrolysates with alcalase

Sunflower protein isolate (SFPI) was hydrolyzate according to Dabbour *et al.* (2018). Briefly, sample was diluted to 5% in water and sonicated (37 kHz – Unique ultrasonic cleaner / 8000) at 50°C, pH 9.0 for 20 min. Hydrolysis was carried out by addition of Alcalase® (0.3 U/g protein) at pH 9.0 for 90 min at 50ºC; reaction was stopped by pH reduction to 5. The hydrolysates were fractionated and filtered using an ultrafiltration system (Labscale TFF 29751 model - Millipore) and cellulose membrane (<5kDa). All the fractions obtained (filtered (<5kDa) and retained (>5kDa)) were lyophilized. The alcalase concentration was corrected by its enzymatic activity measured by (CASTRO; SATO, 2013) with azocasein as substrate. The degree of hydrolysis (DH) was determined according to Adler-Nissen (1986), with pH-stat employing the following equation:

$$\text{DH (\%)} = \frac{\text{BNb}}{\alpha\text{Mphtotal}}\ x\ 100$$

Where: DH= degree of hydrolysis; B= volume of consumed base (mL); Nb= normality of the base; α= average degree of dissociation; Mp= mass of protein; htotal= total peptide bond content (mmol/g).

2. 3 Molecular exclusion chromatography

Samples were analyzed by size exclusion chromatography (SE-FPLC) using Fast Protein Liquid Chromatograph (Akta Pure Chromatograph, GE Healthcare, Chicago, Illinois, USA) with gel filtration columns connected in series (models Superdex 200 and Superdex 30). Samples (5mg/mL) were diluted and sonicated for 20 min. Both the sample and standard (1 mg/mL) were diluted in 25 mM sodium phosphate buffer (pH 7.4 with 150 mM NaCl) and filtered in a hydrophilic polytetrafluoroethylene (PTFE; 0.45 µm) membrane. Samples were injected (0,5 mL/min, 120 min) and readings at 280 nm UV detection. The standards were α-Lactalbumin (14178 Da), Insulin (5807.6 Da), Vitamin B12 (1355.4 Da), L-β-4-Dihydroxyphenylanine (197.2 Da) or L-Norleucine (131.17 Da). The wavelength of 280 nM was employed (VANDER HEYDEN, 2002; BISINOTTO *et al.,* 2020).

2. 4 Electrophoresis

Protein samples were analyzed in sodium dodecyl sulfate-polyacrilamide gel (12%) (SDS-PAGE). Samples at 2 mg/mL were homogenized in a sample buffer (0.0625M tris–HCl, pH 6.8, 2% (w/v) SDS, 5% (w/v) b-mercaptoethanol, 10% (v/ v) glycerol, 0.002% (w/v) bromophenol blue) and subsequently boiled for 5 min and centrifuged at 10.000 g. Protein content was measured by Lowry's method, modified by Peterson, using bovine serum albumin as standard (PETERSON, 1979). Equal amounts (20 µg) of total protein was electrophoresed in a 10% (w/v) SDS–polyacrylamide gel. Gel was run in 120 V constant for 1,5 h and protein fixed and stained with 0.006% (w/v) de Coomassie Blue R e 6% (v/v) of acetic acid. Molecular weights of the polypeptides were estimated by using low molecular weight standards (Sigma) mol wt 6,500-66,000 Da.

2. 5 Hydrophobic profile

Reverse phase high performance liquid chromatography (RPHPLC) was performed on an Agilent liquid chromatograph with a semipreparative and analytical quaternary pump system and a diode array detector (DAD) (Agilent,

Waldbronn, Germany) on a Microsorb – MV™ C18 column (4.6 mm× 250 mm; 5 µm particle size) (Rainin, Woburn, MA, USA) following Caetano-Silva *et al.* (2017). Composition of solvent A – 0.04% TFA in ultrapure water and solvent B – 0.03% TFA in acetonitrile. The gradient Elution conditions were 100% A from 0 to 40 min, from 40 to 45 min 70% B and 30% A, and 45 to 60 min, 100% A. Detection was at 214 nm, and the sample injection volume was 50 µL (3 mg protein/mL for the samples).

2. 6 Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay was performed as described by Corrochano *et al.* (2019). Samples and standard was diluted in 75 mM potassium phosphate buffer (pH 7.4). In a microplate: 20 µl of sample or standard, 120 µl of fluorescein solution (0.17 µM), 60 µl of AAPH solution (40 mM) were added and monitored for 2 h at 90-second intervals at 485 nm excitation and 520 nm emission wavelength in a microplate reader (Varioskan Lux, Thermofisher, Singapore). Trolox standard curve (12.5–400 µM) was used to express the results as µmol Trolox equivalent/g sample.

2. 7 DPPH antioxidant activity assay

The DPPH assay was performed as described by Torres (2021), with minor modifications. A solution of 80 µM of 2,2-diphenyl-1-picrylhydrazyl was prepared in a dark bottle using methanol. It was mixed with diluted concentrations in standard Trolox series (1.5 to 100 µM). The mixture was stirred and incubated at room temperature for 30 minutes. Then, a volume of 200 µL was dispensed into a 96-well microplate and read at an absorbance of 517 nM. The elimination activity of the tested samples was extrapolated into the DPPH inhibitory percentage using the equation; AA (%) = ((Abs White-(Abs Sample)/(Abs White)) x 100). A standard Trolox curve was used to express the DPPH values of the samples at different concentrations. The results were expressed as µmol equivalents of Trolox/g of sample, in triplicate.

2. 8 Antioxidant activity assay – ABTS

The ABTS assay was performed as described by Meera *et al,* (2019). A mixture of potassium persulfate in 2.45 mM aqueous solution and 7 mM ABTS was prepared (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid radical

solution (ABTS+)). This was left to stand in the dark for 12 hours. Its absorbance was adjusted to 0.7 ± 0.02 at 734 nm using a UV-Vis spectrophotometer before analysis. Then, 10 µL aliquot of the sample was placed in a microplate and with subsequent addition 190 µL of ABTS+ solution (Abs 0.7 ± 0.02) was added. After six minutes, the absorbance was read at 730 nm on the microplate. The results were expressed as µmol/Trolox equivalent/g sample in triplicate or by the percentage of antioxidant activity (AAT), calculated and expressed according to the equation (DRYÁKOVÁ *et al.,* 2010). AAT (%) = ((Abs White-(Abs Sample)/(Abs White)) x 100).

2. 9 In vitro digestion assay

The fractions SFPi, SPHr and SPHf were submitted to the INFOGEST assay (BRODKORB *et al.,* 2019), obtaining the digested fractions. No enzyme was used to digest starch and fat (amylase, bile and pancreatic lipase) since the sample was protein isolates. The enzymatic activity of the enzyme's pepsin and pancreatin was measured prior to the digestion process, according to methods suggested in INFOGEST supplementary material. For the oral phase, the sample was mixed with salivary fluid in a ratio of (1:1 w/v) with 10 mL of total volume. $CaCl_2$ 0.3 M was added to the salivary fluid immediately before the test, in a bath at 37 ◦C with stirring, for 2 min. In the gastric phase, a 1:1 w/v ratio of oral bolus was mixed with simulated gastric fluid containing 0.3 M $CaCl_2$ freshly added, pH adjusted to 3.0 and sequentially added pepsin enzyme (2000 U/mL), with the volume adjusted to 20 mL. This remained in a bath at 37 ◦C under stirring for 2 h. For the intestinal phase, the gastric chyme was mixed in a ratio of 1:1 w/v with intestinal fluid with $CaCl_2$, pH adjusted to 7.0 with 5 M NaOH and addition of pancreatin dilution to reach the final concentration final of 100 U/mL in the intestinal phase. The total volume was adjusted to 40 mL at this stage. The intestinal mixture was placed in a bath at 37 ºC for 2 h. Enzyme activity was inhibited by sample incubation in a bath at 85 ºC for 10 min/cold bath and centrifuged at 7000 g for 20 min at 4 ºC. Supernatant was collected and lyophilized and stored under freezing conditions for later analysis.

2. 10 Identification of sequences by LC/MS/MS (Nano-coupled liquid chromatography QExactive mass)

For the test, SPHf and digested samples were analyzed. First, the samples were resuspended in LC/MS water with 0.1% formic acid and their protein content quantified by the Qubit Protein Assay method. Subsequently, chromatography was performed on a PicoChip source (Model 1PCH-550; 75 µm ReproSil Pur C18 3 µm silica matrix; New Objective, USA) at a continuous flow rate of 0.300 µl/min. 1 ug of sample was injected into an Acclaim PepMap 100 trap (75 µm ID, C18 3 µm; Thermo Fisher Scientific). Sample separation took place in a gradient of 2-40% mobile phase B for 120 min, followed by 10 min in 80% mobile phase B and column reequilibration for 10 min in 2% mobile phase B. The mobile phases of the chromatography included: mobile phase A water/0.1% formic acid, and phase B acetonitrile/0.1% formic acid. Mass spectra were acquired from a QExactive mass spectrometer (Thermo Fisher Scientific) by the DDA method (data-dependent acquisition, Full MS/MS) with selection of the 10 best counts. The precursor ion search was performed at 300-1,750 m/z at a resolution of 70,000. An isolation window of 2 m/z was selected, with collision energy NCE 15 and 30, followed by MS/MS acquisition at 17,500 resolutions. The automatic gain control (AGC) target of 1 and 4 and maximum injection time of 100 ms. Loads 1 and greater than 5 were excluded. A dynamic exclusion time of 30 s was used. Samples were analyzed in duplicate. The spectra were analyzed with PatternLab for Proteomics (CARVALHO *et al.,* 2015) using the *Helianthus annuus* database from the UNIPROT database (https://www.uniprot.org). The search for peptides was carried out with the NOVOR software (MA, 2015) (available at https://novor.cloud/), using predefined definitions and the *Helianthus annuus* database as a reference. The sequencing results were checked against bioactivity in the BIOPEP-UWM database (https://biochemia.uwm.edu.pl/en/biopep-uwm-2/). The potential antioxidant activity of peptides identify was also evaluated in silico using AnOxPePred webserver (https://services.healthtech.dtu.dk/service.php?AnOxPePred-1.0). This server uses deep learning method for prediction of free radical scavenging (FRS) and metal chelating properties of peptides (OLSEN *et al.,* 2020).

2. 11 Statistical analyses

Statistical analysis was performed using SPSS 16.0 software (SPSS Inc., Chicago IL). An ANOVA was performed followed by Duncan post-test to assess

differences between groups. The mean ± standard error was used to represent the data from each in vitro experiment, which was repeated at least five times. Statistical significance was determined as $p < 0.05$.

3. Results and Discussion

3.1 Production of sunflower protein isolates with low phenolic content

Protein content increased from 48 to 92 % in sunflower protein isolates (SFPi). In hydrolyzed fractions filtered (SPHf) and retented (SPHr) protein content reduced to 71 and 56%, respectively, probably because of a dilution in the hydrolysis process. The phenolic compounds in SFPi content was eliminated a 95% compared to the original flour (Table 1). However, SPHf and SPHr showed 76 % of phenolic content eliminations. In this study phenolic compounds reduction in isolate protein was similar to results obtained by Alexandrino *et al.* (2017). Nevertheless, a residual fraction of phenolics remains attached to the protein isolates, which are released and detected only after hydrolysis. The alkaline environment in the protein isolation process favors the interaction between phenolics and protein (LI *et al.,* 2021). However, during hydrolysis occurs protein cleavage exposing internal sites and releasing the phenolics previously hidden.

Numerous processes can be used for removal of phenolic compounds, such as extraction with methanol, ethanol, adsorption resin, white acid extraction, ion exchange and others (SINGH *et al.,* 2012; ARRUDA *et al.,* 2016). Among the methods of phenolics extraction, the use of ethanol was shown to be more effective to the removal of chlorogenic acid from protein isolates, since disrupt bonds between phenolic and protein (ALEXANDRINO *et al.,* 2017; SALGADO *et al.,* 2012). A complete phenolic removal its hardly achieved, since the interaction between polyphenol and protein can be of several forms, such as covalent and non-covalent, between hydrophobic and hydrophilic amino acids and other factors (KANAKIS *et al.,* 2011; OZDAL *et al.,* 2013; YILMAZ *et al.* 2022; HAN *et al.,* 2020; QUAN *et al.,* 2019). In this study antioxidant bioactivity was evaluated in sunflower protein hydrolysate with reduced content of phenolic compounds.

Table 1. Protein content and quantification of total phenolic compounds of the samples

Sample	Protein content (%)	Phenolic content (mg AG /100g)	Total phenolic content elimination (%)
Deffated sunflower (DS)	48 ± 1.1	488 ± 4.7	-
Sunflower protein isolates (SFPi)	92 ± 0.1	26 ± 2.4	95
Sunflower protein hydrolysate filtered (SPHf)	71 ± 0.8	118 ± 1.50	76
Sunflower protein hydrolysate retented (SPHr)	56 ± 0.3	116 ± 0.82	76

3.2 Protein hydrolysis and molecular size evaluation

Since low molecular weight peptides have been related with better bioactivity, two fractions of hydrolysates were prepared: SPHf, smaller than 5kDa; and SPHr. The hydrolysis with alcalase induced 30.3 ± 2.6 % of degree hydrolysis (DH). This value was in accordance to previously published data (10 to 30% of DH) employing the same enzyme at equivalent concentration (PRADO *et al.,* 2020; UGOLINI, *et al.,* 2015; REN *et al.,* 2016). The effective hydrolysis obtained for sunflower samples may be related to the ultrasound treatment (20 min), which induced the opening of the protein structure and consequently the larger contact area of the enzyme during hydrolysis (YANJUN *et al.,* 2014).

Electrophoretic profiles of the sunflower isolate and hydrolyzed fractions are shown in Figure 1. No bands were observed in the hydrolyzed fractions, suggesting the predominance of low molecular weight peptides, which migrated rapidly through the electrophoresis and lost in the gel. SFPi sample shows bands in the range of 10 and 50 kDa. Two main protein groups are present in sunflower seeds: globulins, which correspond to the range of 20-60 kDa and 40-90% of total proteins, and albumins with 11-20 kDA, which represent 10-30% of total proteins. Among the globulins classified by sedimentation coefficient, 11S globulin (or heliantinin) is the main class; and for albumin, 2S albumins prevail (GONZÁLEZ-PEREZ *et al.,* 2002 and 2007).

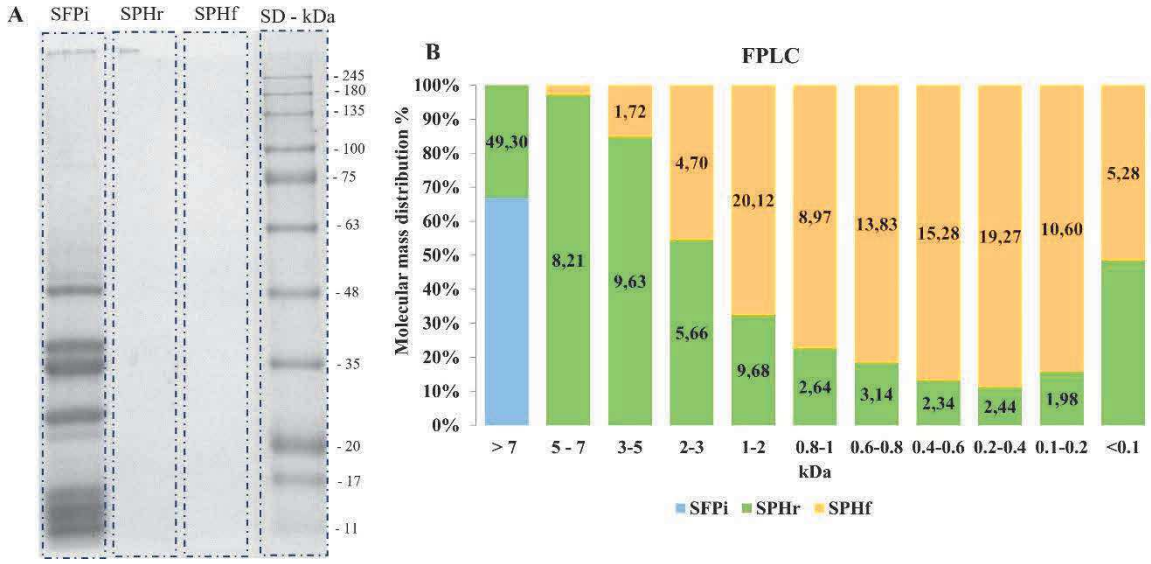

Figure 1. Electrophoretogram and molecular size distribution profile of samples. A: SDS-PAGE gel of SFPi and hydrolysates retented (SPHr) and filtered (SPHf) (SD=molecular mass standard) and B: Molecular weight (MW) distribution profile of SFPi, SPHf and SPHr in molecular size exclusion chromatography.

To evaluate with better precision, the molecular size range of each hydrolyzed fraction, samples were analyzed by molecular size exclusion chromatography, showed in Figure 1B. All proteins in SFPi were above 7 kDa, which corroborates with electrophoresis analysis and corresponds to albumin and globulins, the main proteins present in sunflower (CARRÃO-PANIZZI; MANDARINO, 1994). SPHr presented a wide distribution in different molecular weights, but still showed a large proportion in the range above 7 kDa. This data indicates that alcalase hydrolysis was not complete and some proteins remained intact after the hydrolysis process. Interestingly, this large molecular weight proteins were not observed in electrophoresis analysis, probably because low assay sensitivity. In the other hand, the SPHf fraction showed all peptides with smaller size than 3 kDa. Most peptides were found in the range smaller than 600 Da (REN *et al.,* 2016, UGOLINI *et al.,* 2015; DABBOUR *et al.,* 2019a and 2019b). The molecular size of peptides has been extensively associated with better bioactivity. In previously review it was shown that of 42 antioxidant peptides from different food sources, 70% presented a molecular size range from 400 to 650 Da (ZOU *et al.,* 2016).

3.6 Amino acid composition and hydrophobicity profile

Although the SFPi sample presents all the indispensables amino acids, as expected for a pseudo-cereal, lysine appears as the limiting amino acid (Table 2), following the FAO reference standard (CARRÃO-PANIZZI; MANDARINO, 1994). The hydrolysis process impaired the distribution of total essential amino acids regarding Met + Cys and Leu, probably being lost through their release into free amino acids. Most amino acids of SFPi were classified into hydrophobic and acids, which may directly implicate in the bioactivity of the formed peptides. Despite limited in lysine, the sunflower protein can be considered of good quality, and this low lysine levels can be corrected by adding other protein sources that have surplus values for the limited amino acid (LIN *et al.* 1974; GILBERT *et al.,* 2011; RAMACHANDRAN *et al.,* 2007; SALGADO *et al.,* 2010 and 2012; ALEXANDRINO *et al.,* 2017).

Table 2**.** Total amino acid composition of sunflower protein isolates and hydrolysates

Amino acids (AA)		**WHO/FAO/UNU mg/g ref. protein**	**SFPi**	**SPHf**	**SPHr**
			mg/g protein	**mg/g protein**	**mg/g protein**
Indispensable	Lys	45	25.0 ± 0.00	16.2 ± 0.00	17.3 ± 0.00
	Trp	6	31.0 ± 0.00	24.3 ± 0.00	23.3 ± 0.06
	Phe + Tyr	38	82.8 ± 0.00	64.4 ± 0.01	54.7 ± 0.01
	Met + Cys	22	28.6 ± 0.01	15.2 ± 0.01	19.04 ± 0.01
	Thr	23	31.0 ± 0.01	24.3 ± 0.00	23.3 ± 0.06
	Leu	59	60.9 ± 0.0	46.6 ± 0.00	42.2 ± 0.00
	Ile	30	40.1± 0.00	31.4 ± 0.00	30.3 ± 0.00
	Val	39	49.0 ± 0.01	38.8 ± 0.01	35.5 ± 0.01
	His	15	26.9 ± 0.01	20.2 ± 0.00	17.9 ± 0.01
AA distribution (%)	Hydrophobic		27.91	15.58	14.81
	Hydrophilic		10.21	5.53	5.50
	Ácids		28.75	15.36	15.60

	Básics		13.28	6.92	7.40

Source: Values are means ± standard deviations of three measurements. Protein determinate by Kjeldahl. Abbreviations includes: Asp: Aspartic acid; Ala: Alanine; Arg: Arginine; Glu: Glutamic acid; His: Histidine; Gly: Glycine; Cys: Cysteine; Ile: Isoleucine; Leu: Leucine; Lys: Lysine; Met: Methionine; Phe: Phenylalanine; Pro: Proline; Ser: Serine; Thr: Threonine; Glu: glutamic acid; Tyr: Tyrosine; Val: Valine. Hydrophobic (Ala, Val, Met, Phe, Leu, Ile, Pro), Hyrophilic (Gly, Thr, Tyr, Cys) Basics: Arg, His, Lys) and Acids AA (Glu; Asp). Score to adults > 18 years (FAO, 2007).

The hydrophobicity profile of SFPi and the hydrolyzed fraction (SPHr and SPHf) were analyzed and shown in Figure 2. For a better interpretation of the results, the area of the chromatogram was divided into three zones, corresponding to the concentration of the mobile phase at 60 min: 1 to 20 min, hydrophilic zone; 20 to 40 min, intermediate zone and 40 to 60 min, hydrophobic zone (LEGAY *et al.,* 1997; NATRELLA *et al.,* 2023). It is possible to observe a reduction in hydrophobicity after the hydrolysis process with alcalase, since chromatographic picks of SPHr and SPHf are shifted to the left, in the zone I and II of medium and low hydrophobicity. A possible explanation could be the reduction of hydrophobic amino acids (Val, Met, Phe, Leu and Ile) in the peptide sequence after the hydrolysis process, as observed in Table 2 in SPHr and SPHf. Alternatively, the hydrophobicity profile of the peptide may be related to the DH. Previous work with sunflower protein showed an increase in hydrophobicity with lower DH (0 and 18%), but reduced hydrophobicity with an increase in DH (18 and 24%). This was attributed to protein-protein interaction and restriction of hydrophobic zones to aggregates (DABBOUR *et al.,* 2019a). In fact, these results are in accordance with our higher DH (>27%).

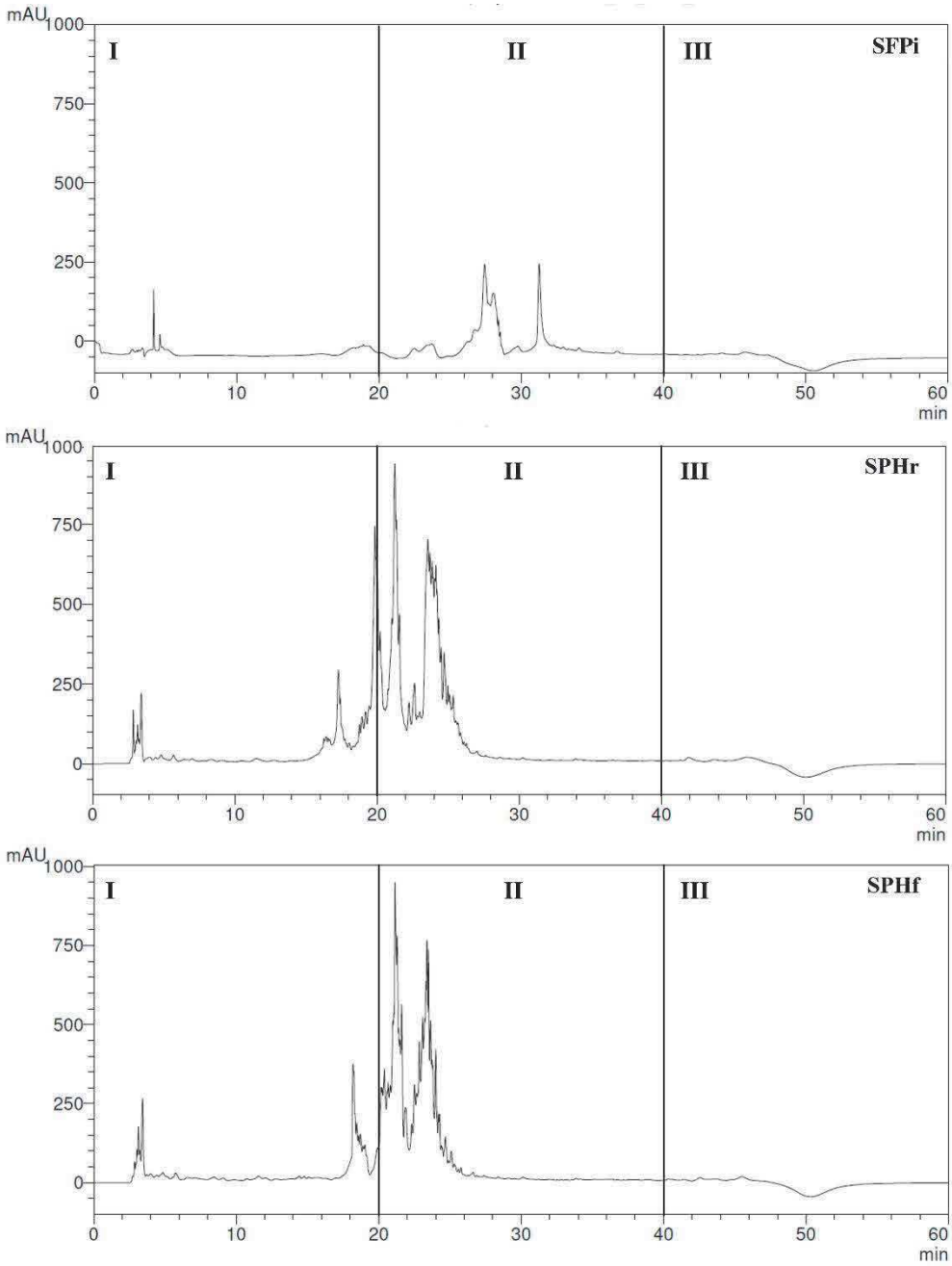

Figure 2. Hydrophobic profile of sunflower protein isolates and hydrolyzed fractions by alcalase. Chromatograms are divided in hydrophobicity zones, related to retention time and mobile phase concentration where: 0–25% Mobile Phase B corresponds to low hydrophobicity (I), 25–50%

medium hydrophobicity (II), 50–100% and high hydrophobicity (III). SFPi=sunflower protein isolates; SPHr= sunflower hydrolysates retented and SHPf = sunflower protein filtered.

3. 4 Antioxidant activity

Three antioxidant assays with different mechanism of action were used to evaluate the potential antioxidant: ABTS, DPPH and ORAC. Since the peptides obtained from hydrolysis by alcalase must have their physiological effect confirmed only after the gastrointestinal digestion, the resistance of the antioxidant bioactivity was also evaluated after the simulated digestion process. The results are summarized in Figure 3 (before and after digestion).

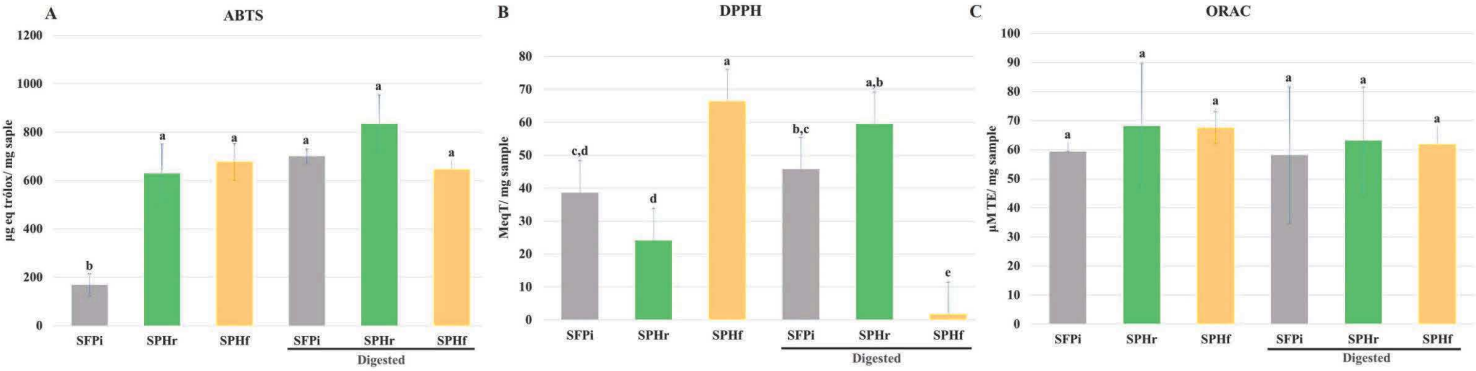

Figure 3. Antioxidant activity of the sunflower protein hydrolysates before and after digestion. A: ABTS; B: DPPH and, C: ORAC. Samples were analyzed in 0.6 mg/mL. Each value is the mean of standard error of at least 5 experiments. Statistical analysis was

performed using one-way ANOVA followed by Duncan's test, with $p<0.05$. SFPi=sunflower protein isolates; SPHr= sunflower hydrolysates retented and SHPf = sunflower protein filtered.

Although the bioactivity of peptides is related to their molecular size, this analysis has not previously been reported for sunflower peptides. It is noticeable that after alcalase hydrolysis (before digestion), the antioxidant activity increased more than 350 % in ABTS and 150 % in DPPH assay in filtered fraction (SPHf), confirming that low molecular size of the peptides may confer greater antioxidant capacity than larger fractions. An improvement in antioxidant bioactivity after the hydrolysis process with alcalase corroborates with previous results in sunflower hydrolysates treated with dual-frequency ultrasonic (DABBOUR *et al.,* 2019b). The production of bioactive peptides must be enzyme-dependent since sunflower protein hydrolysis with other commercial enzymes such as Neutrase and Flavourzym was not as effective for antioxidant peptide production (PRADO *et al.,* 2020).

The antioxidant activity by ORAC was similar for the samples, despite the tendency for hydrolyzed fractions to increase. This difference in antioxidant results can be explained by the specificity of each method. The ORAC assay measures the ability of the antioxidant compound to inhibit oxidation induced by peroxyl radical through proton transfer, while ABTS and DPPH is focused on antioxidant activity through electron transfer (PRIOR *et al.,* 2005).

After digestion the SFPi increased antioxidant activity in ABTS assay, probably by an increase in hydrolysis of integral protein and formation of peptide during digestion process. This suggests that the consumption of integral sunflower protein would be sufficient to obtain bioactive peptides with antioxidant potential, without the need for their prior hydrolysis with commercial enzymes, such as alcalase, as verified in recent work by our group (BISINOTTO *et al.,* 2023).

The antioxidant activity was preserved in digested SPHf in ABTS and ORAC, but drastically reduced in DPPH analysis. In the other hand, in digested SPHr the antioxidant activity was maintained or even increased, probably due to the hydrolysis of proteins

that remained intact after alcalase hydrolysis. The reduction in the antioxidant bioactivity of SPHf after digestion with the DPPH assay and the persistence of bioactivity in digested SPHr may indicate a greater susceptibility of the smaller peptides to hydrolysis promoted by gastrointestinal enzymes. Like our results, copper chelating sunflower peptides produced by hydrolysis with alcalase were partially resistant to incubation with simulated gastric and intestinal fluids (MEGIAS *et al.,* 2009).

3. 5 Peptide identification with mass spectrometry

To better understand structure-function relationship of generated peptides after alcalase hydrolysis and after digestion, mass spectrometry of the SPHf and digested fractions samples was performed. The data regarding the peptide sequences of the fractions are shown in Table 1 (supplementary material 1). Spectra analysis of SPHf and digested was performed with Pattern lab using *Helianthus annuus* database from Uniprot, to obtain exclusive peptides from sunflower. Several 196 peptide sequences were identified for SPHf and only 5 for digested fraction. This result can have two interpretations: (1) a significant reduction in peptides variability after the digestive process, that is, few peptides remain intact after the action of digestive enzymes, so less sequence were identified; (2) most of peptide size formed after digestion were smaller than 400 Da. These molecular sizes are not detected in the analyzed equipment since it is below the ionization range threshold. Given that bioactivity of the digested fraction is maintained (at least in the ABTS and ORAC assay) and the SE-FPLC analysis of the SPHf fraction showed low molecular weight peptides, the second option seems to be more feasible. According to Zou *et al.* (2016), peptides with antioxidant bioactivity have an average size of 400 to 650 Da. Interestingly, three sequence were identified in both fraction (IERGRGIQ, IIRPPQ and VVRPPIRIQ), which means that these peptides were resistant to digestion in an intact form.

The sequenced peptides of the SPHf fraction were originated from 10 proteins, mostly represented by 11S globulin (73%), followed by glutein (10%). This result correlate with our sample, since globulins are predominantly found in seeds and constitute most of the sunflower proteins, ranging from about 40 to 90% (GONZALES-PEREZ *et al.,* 2007). Homologous peptides in 11S globulin derived peptides are observed, which vary in lengths (in few amino acids

upstream or downstream protein sequence) (subscript numbers in supplementary material Table 1). The peptide sequences obtained from sunflower hydrolysate are derived from different parts of 11S globulin protein sequence, showing that most of the protein was fragmented (Box 1 of the supplementary material). Although the alcalase enzyme has broad specificity, it prefers to cleave the carboxylic region of the amino acids Glu (E), Met (M), Leu (L), Tyr (Y), Lys (K) and Gln (Q) (ADAMSON; REYNOLDS, 1996). In fact, big part of sequenced amino acids has these amino acids in their C-terminal region.

Peptide sequences identified were cross-checked with BIOPEP-UWM database, which contain mainly food peptide sequences with bioactivity already reported in *in vivo* or *in vitro* previous studies (MINKIEWICZ *et al.,* 2022). However, no sequence identified in our sample showed bioactivity with 100% similarity. This result was expected, since there is little data in the literature relating functionality with sequence structure of plant derived peptide. In fact, our study seems to be the first to relate the structure of hydrolyzed sunflower peptides to antioxidant bioactivity.

Although we did not find direct bioactivity in the intact sunflower peptide sequence, we found several di- and tri-peptides contained within the sequence that were previously related to antioxidant activity (Table 3). Note that several antioxidant peptides have been found in plant sources, like sunflower in protein content and rich in homologous proteins, such as soybeans. Therefore, it is possible that the smaller peptides, not identified in our sample because of the ionization threshold, may be also associated with antioxidant activity, as observed in the *in vitro* antioxidant analysis (Figure 3). Interestingly, antioxidant fragments are reach in Tyrosine (Y), leucine (L) and proline (P) which have been previously associated with antioxidant activity (ZOU, 2016).

Finally, the potential antioxidant activity of peptides identify was also evaluated *in silico.* The AnOxPePred web server use a deep learning method for prediction of free radical scavenging (FRS) and metal chelating properties of the whole peptides (OLSEN *et al.,* 2020). The top scoring peptides were considered as potential antioxidants (Table 3). Thirteen peptides from our sample had FRS above 0,5 score, which were considered as potentially bioactive when compared in experimental assays. These results are also in agreement with those published

by Deka and Saikia (2023) who studied the potential in silico activity of different 11S globulin including sunflower. However, the antioxidant potential of the 3-digestion resistant peptide (supplementary material with asterisks) presented FRS under 0,5 score: IERGRGIQ (0,36); IIRPPQ (0,44); VVRPPIRIQ (0,43).

Table 3. Identification of sunflower peptides with potential shorter sequences with antioxidant activity in previous studies

Peptide sequence Identified	Predicted free radical scavenger (FRS) score	Predicted chelation (CHEL) score	Biopeptide extracted from original peptide sequence	Origin of biopetide extracted	Reference
AEKG**HL**QPN	0.46477	0.25367	HL	**Synthetic peptides of soybean protein**	10.1021/jf950833m
SFKFPILE**HL**	0.38542	0.24877			
AIQS**PHW**TIN	**0.50619**	0.22474	PHW		10.1021/jf021191n
IQS**PHW**TIN	0.48768	0.23813			
LL**PYY**PNTPE	**0.60884**	0.20892	PYY		10.1021/jf950833m
TG**RHQ**QSQRPGWE	0.33960	0.22997	RHQ		10.1021/jf021191n
TG**RHQ**QTQRPSWE	0.36449	0.22708			
GERLPFDED**RHQ**	0.33966	0.25427			
RLPFDED**RHQ**	0.40462	0.24493			
RLPFDED**RHQ**KVE	0.44578	0.22719			
TD**RHQ**KIH	0.38660	0.25712			
GGRRGGGEGNQD**RHQ**	0.47151	0.25095		Hydrolysetes of Sardinelle by-products proteins (*Sardinella aurita*)	10.1016/j.foodchem.2009.05.021
GLLL**PYY**PNTPELVY	**0.61410**	0.20455	PYY	**Hydrolysates of potato protein**	10.1021/jf101556n
				Synthetic peptides of soybean protein	10.1021/jf950833m
AERG**EL**RPN	0.38660	0.25712	EL	Synthetic casein protein	10.1016/s0955-2863(99)00083-2
GNSVFDN**EL**RE	0.34326	0.22190			
GNSVFDN**EL**REG	0.31211	0.21325			

HNDGNT**EL**	0.38281	0.25362			
HNDGNT**EL**VVV	0.34314	0.20508			
NNGQD**EL**VII	0.30653	0.19021			
SADRG**EL**RPN	0.35424	0.21353			
TRDN**VY**AGF	0.38825	0.22466	VY	**Hydrolysates of potato protein**	10.1021/jf101556n
DN**VY**AGF	0.45668	0.21403			
LTRDN**VY**AGF	0.39129	0.22000			
ENIDNPSH**ADF**VNPQ	0.44330	0.28437	ADF	**Synthetic peptide from Okara protein**	10.3136/fstr.8.357
H**ADF**VNPQ	0.38009	0.28488			
IDNPSH**ADF**VNPQ	0.47559	0.26434			
NIDNPSH**ADF**VNPQ	0.46417	0.25458			
PSH**ADF**VNPQ	0.35244	0.26510			
LLPSYVNTPI**LAF**	0.44343	0.20053	LAF	Synthetic of mung bean meal protein	10.3390/molecules26061515
RIQPGGLLLPSYVNTPI**LAF**	**0.52577**	0.21034			
NIDDPSNAD**LY**NPQ	0.39043	0.25190	LY	**Hydrolysates peptide from soybean protein**	10.1007/s00217-009-1093-1
RDN**VY**AGF	0.44729	0.22303	VY	**Hydrolysates of potato protein**	10.1021/jf101556n
VVL**AY**EPVWAIGTGK	0.48772	0.15001	AY	**Synthetic peptide from Okara protein**	10.3136/fstr.8.357
				Hydrolysates fraction of marine bivalve peptides (*Mactra veneriformis*)	10.1016/j.foodchem.2014.06.113
RAGEQGS**RW**VSF	0.43841	0.17751	RW	Hydrolyzed of egg protein	10.1016/j.foodchem.2010.04.083
TNRAP**LK**SPL	0.30750	0.26199	LK		
NRAP**LK**SPL	0.31359	0.25321			
GVDF**IR**H	0.35857	0.21452	IR		
I**IR**PPQ	0.44182	0.27872			
I**IR**PPQAR	0.36838	0.28227			
QI**IR**PPQAR	0.41122	0.25741			
N**NEN**QLDEY	0.34481	0.23910	NEN	Synthetic from β-Lactoglobulin	10.1007/s13594-015-0226-5
RGFQD**RHQ**KIR	0.45211	0.23101	RHQ	**Synthetic peptides of soybean protein**	10.1021/jf021191n
K**LPL**LQ	0.35917	0.29142	LPL	**Synthetic peptide from hemp seed (*Cannabis sativa L.*)**	10.1016/j.jff.2013.11.005
QVVRPP**IR**IQ	0.41680	0.22782	IR	Hydrolyzed of egg protein	10.1016/j.foodchem.2010.04.083
RGLQVVRPP**IR**IQ	0.44215	0.18801			
VERGLQVVRPP**IR**IQ	0.44334	0.16774			
VVRPP**IR**IQ	0.43123	0.23234			
DI**PW**PF	**0.53734**	0.28542	PW	**Peptide fraction of buckwheat protein**	10.1016/j.foodchem.2009.05.024
DMPFDI**PW**PFRPS	**0.61714**	0.26588			

EIPFDMPFDI**PW**PFRPS	**0.56245**	0.23478			
FDI**PW**PFRPS	**0.63753**	0.26920			
FDMPFDI**PW**PFRPS	**0.59280**	0.26175			
MPFDI**PW**PFRPS	**0.55048**	0.26555			
PFDI**PW**PF	**0.57696**	0.26467			
PFDI**PW**PFRPS	0.41910	0.19481			
AGEDKGR**LW**PF	**0.63753**	0.26920	LW	Hydrolysates fraction of marine bivalve peptides (*Mactra veneriformis*)	10.1016/j.foodchem.2014.06.113
KDDDLK**AY**	0.27976	0.24703	AY	**Synthetic peptide from Okara protein**	10.3136/fstr.8.357
				Hydrolysated of tuna (Katsuwonus pelamis)	10.2331/suisan.65.92
				Hydrolyzed of egg protein	10.1016/j.foodchem.2010.04.083
SEDK**AD**FR	0.28384	0.22623	ADF	**Synthetic peptide from Okara protein**	10.3136/fstr.8.357
ADRG**EL**RPN	0.36833	0.21247	EL	Synthetic casein peptide	10.1016/s0955-2863(99)00083-2
WLPSPFFPI	**0.50706**	0.26398	WLP	Peptides from sea squirt (*Halocynthia roretzi*)	10.1039/d2fo00729k rsc.li/food-function
VVRPPI**IR**IQ	0.43123	0.23234	IR	Hydrolyzed of ovotransferrin	10.1016/j.foodchem.2010.04.083
I**IR**PPQ	0.44182	0.27872		Hydrolyzed of ovotransferrin	10.1016/j.foodchem.2010.04.083

Source: A = Ala; C = Cys; D = Asp; E = Glu; F = Phe; G = Gly; H = His; I = Ile; K = Lys; L = Leu;M = Met; N = Asn; P = Pro; Q = Gln; R = Arg; S = Ser; T = Tre; V = Val;W = Thr; Y = Tyr. In red are the di- and tri-peptides contained in the sequence that have previously been linked to antioxidant activity.

4 Conclusion

Protein hydrolysates from sunflower isolate with low phenolic content was obtained, as well as peptides with low molecular size have been generated by hydrolysis with alcalase enzyme. However, part of the proteins after hydrolysis remained intact, as observed in the retained fraction. Despite the high content of hydrophobic amino acids composing the sample, the hydrophobicity profile of the peptides was reduced after hydrolysis. The low molecular size of the filtered fraction of sunflower hydrolysate was associated with a greater antioxidant bioactivity by the ABTS and DPPH methods. However, the resistivity to the digestive process was partially maintained in low molecular weight peptides. For the first time the sequence of sunflower bioactive peptide was identified and cross-checked with bioactive database. These results contribute to a better understanding between the structure and bioactivity of sunflower peptides. Sunflower protein hydrolysates may be an excellent protein source for human nutrition and for application in

plant-based products because of its excellent functionality. If enzymatically processed, it is suggested that generated peptides may be protected by microencapsulation to preserve bioavailability.

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Declaration of Competing Interest

The authors declare there was no conflict of interest to declare.

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CAPÍTULO 3

SUNFLOWER PROTEIN HYDROLYSATE WITH LOW PHENOLIC CONTENT PROMOTE ANTIOXIDANT EFFECT ON CACO-2 CELLS

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ABSTRACT

Recent studies have shown that gastrointestinal diseases are associated with oxidative stress, caused by the dysregulation between the formation of pro-oxidant and antioxidant substances. Sunflower meal, a co-product of biodiesel production, is rich in proteins with unknown functionality. Studies show that the peptides, generated after a hydrolysis process, may have an antioxidant action beneficial to health, however little explored for sunflower peptides. This study evaluated the antioxidant potential of sunflower protein hydrolysates with low phenolic content in Caco-2 intestinal cells and the mechanism of action of this process. The protein from the defatted flour was isolated by isoelectric pH (4.5) and the phenolic compounds were removed by alcohol extraction (70%). The sunflower protein isolate (SI) was hydrolyzed by the enzyme alcalase (pH 9, at 50ºC/90 min) and ultrafiltered <5kDa, generating the filtered fraction of sunflower hydrolysate (SPHf). SPHf did not show antimicrobial activity against major foodborne pathogens. However, SPHf prevented hydrogen peroxide-induced oxidative stress in Caco-2 gastrointestinal cells. Sunflower hydrolysate was able to attenuate reactive oxygen species production and reverse the increase in GSH content and CAT activity induced by the stimulus with H_2O_2. A total of 189 peptide sequences from the SPHf fraction were identified, with an average number of 10 amino acid per peptide, and low molecular weight ranging from 500 - 2000 Da. Most peptides presented hydrophobic property and C and N-terminal regions showed higher incidence of hydrophobic and acid amino acids. These structure characteristics may influence the capacity of compounds to act as an electron donor to antioxidant bioactivity. This study brings for the first-time data on the mechanism of antioxidant action of sunflower peptides in gastrointestinal cells and and associate important structure relation properties. These data support new physiological application for sunflower products.

Keywords: sunflower; oxidative stress; bioactive compound; bioactive peptides; antioxidant peptide

1 Introduction

Defatted sunflower meal, a rich protein by-product of oil extraction, can be used as a raw material to increase the nutritional value of foods, contributing for the alternative protein market. However, the application of this ingredient in the

production of new functional foods has been ineffective, largely due to the lack of knowledge about its physiological properties.

Once hydrolyzed, proteins can present new physiological bioactivities, not presented in the original structure, that can beneficially impact health (PRADO *et al.,* 2020). During hydrolytic process, smaller polypeptide chains are formed, exposing previously hidden protein sites. These compounds with smaller size and new properties may favors the interaction with receptors and other regulatory molecules in the cell, emerging various bioactive properties, such as antioxidant capacity (AGYEI *et al.,* 2016).

The human intestine, an organ that has several functions, including that of a barrier, can become susceptible to oxidative stress (OS). This can potentiate the onset of inflammatory bowel diseases, for example (WAN *et al.,* 2015). Due to their antioxidant properties and their potential for the treatment/prevention of diseases associated with OE, food-derived peptides have been studied (TONON *et al.,* 2016). However, the potential antioxidant effect of sunflower peptides, obtained by enzymatic hydrolysis with the commercial enzyme alcalase, has been little explored *in vitro* and especially in biological systems. Caco-2 cells refer to a colonocyte cell lineage model for the study of enterocytes, which mimic the physiological responses of mature human epithelial cells (WAN *et al.,* 2015).

Oxidative stress (OS), triggered by the imbalance between the production and elimination of reactive oxygen species (ROS), nitrogen (RNS). Under these conditions, endogenous antioxidants are not enough to minimize the pathological effects (AHMADINEJAD *et al.,* 2017).

Therefore, this study evaluated the antioxidant potential of sunflower protein hydrolysates in Caco-2 intestinal cells and the mechanism antioxidant action of this process. In addition, it was evaluated the potential antimicrobial action against the main food-borne pathogens. Finally, the identified peptides were analyzed for their structure-function relationship.

2 Materials and Method

2. 1 Chemical reagents and characterization

- *Sunflower protein preparation:* Defatted sunflower meal (*Helianthus annuus L*.) was produced and donated by Sunbloom protein Company. Sunflower protein

was isolated by sequential extractions using 70% ethanol, according to Salgado *et al.* (2010). Briefly, sunflower meal at 1:15 (w/v) ratio, pH 5 was stirred for 1 h at room temperature, centrifuged at 11000×g for 20 min at 20°C. The precipitate (fibers and proteins) was dispersed in distilled water (5 mL) and pH adjustment to 9 (NaOH 1 mol/L), stirred for 1 h, centrifuged at 11000×g for 20 min at 20ºC. Supernatants were submitted to isoelectric precipitation (pH 4.5/ HCl 1 mol/L), resting overnight, and centrifuged at 11000×g for 20 min at 4°C. This procedure was done twice to optimize phenolic extraction. Decanted sunflower isolate protein were centrifuged at 11000×g for 20 min at 4°C and lyophilized.

2. 1. 1 Preparation of sunflower hydrolysates with alcalase

Sunflower protein isolate (SFPi) was hydrolysate according to Dabbour *et al.* (2018). Briefly, sample was diluted to 5% in water and sonicated (37 kHz – Unique ultrasonic cleaner / 8000) at 50°C, pH 9.0 for 20 min. Hydrolysis was carried out by addition of Alcalase® (0.3 U/g protein) at pH 9.0 for 90 min at 50ºC; reaction was stopped by pH reduction to 5. Hydrolysates were fractioned by ultrafiltration (<5kDa) and filtered and retained fractions were freezedried. The alcalase concentration was corrected by its enzymatic activity measured by (CASTRO; SATO, 2013) with azocasein as substrate. The degree of hydrolysis (DH) was determined according to Adler-Nissen (1986), with pH-stat employing the following equation:

$$\text{DH (\%)} = \frac{\text{BNb}}{\alpha \text{Mphtotal}}\ x\ 100$$

Where: DH= degree of hydrolysis; B= volume of consumed base (mL); Nb= normality of the base; α= average degree of dissociation; Mp= mass of protein; htotal= total peptide bond content (mmol/g).

2. 2 Caco-2 cell culture

Human adenocarcinoma cells derived from human intestinal epithelium (Caco-2) were obtained by Cells Bank in Rio de Janeiro (code 0059) and donated from Dr. Juliana Alves Macedo Laboratory. These cells were seeded in 25 cm^2 flasks and cultured in DMEM containing 10% fetal bovine serum, 1% Penincillin/streptomycin, 8.4mM Hepes, 1% sodium pyruvate, 1% non-essential amino acids and 1% L-glutamineunder 5% CO_2 and 37°C condition. Medium was change every 2 days. The exponentially growing cells were trypsinized and seeded

in 24-well plates (with a cell density of $10x10^4$ cells/well) or 96-well plates ($2x10^4$ cells/well) depending on the type of treatment adopted.

2. 2. 1 Treatment

Caco-2 cells were submitted to a pretreatment with or without sunflower hydrolysate for 1h, followed by stimulation in the presence or absence of hydrogen peroxide H_2O_2 (1 mM) for 3h. For this evaluation, the following concentration curve of peptide hydrolysates were adopted: 0.6; 0,3 and 0,015 mg/mL.

2. 3 Assessment of cell viability by MTT

The effect of the metabolic activity of cell internalization of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazoline bromide or MTT (M5655, Sigma-Aldrich St. Louis, MO, USA) was investigated. The dissolution of the crystals, resulting in the conversion of MTT into insoluble purple crystalline formazan, was measured by reading the absorbance in a microplate reader (Varioskan Lux, Thermofisher, Singapore) at wavelengths of 560 and 650 nm, respectively (LEITE, *et al.,* 2009). cell internalization of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazoline bromide or MTT (M5655, Sigma-Aldrich St. Louis, MO, USA), which results in the dissolution of the crystals.

2. 3 Evaluation of reactive oxygen species by DCF

The formation of DCFH from the action of intracellular esterases was assessed after the entry of A 2',7'-Dichlorofluorescein or DCF-DA (D6883, Sigma Aldrich, St. Louis, USA), by passive diffusion (WAN *et al.,* 2015). The cells were seeded in black 96-well plates (density 2x104 cells/well) and treated for 3 hours. In the final 30 minutes of treatment, DCF-DA was incubated at a concentration of 20 µM per well. At the end of 3 hours, the wells were washed with PBS and incubated with 1 mM hydrogen peroxide, which aims to stimulate the oxidation of DCFH to dichlorofluorescein (DCF). Once this was done, the fluorescence was read in kinetic time with readings every 5 minutes for a total time of 1 hour at 37°C (Varioskan Lux, Thermofisher, Singapore) at a wavelength of 485 nm excitation and 520 nm emission. The result was expressed as an arbitrary unit of fluorescence per microgram of protein (UF/mg of protein).

2. 4 Nitric oxide production: NO

The production of nitric oxide was evaluated by Griess assay (SOLIMAN; MAZZIO, 1998). After treatment of the cells, the supernatant was collected and incubated for 10 minutes with the same volume of Griess reagent in a transparent 96-well plate under the cover of light at 25ºC. Then, the absorbance was read at a wavelength of 550 nm in a microplate reader (Varioskan Lux, Thermofisher, Singapore). For the standard curve, sodium nitrite (1 mM NaNO2 1 mM NaNO2) (N1015.01.AG, Synth, São Paulo, Brazil) is used at the following points: 40; 20; 10; 5; 2.5 and 1.25 µM/mL. Data were expressed as nM nitrite/mg protein/h.

2. 5 Determination of glutathione content: GSH

Reduced glutathione content was determined as previously described (BROWNE; ARMSTRONG, 1998) with fluorogenic assay. Briefly, samples were homogenized in sodium phosphate buffer (0.1 M, pH 8.0) containing 5 mM EDTA. Proteins were precipitated with 1.7% acid meta-phosphoric and centrifuged at 1000xg for 10 min at 4°C. The supernatant is incubated with o-phthaldialdehyde (1 mg/mL methanol) at room temperature for 15 min. Fluorescence is measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve is employed using standard GSH solutions (G-6529, Sigma Aldrich, St. Louis, USA) (0-500 µM). Readings were discounted from the blank and the result expressed as µmolGSH/mg protein.

2. 5. 1 Soluble protein content: Lowry

The quantification of soluble proteins used in cell culture was quantified according to the Lowry method (PETERSON, 1979). Bovine albumin (1 mg/mL) (A4503, Sigma Aldrich, St. Louis, USA) were used as standard at concentrations of 0; 2; 5; 10; 15; 20; 25; 30; 35 and 40 mL/mL. The amount of protein was expressed in µg/µL.

2.6 SOD activity

The SOD activity of the previously treated Caco-2 cells was evaluated using the Cayman Chemical kit (*706002) and the instructions according to the manufacturer were followed. The intracellular content of SOD was obtained by lysis each well (24 well plates) in 300 µl of kit indicated solution.

2. 7 CAT activity

Measure the CAT activities in each sample with Cayman Chemical kit (*707002). The assay principle is based on the reaction of catalase to decompose hydrogen peroxide (H_2O_2). The excess H_2O_2 can form complexation with ammonium molybdate to produce light yellow solution. The solution's adsorption at 450 nm can be used to calculate the concentration of H_2O_2 and therefore to indirectly indicate the CAT activities.

2. 8 Antimicrobial activity

The antimicrobial activity of the sunflower fraction SPHf was analyzed against the following bacteria *Salmonella Enteretidis* ATCC 13076; *Escherichia coli* ATCC 25922; *Bacillus Cereus* ATCC 14575 and *Staphylococcus aureus* ATCC 1901. The bacteria were incubated at 37ºC for 24h in brain heart infusion (BHI) culture medium. For the analyses, the cell suspensions were centrifuged, and the pellet was washed three times with saline solution (0.85%). Subsequently, the suspensions were diluted to 10^5 CFU/mL using BHI medium. The value for the lowest inhibitory concentration (MIC) was calculated in triplicate using 96-well cell culture microplates, where each well contained 100 µL of culture medium previously inoculated with each bacterium. Based on the quantity of samples available, dilutions of 30 mg/mL (initial test) and 140 mg/mL (control sample only) were prepared. Serial dilutions were then prepared and 100 µL of each were transferred to each well. The plates were incubated at 37°C for 24h. The MIC was defined as the lowest concentration of each sample capable of inhibiting microbial growth and was expressed as mg/mL.

2. 9 LC/MS/MS analysis (Nano-coupled liquid chromatography QExactive mass)

In this study, the SPHf sample was analyzed. SPHf was resuspended in water with 0.1% formic acid and its protein content quantified using the Qubit Protein Assay method. Chromatography was then carried out on a PicoChip source (model 1PCH-550; ReproSil Pur C18 silica matrix of 75 µm and 3 µm; New Objective, USA) at a continuous flow rate of 0.300 µl/min. 1 g of sample was injected into an Acclaim PepMap 100 trap (75 µm ID, C18 3 µm; Thermo Fisher Scientific). Sample separation took place in a gradient of 2-40% mobile phase B for 120 minutes, followed by 10 minutes in 80% mobile phase B and re-equilibration of the column for 10 minutes in 2% mobile phase B. The mobile phases of the chromatography

included: mobile phase A water/0.1% formic acid and phase B acetonitrile/0.1% formic acid. The mass spectra were acquired on a QExactive mass spectrometer (Thermo Fisher Scientific) using the DDA method (data-dependent acquisition, Full MS/MS) with selection of the 10 best counts. The search for the precursor ion was carried out at 300-1,750 m/z with a resolution of 70,000. An isolation window of 2 m/z was selected, with collision energy NCE 15 and 30, followed by MS/MS acquisition at 17,500 resolutions. The automatic gain control (AGC) target was 1 and 4 and the maximum injection time was 100 ms. Loads 1 and greater than 5 were excluded. A dynamic exclusion time of 30 s was used. The samples were analyzed in duplicate. The spectra were analyzed with PatternLab for Proteomics (CARVALHO *et al.,* 2015) using the *Helianthus annuus* database from the UNIPROT database (https://www.uniprot.org). The search for peptide sequences was carried out using the NOVOR software (MA, 2015) (available at https://novor.cloud/), using predefined definitions and the Helianthus annuus data base as a reference. The percentage of amino acids in the N- and C-terminal regions, molecular size, isoelectric pH, number, and sequence of AA were analyzed using the Peptide property calculator software (https://pepcalc.com/). The percentage of hydrophobicity, acidic, basic, and neutral AA were analyzed using the Peptide 2.0 software (https://www.peptide2.com/N_peptide_hydrophobicity_hydrophilicity.php).

2.10 Statistical analyses

Statistical analysis was performed using SPSS 16.0 software (SPSS Inc., Chicago IL). An ANOVA was performed followed by Duncan's post-test to assess differences between groups. The mean ± standard error was used to represent the data from each in vitro experiment, which was repeated at least five times. Statistical significance was determined with $p < 0.05$.

3 Results and discussion

3. 1 Protein hydrolysis

In preliminary studies by our research group (manuscript submitted under evaluation), a protein isolate was obtained with 92% protein and with a 95% reduction in its phenolic content (488 to 26 mg of gallic acid/100g). In addition, was obtained a hydrolyzed fraction with alcalase, ultrafiltrate with <5 kDa membrane,

forming the sunflower protein hydrolysate fraction (SPHf). A degree of hydrolysis (DH) of 30.3 ± 2.6 % was recorded.

3. 2 Treatment with protein hydrolysates on Caco-2 cells

The antioxidant capacity of sunflower peptides with low phenolic content, was evaluated in Caco-2 cell cultures under oxidative stimulation with hydrogen peroxide (H_2O_2).

To assess the best treatment concentration for the sunflower peptide and avoid cytotoxicity, SPHf was tested at 0.15, 0.3 and 0.6 mg/mL with and without peroxide stimulation. Figure 1A shows the cell viability test with MTT, showing a trend towards reduced cell viability at the highest concentrations (0.3 and 0.6 mg/mL) in the treatments with the oxidative stimulus. Therefore, the dose of 0.15 mg/mL was used for the next analyses. Figure 1B shows that stimulation with H_2O_2 (1 mM) induced an increase in the production of reactive oxygen species (ROS). However, this induction was avoided in the treatment with SPHf and H_2O_2 (1 mM). This result indicated that SPHf had an antioxidant action on oxidized Caco-2 cells. It is believed that SPHf may have acted in parallel in the donation of electrons to free radicals and protection of antioxidant engogenous molecules to the cell (Figure 2A and C). (CORROCHANO *et al.,* 2019; TONOLO *et al.,* 2024).

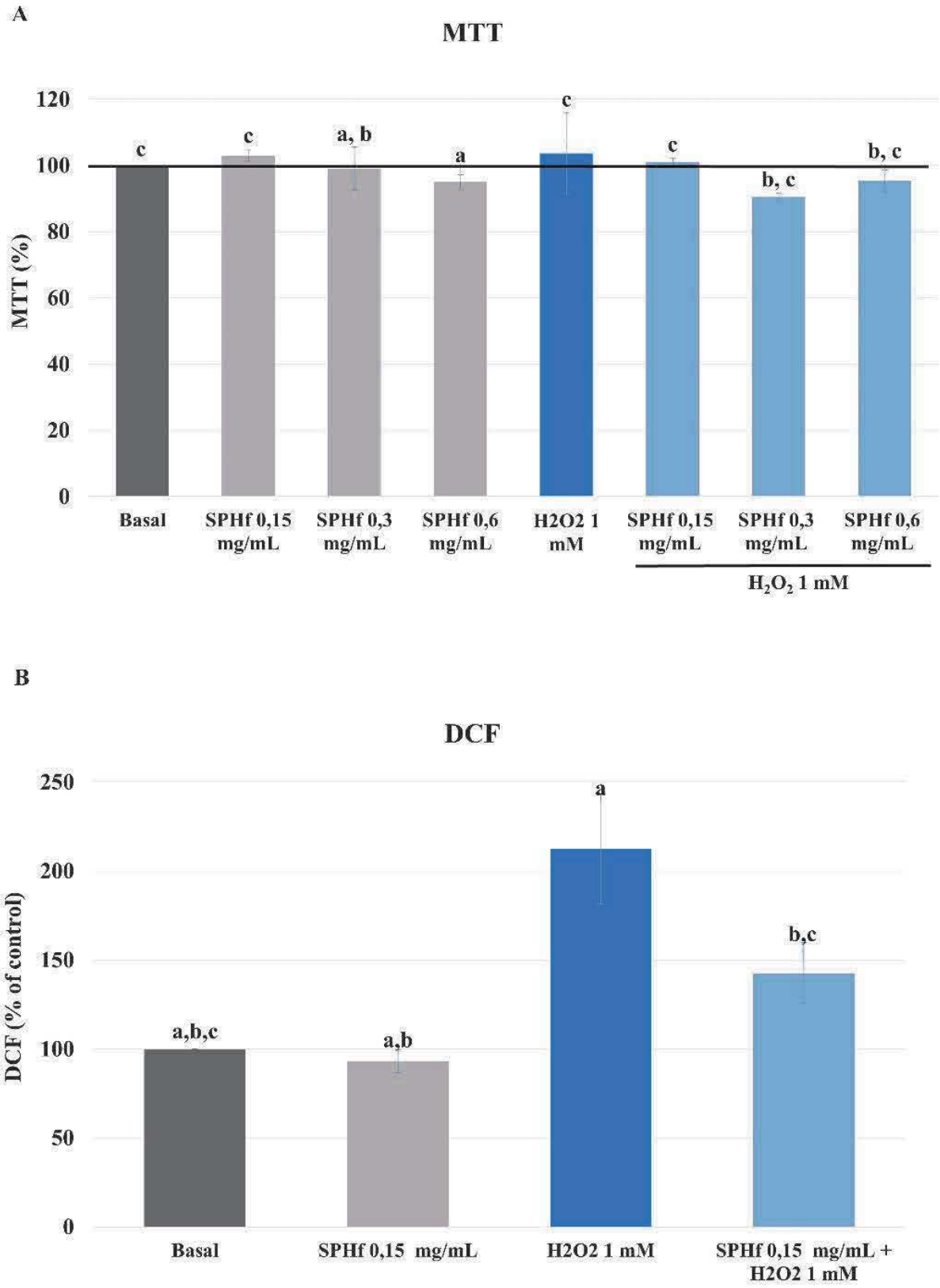

Figure 1. SPHf prevents ROS production in Caco-2 cells stimulated with peroxide. A: The viability of the cells was tested with different concentration of SPHF with and without H2O2 for 3 h. B: The lowest concentration of SPHf attenuate the production of ROS stimulated by peroxide in Caco-2 cells.

3. 2 Activity of antioxidant endogen enzymes

Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are endogenous enzymes that represent the first line of cellular antioxidant defense, especially in relation to the superoxide anion radical (O_2^-). SPHf was able to modulate these compounds as observed in Figure 2. Sunflower hydrolysate was able to reverse the increase in GSH content and CAT activity induced by the H_2O_2 stimulus (Fig 2A and C). Although not significant, the same profile was observed with SOD activity. An increase in the content and activity of antioxidant compounds induced by H_2O_2 can be interpreted as an attempt by the cell to combat the oxidative stress generated by the oxidant. However, in the presence of sunflower hydrolysates, this increase was prevented by the effect of the peptides themselves and their ability to donate electrons to free radicals. No change was observed in the secretion of nitric oxide by Caco-2 cells (Fig 2D).

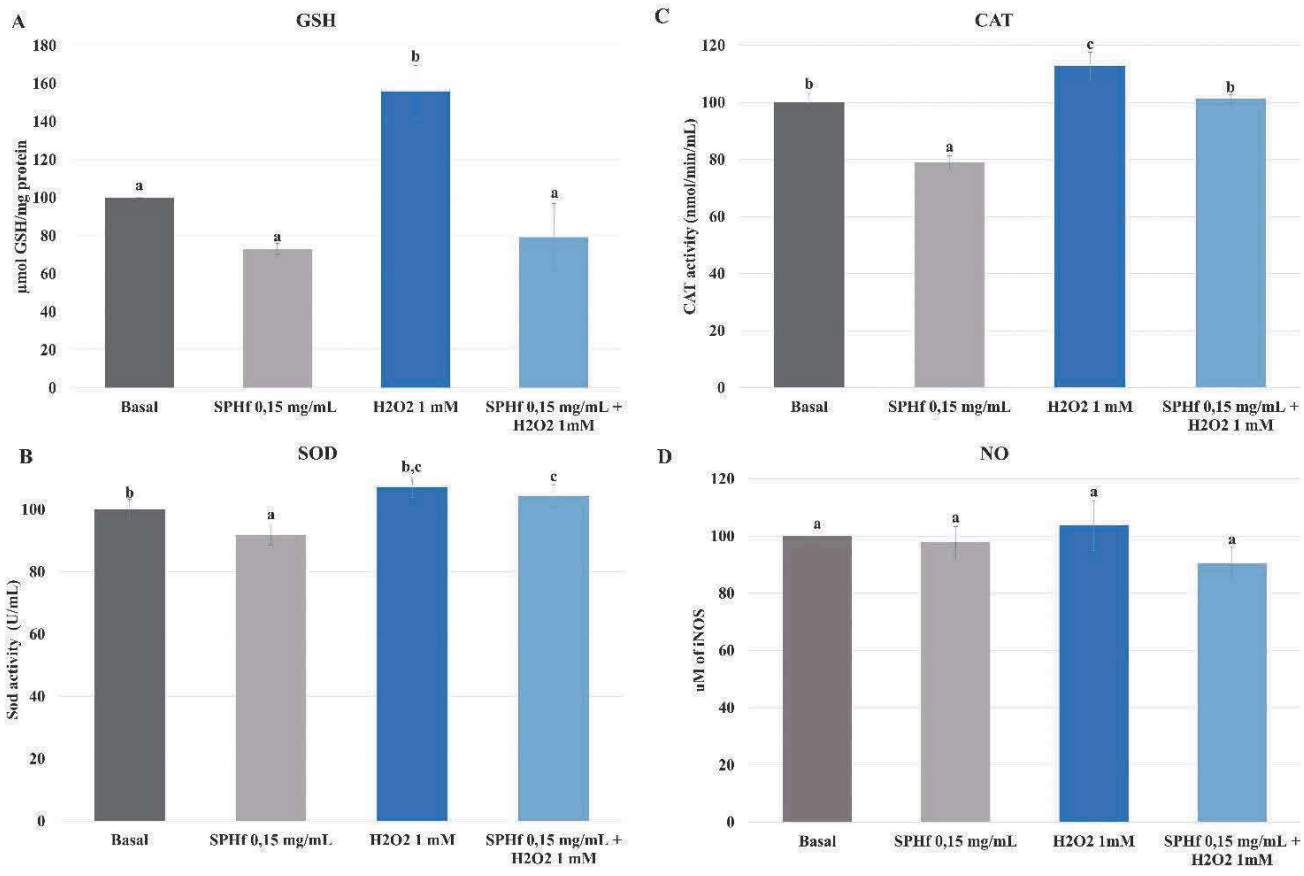

Figure. 2. SPHf modulate GSH and CAT increase after peroxide stimulus. A: GSH content; B: SOD activity, C: CAT activity; D: NO secretion after treatment in 3h with SPHf and hydrogen peroxide

In the literature, there are several studies that evaluated antioxidant activity of sunflower protein hydrolysates using chemical models (ABTS, DPPH and ORAC). In the study by Tonolo *et al.* (2024), sunflower protein hydrolysates obtained by (simulated) gastrointestinal hydrolysis were able to reduce the levels of NF-κB (pro-inflammatory gene) in the cytosol of Caco-2 cells. In addition, inhibition of NRF2 (signaling molecule for the antioxidant response) was also not observed. Finally, antioxidant activity by molecules endogenous to the cell (GSH, SOD and CAT) was not verified. However, none of them showed bioactivity in Caco-2 cells. Furthermore, few of these analyses reduced the phenolic content in protein hydrolysates which may contribute for antioxidant action (DABBOUR *et al.,* 2019; HABINSHUTI *et al.,* 2019; PRADO *et al.,* 2020; BISINOTTO *et al.,* 2023). Therefore, our study is the first to evaluate the antioxidant bioactivity in Caco-2 cells, with reduced phenolic content, demonstrating that peptide can act as antioxidant agent by itself, with now phenolic intereference. Moreover, we pointed out some mechanism of action of peptides showing modulated action in important antioxidant enzymes of Caco-2 metabolism.

3. 3 Peptide identification with mass spectrometry

A total of 189 peptide sequences from the SPHf fraction were identified (Table 1). It was observed that the sequences identified had an average number of peptides per fragment of 10 AA, as well as a molecular weight ranging from 500 - 2000 Da. Lower peptide weigh has been associated with better bioactivity than higher. In a study with residual material of olive oil, showed that alcalase hydrolyzed peptides with low molecular weight peptides presented better antioxidant action than long chain peptides (ESTEVE *et al.,* 2015).

The antioxidant potential of the peptide may be associated not only with its low molecular weight, but also with its amino acid composition and sequence. Half of the identified peptides presented hydrophobicity greater than 50%. A possible explanation is the increase exposure of internal hydrophobic amino acids during hydrolysis. High proportion of hydrophobic amino acids has been reported in peptides with high antioxidant activity. The hydrophobicity of amino acids may favor antioxidant protections is mainly related to the chemical structure: presence of aromatic rings which serve as scavengers of hydroxyl radicals (ZOU *et al.,* 2016) or as hydrogen atom and electron donors (TAHA *et al.,* 2023; PRADO *et al.,* 2020).

Furthermore, hydrophobic peptides tend to have better penetration of cell plasma membranes.

The properties of amino acids present in the N- and C-terminal regions of the peptides may also be related with antioxidant predictability. A quantitative structure-activity relationship (QSAR) bioinformatic modeling showed that the electronic donating ability of amino acids in the C and N-terminal regions significant influence the antioxidant activity, such as acidic (Glu and Asp) and hydrophobic (Ala, Val, Met, Phe, Leu, Ile, Pro, Trp) amino acids (LI; LI, 2013; ZOU *et al.,* 2016).

Figure 3 show the percentage of amino acid composition in terminal regions. AAs with an incidence above 15 per region were considered to have a high prevalence. In C-terminal regions, there was a prevalence of: Glu (acidic), Ala and Leu (hydrophobic), Gln (neutral) and phe (aromatic). As for the N-terminal region, a high incidence of: Gly, Leu and Val (hydrophobic); Asn and Ser (polar) and Arg (basic) was observed. The presence of aromatic and hydrophobic amino acids in C-terminal regions were associated with metal chelating capacity (CANABADY-ROCHELLE *et al.,* 2015). Val or Leu at the N-terminus favor the interaction of peptides with the cell plasma membrane and protection against the effect of oxidative stress on mammalian epithelial cells, respectively (LI *et al.,* 2011). The presence of the amino acids Asp, Glu, Gly, Lys, and Val in the terminal regions of the amino acid may also be associated with a reduction in the formation of free radicals from the oxidation of fatty acids present in the plasma membrane (TONOLO *et al.,* 2024). In addition, due to the aromatic rings present in their structures, these AA serve as proton donors to free radicals from oxidative reactions arising from enzymatic reactions. Another important factor is that these AA promote an interaction with the cell plasma membrane, favoring their antioxidant protective effect on the cell (ZOU *et al.,* 2016). Furthermore, the amino acid Leu can be transaminated to glutamate and can serve as a substrate for GSH synthesis. Therefore, the incidence of Pro, Val and Leu in the terminal regions of the peptide may favor a reduction in the accumulation of ROS in intestinal cells by preserving the content of GSH (tripeptide composed of glutamate) and the activity of SOD and CAT (ZHAO *et al.,* 2023; TONOLO *et al.,* 2024). Not only hydrophobic AA, but the incidence of acidic amino acids in the terminal region of the peptide can favor antioxidant activity. Our sequences showed a high incidence of the Glu AA (±12%).

Its presence in the C-terminal region of the peptide favors greater donation of electrons to free radicals, as it has an excess of electrons (ZOU *et al.,* 2016).

Table 1. Sunflower peptide sequences identified.

Peptide sequence Identified	Nº AA	Molecular weight (Da)	Iso-electric point (pH)	Hydrophobicity (%)	Acid (%)	Basic (%)	Neutral (%)
SRGDIFPVPQ	10	1115.24	6,38	50	10	10	30
AEKGHLQPN	9	993.08	7,86	34	11	22	33
DNVYAGF	7	784.81	0,76	43	14	0	43
ENIDNPSHA[1]	9	995.99	3,92	34	22	11	33
ENIDNPSHADFVNPQ[1]	15	1696.73	3,54	40	20	7	33
LTRDNVYAGF	10	1155.26	6,59	40	10	10	40
NRAPLKSPL	9	995.18	11,39	56	0	22	22
RDNVYAGF	8	941	6,34	38	12,5	12,5	37
TNRAPLKSPL	10	1096.28	11,39	50	0	20	30
TRDNVYAGF	9	1042.1	6,32	34	11	11	44
VVLAYEPVWAIGTGK	15	1602.87	6,77	60	6	6	28
AIIVDDVNNPANQL	14	1495.63	0.54	57	14	0	29
APRLGSPE	8	825.91	6.93	50	12,5	12,5	25
DDVNNPANQL[2]	10	1099.11	0,54	40	20	0	40
DVNNPANQL[2]	9	984.02	0,69	44	12	0	44
DNQREADVF	9	1093.1	3,54	33	33	12	22
EHKLPILSLMDL	12	1408.71	5,17	58	17	17	8
EQVSRGDIFPVPQFF	15	1765.96	3,93	53	13	7	27
LLPSYTNTPILF	12	1378.61	3,64	58	0	0	42
IERGRGIQ	8	928.05	10,69	25	12,5	25	37,5
KGYLQPN	7	818.92	9,55	29	0	14	57
FVTPDEGQEQM[3]	11	1280.36	0,66	36	28	0	36
FVTPEEEQQQM[3]	11	1365.47	0,76	36	28	0	36

FVTPEEEQQQMH[3]	12	1502.61	3,84	33	25	9	33
GDIFPVPQ[4]	8	871.98	0,76	62,5	12,5	0	25
GDIFPVPQFF[4]	10	1166.32	0,76	70	10	0	20
GDIFPVPQFFA[4]	11	1237.4	0,69	73	9	0	18
GDIFPVPQFFAA[4]	12	1308.48	0,69	75	8	0	17
GERGMDSSADSH	12	1248.24	4,16	17	25	16	42
FLPSFQPFPRLL	12	1461.75	10,59	75	0	8	17
SREDAQKV	8	931.99	6,66	25	25	25	25
FREGDIIAIPA	11	1201.37	3,9364	64	18	9	9
HEQQPR	6	793.83	7,57	17	17	33	33
HKLPILSLMDL	11	1279.59	7,56	64	9	18	9
IFPVPQ	6	699.84	3,71	83	0	0	17
IIVDDVNNPA[5]	10	1069.17	0,54	60	20	0	20
IIVDDVNNPAN[5]	11	1183.27	0,62	54	18	0	28
IIVDDVNNPANQL[5]	13	1424.56	0,54	54	15	0	31
KLPILSLMDL	10	1142.45	6,43	70	10	10	10
MEFVTPEEEQQQM[6]	13	1625.78	0,69	38	31	0	31
MEFVTPEEEQQQMH[6]	14	1762.92	3,67	36	29	7	28
NEQVSRGDIFPVPQ[7]	14	1585.72	3,93	43	14	7	36
NEQVSRGDIFPVPQFF[7]	16	1880.06	3,93	50	12,5	6,25	31,25
QVSRGDIFPVPQ[8]	12	1342.5	6,35	50	8	8	34
QVSRGDIFPVPQFF[8]	14	1636.85	6,35	58	7	7	28
RFREGDIIAIPA	12	1357.56	6,64	58	17	17	8
RGDIFPVPQFF[9]	11	1322.51	6,35	64	9	9	18
RGDIFPVPQFFAA[9]	13	1464.66	6,35	69	8	8	15
RGDIFPVPQFFAAT[9]	14	1565.77	6,35	64	7	7	22

RGDIFPVPQFFAT[9]	13	1494.69	6,35	61	7	7	25
PSHADFVNPQ	10	1111.16	4,87	50	10	10	30
QIIRPPQAR	9	1078.27	12,1	56	0	22	22
FREGDMIIIPA	11	1261.49	3,93	64	18	9	9
RLPFDEDRHQ	10	1312.39	5,23	30	30	30	10
HSVSVPGDL	9	909.98	4,87	44	12	12	32
GRPQQQF	7	859.93	10,84	28	0	14	58
IERGRGIQ	8	928.05	10,69	25	12,5	25	37,5
AIQSPHWTIN	10	1166.29	7,88	50	0	10	40
DVSRPDFFNPE	11	1322.38	3,54	45	27	10	18
SADRGELRPN	10	1114.17	6,67	30	20	20	30
ALEPIEVIQA	10	1082.25	0,76	70	20	0	10
FAPSFSRGQ	9	996.08	10,59	44	0	12	44
GGRAPGRGW	9	913	12,1	34	0	22	44
LLPSYVNTPILAF	13	1447.72	3,64	70	0	0	30
ALEPNERVEAE	11	1256.32	3,67	46	36	9	9
NALEPIERVQ	10	1168.3	4,15	60	10	10	20
QEGWDNILR	9	1130.21	3,93	33	22	11	34
RQGDVV	6	672.73	6,35	33	17	17	33
TEQFEGR	7	865.89	4,15	14	28	14	44
WKAALDEALANAP	13	1369.52	3,93	69	15	8	8
SYPTLPGWIPSPF	13	1461.66	3,42	62	0	0	38
WLPSPFFPI	9	1103.31	3,54	89	0	0	11
RQGDIIAIPA	10	1053.21	6,35	60	10	10	20
SLFLPSFQSYPRLL	14	1667.94	9,57	57	0	7	36
TSQPNQRLE	9	1072.13	6,55	22	11	11	56

VDDVNNPANQL[10]	11	1198.24	0,54	46	18	0	36
VIVDDVNNPA[10]	10	1055.14	0,54	60	20	0	20
VIVDDVNNPANQL[10]	13	1410.53	0,54	54	16	0	30
VSRGDIFPVPQ[11]	11	1214.37	6,61	54	9	9	28
VSRGDIFPVPQF[11]	12	1361.54	6,61	58	8	8	26
VSRGDIFPVPQFF[11]	13	1508.72	6,61	61	7	7	25
VSRGDIFPVPQFFA[11]	14	1579.79	6,61	64	7	7	22
VSRGDIFPVPQFFA[11]	15	1579.79	6,61	64	7	7	22
VSRGDIFPVPQFFAA[11]	15	1650.87	6,61	66	7	7	20
VTPEEEQQQMH	11	1355.43	3,84	27	27	10	36
VVAIIVDDVNNPANQL	16	1693.9	0,54	62,5	12,5	0	25
YTSQPNQRLE	10	1235.3	6,58	20	10	10	60
AERGELRPN	9	1041.12	7,08	34	22	22	22
GERLPFDED[12]	9	1077.1	3,43	34	44	11	11
GERLPFDEDRHQ[12]	12	1498.56	4,42	25	33	25	17
HADFVNPQ	8	926.97	4,87	50	12,5	12,5	25
IDNPSHA[13]	7	752.77	4,87	44	14	14	28
IDNPSHAD[13]	8	867.86	3,71	37,5	25	12,5	25
IDNPSHADFVNPQ[13]	13	1453.51	3,71	46	15	8	31
IIRPPQ	6	722.88	10,85	66	0	17	17
IIRPPQAR	8	950.14	12,1	62,5	0	25	12,5
NIDNPSHADFVNPQ	14	1567.61	3,71	43	14	7	36
NNENQLDEY	9	1138.1	0,62	11	0	33	56
DIPWPF	6	773.87	0,76	83	17	0	0
DMPFDIPWPFRPS	13	1604.83	3,71	69	15	8	8
EIPFDMPFDIPWPFRPS[14]	17	2091.39	3,54	70	18	6	6

FDIPWPFRPS[14]	10	1261.43	6,4	70	10	10	10
FDMPFDIPWPFRPS[14]	14	1752	3,71	72	14	7	7
MPFDIPWPFRPS[14]	12	1489.74	6,39	76	8	8	8
PFDIPWPF[14]	8	1018.16	0,76	87,5	12,5	0	0
PFDIPWPFRPS	11	1358.54	7,08	73	9	9	9
FEIPFDMPF	9	1142.32	0,7	78	22	0	0
GGRRGGGEGNQDRHQ	15	1580.58	10,59	0	13	27	60
GNSVFDNELRE[15]	11	1279.31	3,69	27	27	10	36
GNSVFDNELREG[15]	12	1336.36	3,69	25	25	8	42
GVDFIRH	7	842.94	7,81	44	14	28	14
HNDGNTEL[16]	8	898.87	3,92	12,5	25	12,5	50
HNDGNTELVVV[16]	11	1196.27	3,92	36	18	10	36
SFKFPILE[17]	8	980.16	6,59	62,5	12,5	12,5	12,5
SFKFPILEHL[17]	10	1230.45	7,54	60	10	20	10
SVFDNELRE[18]	9	1108.16	3,69	33	33	12	22
SVFDNELREG[18]	10	1165.21	3,69	30	30	10	30
IQSPHWTIN	9	1095.21	7,82	44	0	12	44
IVRPPQDR	8	980.12	10,69	50	12,5	25	12,5
KEGDVVAIPT	10	1028.16	3,93	50	20	10	20
KTNDNAMIA	9	977.1	6,43	44	11	11	34
NFAVIK	8	690.83	9,7	66	0	17	17
INDVSRPDFFNPE	13	1549.64	3,54	43	23,5	10	23,5
RALPVDVL	8	882.06	6,35	75	12,5	12,5	0
RAGEQGSRWVSF	12	1379.48	10,39	33	8	17	42
NIDNPSHA	8	866.88	4,87	38	12,5	12,5	37
RLPFDEDRHQKVE[24]	13	1668.81	5,34	31	31	31	7

AGEDKGRLWPF	11	1275.41	6,97	46	18	18	18
DFPDLRDINVAF	12	1421.55	3,41	58	26	8	8
QVVRPPIRIQ	10	1205.45	12,1	60	0	20	20
RGFQDR[25]	6	777.83	10,39	17	17	33	33
RGFQDRHQKIR[25]	11	1440.61	11,84	18	9	46	27
RGLQVVRPPIRIQ	13	1531.85	12,4	54	0	23	23
VERGLQVVRPPIRIQ	15	1760.09	11,8	53	7	20	20
RIQPGGLLLPSYVNTPIL	18	1951.31	9,55	56	0	6	38
ALEPNERVE	9	1056.13	3,85	44	33	11	12
RIQPGGL	7	739.86	10,55	43	0	15	42
RQGDVVAIPT	10	1055.19	6,35	50	10	10	30
NHENQLDEN[19]	9	1112.07	3,69	11	33	12	44
NHENQLDENQRRFF[19]	14	1846.91	5,3	21	21	22	36
NIEALEPIEVI	11	1239.41	0,71	64	27	0	9
NLNSFKFPIL[20]	10	1192.41	9,7	60	0	10	30
NLNSFKFPILE[20]	11	1321.52	6,44	54	9	9	28
NLNSFKFPILE[20]	11	1321.53	6,44	54	9	9	28
NSFKFPIL[20]	8	965.14	9,7	62,5	0	12,5	25
NSFKFPILE[20]	9	1094.26	6,44	55	12	11	22
QIVRPPQDR	9	1108.25	10,39	44	12	22	22
GQVVVIPQNFAVIK	14	1511.81	10,12	64	0	8	28
SMPVDVVAN	9	931.07	0,78	67	11	0	22
VVVLPQ	6	653.81	3,67	83	0	0	17
QSPDDTRGH	9	1011.99	5,04	12	22	22	44
REGDMIIIPA[21]	10	1114.32	3,93	60	20	10	10
REGDMIIIPAGA[21]	12	1242.45	3,93	58	17	8	17

RSGQETPEEGSGN	13	1347.3	3,85	8	23	8	61
SFKLPILQ	8	945.16	9,86	62,5	0	12,5	25
NDGQEEIVAI	10	1087.14	0,61	40	30	0	30
KDDDLKAY	8	967.03	4,01	25	37,5	25	12,5
SEDKADFR	8	966.99	4,17	25	37,5	25	12,5
GDPAQGDPTQGK	12	1170.19	3,71	25	17	8	50
GLLLPYYPNTPELVY	15	1752.01	0,95	53	7	0	40
LLPYYPNTPE	10	1206.34	1	50	10	0	40
TGRHGQSQRPGWE	13	1495.56	10,38	15	8	23	54
YTNTPILFF	9	1115.28	3,41	56	0	0	44
GQSQRPGWE	9	1044.08	6,86	22	11	11	56
NSHKLPVLE	9	1036.18	7,39	44	16	16	24
QRQPQSPR[22]	8	996.08	12,1	25	0	25	50
QRQPQSPRLS[22]	10	1196.32	12,1	30	0	20	50
QSQRPGWE	8	987.03	6,58	25	12,5	12,5	50
REEQEWE	7	1005	3,67	14	58	14	14
RSGQTQRPSWE	11	1331.39	10,39	20,5	9	20,5	50
SPGSGSISPIK	11	1029.15	9,86	38	0	8	54
SQRPGWESQRPGWETG	16	1857.94	6,75	25	12,5	12,5	50
TGRPGQSQRPSWE	13	1485.56	10,38	23	8	15	54
TQRPSWE	7	902.95	6,55	29	14	14	43
NIDDPSNADLYNPQ	14	1575.59	0,53	36	21	0	43
NNGQDELVII	10	1114.21	0,66	40	20	0	40
TDRHQKIH	8	1034.13	9,81	12,5	12,5	50	25
GLLLPSYTNTPILF[23]	14	1548.82	3,64	57	0	0	43
GLLLPSYTNTPILFY[23]	15	1711.99	3,57	53	0	0	47

GWDNILRGF	9	1077.19	6,64	44	11	11	34
RGENDQRGHIIF	12	1441.55	7,52	25	17	25	33
GQETPEEGSGN	11	1104.04	0,76	9	27	0	64
NEGDVVAIPT	10	1014.09	0,69	50	20	0	30
RIQPGGLLLPSYVNTPILAF[24]	20	2169.56	9,55	60	0	5	35
ADRGELRPN	9	1027.09	7	34	22	22	22
FFDSGDQQFQ	10	1218.23	0,61	30	20	0	50
KLPLLQ	6	710.9	9,91	66	0	17	17
VVRPPIRIQ	9	1077.32	12,1	67	0	22	11
YGPGGGGGGGRK	12	1019.07	10,41	67	0	22	11

Source: Lopes *et al.* (2024) Adapted. A = Ala; C = Cys; D = Asp; E = Glu; F = Phe; G = Gly; H = His; I = Ile; K = Lys; L = Leu; M = Met; N = Asn; P = Pro; Q = Gln; R = Arg; S = Ser; T = Tre; V = Val; W = Thr; Y = Tyr. Subscript numbers represent homologous peptides sequences.

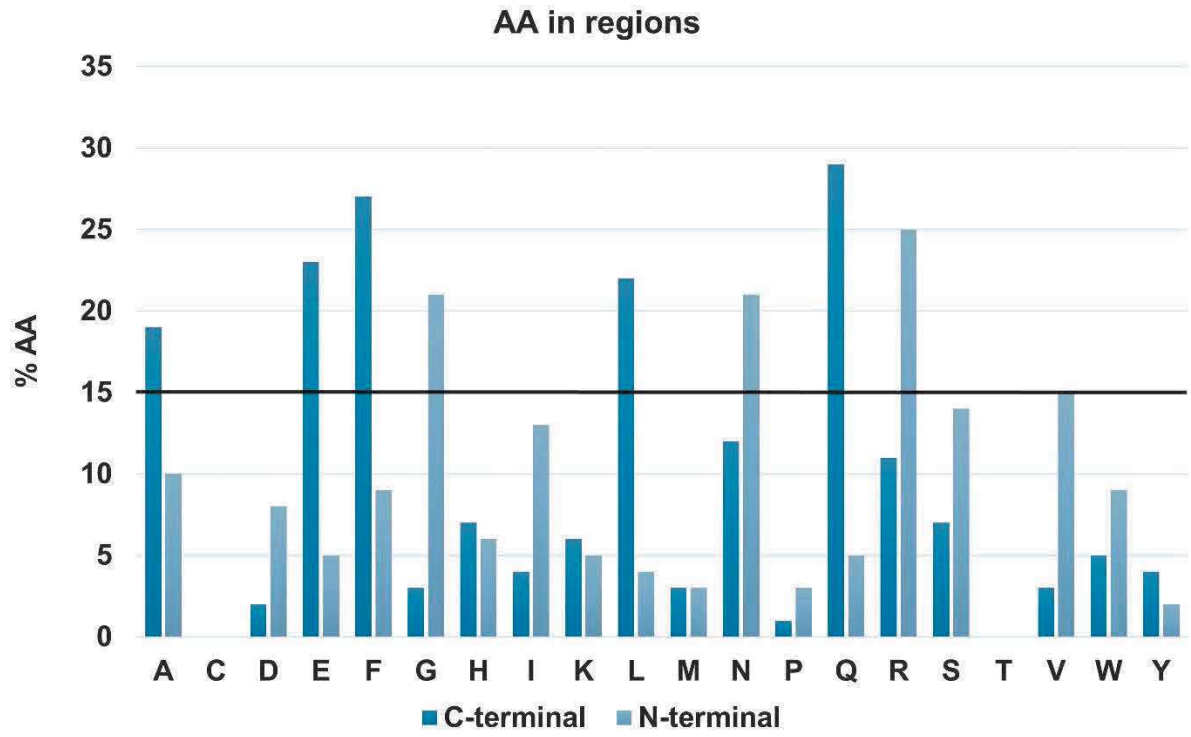

Figure 3. Composition AA% in terminal regions. Abbreviations includes: AA: amino acid; A = Ala; C = Cys; D = Asp; E = Glu; F = Phe; G = Gly; H = His; I = Ile; K = Lys; L = Leu; M = Met; N = Asn; P = Pro; Q = Gln; R = Arg; S = Ser; T = Thr; V = Val; W = Tpr; Y = Tyr.

3. 3 Potential antimicrobial action

In terms of antimicrobial activity, no concentration of SPHf was able to inhibit the growth of the microorganisms evaluated. We did not find a minimum concentration of sunflower peptide with antimicrobial activity against pathogenic microorganisms.

In opposite with our results, the literature presents studies attesting to the antimicrobial activity of sunflower protein hydrolysates (HABINSHUTI *et al.,* 2019; SALGADO *et al.,* 2013), but most of them did not remove phenolic content from the sample. Therefore, our negative results may be related to the lack of phenolic compounds in the sample. In fact, in studies evaluating the action of sunflower phenolic strata, the bactericidal/bacteriostatic effect was observed (ALEXANDRINO *et al.,* 2021; SUBASHINI *et al.,* 2012; MENZEL *et al.,* 2019). Nevertheless, the study by Taha *et al,* (2013) verified that sunflower protein hydrolysates with reduced phenolic content presented antimicrobial activity in Bacillus cereus over four other

pathological bacteria tested. This result may suggest that sunflower peptides, when isolated from phenolic compounds, may have a weaker effect than with both compounds together.

4 Conclusion

Regarding Caco-2 cell cultures under oxidative stimulation with hydrogen peroxide (H_2O_2): the concentration of 0,15 mg/mL of SPHf was not considered toxic and prevent ROS production in Caco-2 cells. Sunflower hydrolysate was able to reverse the increase in GSH content and CAT activity induced by the stimulus of H_2O_2. Although not significant, the same profile was observed with SOD activity. No change was observed in the secretion of nitric oxide. SPHf was unable to inhibit the microbial growth of pathogens. It is believed that this is related to the absence of phenolic compounds in the sample. A total of 189 peptide sequences from the SPHf fraction were identified. The identified sequences had an average number of 10 amino acid per peptide, as well as a low molecular weight ranging from 500 - 2000 Da. Half of peptide presented hydrophobic property. Furthermore, the analysis in the C and N-terminal regions showed higher incidence of hydrophobic and acid amino acids. These structure characteristics may influence the capacity of compounds to act as an electron donor to antioxidant bioactivity. This study provides new insight about sunflower peptides mechanism of action in enterocyte cells, and associate important structure relation properties. Further studies in this area may contribute to better understand the potential application of sunflower peptide in functional food.

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Declaration of Competing Interest

The authors declare there was no conflict of interest to declare.

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a59OtcBh6gBHLGolj4bW99nEGO4Q3YnTWt0ygUwvkWMPNAftWwUnAhbYhFnu uDqBZbugriWoCMkL62PsOOU5wNhVskBRUNzjIvJNPx~ztuwYIBYIeSTOLg__&K ey-Pair-Id=APKAJLOHF5GGSLRBV4ZA.

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CONCLUSÕES

A atividade antioxidante da fração SPHf foi mantida após digestão simulada. Dos 196 peptídeos identificados, 13 apresentaram propriedades antioxidantes previstas utilizando a ferramenta de análise de aprendizagem profunda. Além disso, vários peptídeos continham di- e tri-peptídeos com atividade antioxidante na sua estrutura, com referências cruzadas em bases de dados alimentares. A atividade antioxidante e antimicrobiana dos hidrolisados foi avaliada em linhagem de células Caco-2 estimuladas por peróxido: avaliação da viabilidade celular, produção de espécies reativas de oxigénio, produção de óxido nítrico, teor de glutationa e atividade das enzimas superóxido dismutase e catalase. O SPHf não interferiu na viabilidade celular e protegeu/preveniu as células estimuladas com H_2O_2. O hidrolisado de girassol foi capaz de reverter o aumento do teor de GSH e da atividade da CAT induzido pelo estímulo de H_2O_2. Embora não significativo, o mesmo perfil foi observado com a atividade da SOD. Em ralação as sequências totais da fração SPHf identificadas, houve um número médio de 10 aminoácidos por peptídeo, bem como um baixo peso molecular variando de 500 - 2000 Da. Metade dos peptídeos identificados apresentaram propriedades hidrofóbica. Além disso, a análise nas regiões C e N-terminal mostrou maior incidência de aminoácidos hidrofóbicos e ácidos. Essas características estruturais podem influenciar a capacidade dos compostos de atuarem como doadores de elétrons para a bioatividade antioxidante. Em resumo, o efeito como agente antioxidante dos peptídeos bioativos do girassol com baixo teor fenólico foi atestado, demonstrando que podem ser utilizados como ingredientes na produção de alimentos.

ANEXOS

Supplementary material

Table 1. Peptide sequence identified in sunflower protein hydrolysates with alcalase (SPHf) and digested.

Protein Origin	Peptide sequence identified		
11-S globulin (73%)	SRGDIFPVPQ	RQGDIIAIPA	NHENQLDEN[19]
	AEKGHLQPN	SLFLPSFQSYPRLL	NHENQLDENQRRFF[19]
	DNVYAGF	TSQPNQRLE	NIEALEPIEVI
	ENIDNPSHA[1]	VDDVNNPANQL[10]	NLNSFKFPIL[20]
	ENIDNPSHADFVNPQ[1]	VIVDDVNNPA[10]	NLNSFKFPILE[20]
	LTRDNVYAGF	VIVDDVNNPANQL[10]	NLNSFKFPILE[20]
	NRAPLKSPL	VSRGDIFPVPQ[11]	NSFKFPIL[20]
	RDNVYAGF	VSRGDIFPVPQF[11]	NSFKFPILE[20]
	TNRAPLKSPL	VSRGDIFPVPQFF[11]	QIVRPPQDR
	TRDNVYAGF	VSRGDIFPVPQFFA[11]	GQVVVIPQNFAVIK
	VVLAYEPVWAIGTGK	VSRGDIFPVPQFFA[11]	SMPVDVVAN
	AIIVDDVNNPANQL	VSRGDIFPVPQFFAA[11]	VVVLPQ
	APRLGSPE	VTPEEEQQQMH	QSPDDTRGH
	DDVNNPANQL[2]	VVAIIVDDVNNPANQL	REGDMIIIPA[21]
	DVNNPANQL[2]	YTSQPNQRLE	REGDMIIIPAGA[21]
	DNQREADVF	AERGELRPN	RSGQETPEEGSGN
	EHKLPILSLMDL	GERLPFDED[12]	SFKLPILQ
	EQVSRGDIFPVPQFF	GERLPFDEDRHQ[12]	NDGQEEIVAI
	LLPSYTNTPILF	HADFVNPQ	KDDDLKAY
	IERGRGIQ*	IDNPSHA[13]	SEDKADFR
	KGYLQPN	IDNPSHAD[13]	GDPAQGDPTQGK
	FVTPDEGQEQM[3]	IDNPSHADFVNPQ[13]	GLLLPYYPNTPELVY
	FVTPEEEQQQM[3]	IIRPPQ*	LLPYYPNTPE
	FVTPEEEQQQ	IIRPPQAR	TGRHGQSQRPG

MH[3]		WE
GDIFPVPQ[4]	NIDNPSHADFVNPQ	YTNTPILFF
GDIFPVPQFF[4]	NNENQLDEY	GQSQRPGWE
GDIFPVPQFFA[4]	DIPWPF	NSHKLPVLE
GDIFPVPQFFAA[4]	DMPFDIPWPFRPS	QRQPQSPR[22]
GERGMDSSADSH	EIPFDMPFDIPWPFRPS[14]	QRQPQSPRLS[22]
FLPSFQPFPRLL	FDIPWPFRPS[14]	QSQRPGWE
SREDAQKV	FDMPFDIPWPFRPS[14]	REEQEWE
FREGDIIAIPA	MPFDIPWPFRPS[14]	RSGQTQRPSWE
HEQQPR	PFDIPWPF[14]	SPGSGSISPIK
HKLPILSLMDL	PFDIPWPFRPS	SQRPGWESQRPGWETG
IFPVPQ	FEIPFDMPF	TGRPGQSQRPSWE
IIVDDVNNPA5	GGRRGGGEGNQDRHQ	TQRPSWE
IIVDDVNNPAN[5]	GNSVFDNELRE[15]	NIDDPSNADLYNPQ
IIVDDVNNPANQL[5]	GNSVFDNELREG[15]	NNGQDELVII
KLPILSLMDL	GVDFIRH	TDRHQKIH
MEFVTPEEEQQQM[6]	HNDGNTEL[16]	GLLLPSYTNTPILF[23]
MEFVTPEEEQQQMH[6]	HNDGNTELVVV[16]	GLLLPSYTNTPILFY[23]
NEQVSRGDIFPVPQ[7]	SFKFPILE[17]	GWDNILRGF
NEQVSRGDIFPVPQFF[7]	SFKFPILEHL[17]	RGENDQRGHIIF
QVSRGDIFPVPQ[8]	SVFDNELRE[18]	
QVSRGDIFPVPQFF[8]	SVFDNELREG[18]	
RFREGDIIAIPA	IQSPHWTIN	
RGDIFPVPQFF[9]	IVRPPQDR	
RGDIFPVPQFFAA[9]	KEGDVVAIPT	
RGDIFPVPQFFAAT[9]	KTNDNAMIA	
RGDIFPVPQFFAT[9]	NFAVIK	

Putative rmlC-like (3%)	PSHADFVNPQ	INDVSRPDFFNPE	GQETPEEGSGN
	QIIRPPQAR	RALPVDVL	
	FREGDMIIIPA	RAGEQGSRWVSF	
Lipid Transfer Protein (LTP) (5%)	RLPFDEDRHQ	NIDNPSHA	NEGDVVAIPT
	HSVSVPGDL	RLPFDEDRHQKVE[24]	RIQPGGLLLPSY VNTPILAF[24]
	GRPQQQF	AGEDKGRLWPF	
	IERGRGIQ	DFPDLRDINVAF	
Putative glutelin type-B 1 (10%)	AIQSPHWTIN	QVVRPPIRIQ	ADRGELRPN
	DVSRPDFFNPE	RGFQDR[25]	FFDSGDQQFQ
	SADRGELRPN	RGFQDRHQKIR[25]	KLPLLQ
	ALEPIEVIQA	RGLQVVRPPIRIQ	VVRPPIRIQ*
	FAPSFSRGQ	VERGLQVVRPPIRIQ	
	GGRAPGRGW	RIQPGGLLLPSYVN TPIL	
	LLPSYVNTPILA F	ALEPNERVE	
	ALEPNERVEAE	RIQPGGL	
Plant seed peroxygenase (1%)	NALEPIERVQ	RQGDVVAIPT	
Putative rho GTPase activation protein (1%)		QEGWDNILR	
Putative triosephosphate isomerase (PTI) (1%)		RQGDVV	
Iron-chelate-transporting ATPase (2%)	TEQFEGR	IERGRGIQ	
Oxidoreductase (3%)	WKAALDEALAN AP	IERGRGIQ	
	SYPTLPGWIPS PF	YGPGGGGGGGRK	
Putative NB-ARC (1%)	WLPSPFFPI		
11-S globulin (73%)	IERGRGIQ*	IIRPPQ*	
Putative glutelin type-B 1 (10%)		VVRPPIRIQ*	
Oxidoreductase (3%)		AIGSRSLEKATKFAAN	
Iron-chelate-transporting ATPase (2%)		WTAPKFFWFFFISF	

Subscript numbers represent homologous peptides sequences: * represent sequence found in both fraction (SPHf and digested). Gray lines represent sequenced peptides founded in digested samples.

Box 1. Peptide fragments and their identification of the 11S globulin protein sequence

Protein identification

>tr|A0A251TLE7|A0A251TLE7_HELAN 11-S seed storage protein, plant OS=Helianthus annuus OX=4232 GN=HannXRQ_Chr10g0301931 PE=3 SV=1

Protein sequence and peptide fragments (yellow):

MASKATLLLAFTLLFATCIARHQQRQQQQNQCQLQNIEALEPIEVIQAEAG
VTEIWDAYDQQFQCAGVDFIRHRIQPGGLLLPSYVNTPILAFVERGRGIQ
GVILPGCPETYEYSQEQQFSGEGGRRGGGEGNQDRHQKVENLKEGDV
VAIPTGTAHWLHNDGNTELVVVVFLDTQNHENQLDENQRRFFLAGNPQA
QAQSQQQQQRQPRQQSPQRQRQRQRQRQGQGQNAGNIFNGFTPELIA
QSFNVDQETAQKLQGQNDQRGHIVNVGQDLQIVRPPQDRRSPRQQQE
QRRSPRQQQEQQQGRRGGWSNGVEETICSMKFKVNIDNPSQADFVNP
QAGSIANLNSFKFPILEHLRLSVERGELRPNAIQSPHWTINAHNLLYVTEG
ALRVQIVDNQGNSVFDNELREGQVVVIPQNFAVIKRANEQGSRWVSFKT
NDNAMIANLAGRVSAISSMPVDVVANAYQLSREEAQQLKFSQRETVLFA
PSFSRGQGIRASA

Protein identification

>tr|A0A251UYX1|A0A251UYX1_HELAN Putative 11-S seed storage protein, plant, RmlC-like jelly roll fold protein OS=Helianthus annuus OX=4232 GN=HannXRQ_Chr04g0112711 PE=3 SV=1

Protein sequence and peptide fragments (yellow):

MAATKLILGFFLCLLGTTVLATTRAPRLGSPEFCNLQQLYTSQPNQRLEFE
GGSIEHYWNFNDEQYQCLGTYPSRMTLQPNSLFLPSFQSYPRLLFIEQG
EGIVGIQLPGCAETFDTGVQQQHMQQQQQHQQQQQQQQQQQQPRRM
GERGMDSSADSHQKVHRFREGDIIAIPAGAVYWTYNDRNQEVVAIIVDDV
NNPANQLDFQAKTSFLAGGISSEHIQGQQQFQGQQGRQGQQQIHGQQS
RQQGRQQGRRRSPFGGQEELTRDNVYAGFDTELLAEAFNCDPQIMRAL
QESRNRGVIVQVQQQMEFVTPEEEQQQMHQRRSRGGPSNGVEETICS
AKLVFNLDNQREADVFNRQAGKLNMVNEHKLPILSLMDLSAEKGHLQPN
ALFSPHWTVNSHTVVYVLNGDAQIQVVSNNGEAVLNEQVSRGDIFPVPQ
FFAATARAGQNGFEWVAFKTNRAPLKSPLAGYTSVFRAMPLEVITNSYQ

VSPNQAQSLKLNRETESLLFSPQRQMTLQHNSLFLPSFQPFPRLLFIEQG
EGLVGIQLPGCAETFDTGVQQIWHQHEQQPRRMDPSADSHQKVHRFRQ
GDIIAIPAGAVYWTYNDRNQQVVAVIVDDVNNPANQLDLQAKISFLAGGIS
RKHIQGLQNIQRQQGRKRSPFGGQEEVAKGNVYSGFDTELLAEAFNCEP
YIVRALQESSNRGVIVQVQQQMEFVTPDEGQEQMRQRRSRGGPSNGVE
ETICSAKLVHTVVYVLSGDAQVQVVSNNGEAVLNEQVSRGDIFPVPQFFA
TTARAGQNGLEWVAFKTNRAPLKSPLAGYTSVFRAMPLEVISYSYQVSP
SQAQSLKLNRETESILFSPQSQY

Two proteins were selected as representative images.