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Atividade antiproliferativa de hidrolisados proteicos e frações peptídicas derivados da proteína de soja e do feijão-caupí, sobre linhagens celulares tumorais, *in vitro*

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Dissertação apresentada ao Programa de Pós-Graduação em Ciência de Alimentos, da Faculdade de Farmácia da Universidade Federal da Bahia, como requisito do para a obtenção do título de Mestre em Ciência dos Alimentos.

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À minha avó Lourdes, pelo exemplo de força

e perseverança

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"Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota"

Madre Teresa de Calcutá

RESUMO

Muitos estudos têm demonstrado que peptídeos derivados de proteínas dos grãos de leguminosas exercem efeitos benéficos à saúde humana. Entres estes, destacam-se à ação antioxidante, antilipidêmica, antiglicêmica e antiobesidade. Neste sentido, estudos mais recentes têm sugerido que frações peptídicas parecem modular a proliferação celular de algumas linhagens tumorais. Neste sentido, o presente estudo avaliou a citotocixidade (atividade antiproliferativa) de hidrolisados proteicos e frações peptídicas oriundas das proteínas isoladas da soja (*Glycine max*) e do feijão-caupí (*Vigna unguiculata*), sobre linhagens tumorais, *in vitro*. As proteínas glicinina (11S) e β-conglicinina (7S) da soja, e a β-vignina (7S) do feijão-caupí foram isoladas, através de etapas de solubilização e precipitação no ponto isoelétrico, e depois, foram parcialmente purificadas por processo cromatográfico. Em seguida, as proteínas glicinina e β-conglicinina foram hidrolisadas pela ação sequencial das enzimas pepsina/pancreatina, e a β-vignina por diferentes sistemas enzimáticos: *i*. pepsina, *ii.* tripsina, *iii.* pepsina/pancreatina, e *iv*. alcalase/pepsina. Posteriormente, a citotoxicidade dos hidrolisados foi avaliada. Nenhum dos hidrolisados apresentou efeito citotóxico sobre as células humanas não-tumorais (HUVEC) nas concentrações de 12,5–200 µg/mL. Por outro lado, os hidrolisados proteicos da β-conglicinina e glicinina inibiram a proliferação celular de adenocarcinoma mamário humano (MDA-MB-231), carcinoma hepatocelular humano (Hep-G2) e carcinoma de próstata (DU-145), entre 24% a 54%, e 20% a 45%, respectivamente. Além disso, os hidrolisados da proteína β-vignina, derivados da ação da pepsina (IC_{50}=3,71 µg/mL) e tripsina (IC_{50}=3,02 µg/mL) exerceram uma ação antiproliferativa de -95% e -91%, respectivamente, sobre a linhagem MDA-MB-231. Para a soja, a fração constituída de peptídeos entre 10-3 kDa da β-conglicinina apresentou um efeito antiproliferativo mais significativo sobre a MDA-MB-231 (IC_{50} 7,4 µg/mL) e DU-145 (IC50 6,0 µg/mL), enquanto que a fração < 3 kDa apresentou melhor efeito contra células Hep-G2 (IC_{50} 5,7 µg/mL), ambos com efeito dose-dependente. No feijão-caupí, a fração de peptídeos entre 10-3 kDa apresentou melhor efeito contra as células MDA-MB-231 (IC_{50}=0,62 µg/mL), enquanto a fração de peptídeo de 30-10 kDa teve o melhor efeito inibitório nas células Hep-G2 (IC_{50}=10,63 µg/mL). Os resultados observados neste estudo indicam a presença de peptídeos na fração entre 10-3 kDa, derivados da proteína β-conglicinina da soja e β-vignina do caupí, com ação antiproliferativa sobre linhagens celulares tumorais, sobretudo para adenocarcinoma mamário. Contudo, estudos adicionais são necessários a fim de identificar os peptídeos que exercem este efeito, e esclarecer o(s) mecanismo(s) envolvido(s) na morte celular. Atualmente, estas questões vêm sendo estudadas pelo nosso grupo de pesquisa.

Palavras-chave: *β-conglicinina; β-vignina; peptídeos bioativos; citotoxicidade; MDA-MB-231.*

ABSTRACT

Several studies have shown that peptides derived from proteins in legume grains have beneficial effects on human health. Among these, the antioxidant, antilipidemic, anti-glycemic and anti-obesity action stands out. In this sense, more recent studies have suggested that peptide fractions appear to modulate the cell proliferation of some tumor strains. In this sense, the present study evaluated the cytotoxicity (antiproliferative activity) of protein hydrolysates and peptide fractions from proteins isolated from soy (Glycine max) and cowpea (Vigna unguiculata), on tumor lines, in vitro. Proteins glycine (11S) and β-conglycinin (7S) from soybeans, and β-vignin (7S) from cowpea were isolated, through solubilization and precipitation steps at the isoelectric point, and afterwards, were partially purified by process chromatographic. Then, the proteins glycinin and β-conglycinin were hydrolyzed by the sequential action of the enzymes pepsin / pancreatin, and β-vignin by different enzymatic systems: i. pepsin, ii. trypsin, iii. pepsin / pancreatin, and iv. alkalase / pepsin. Subsequently, the cytotoxicity of the hydrolysates was evaluated. None of the hydrolysates had a cytotoxic effect on human non-tumor cells (HUVEC) at concentrations of 12.5–200 µg / mL. On the other hand, β-conglycinin and glycine protein hydrolysates inhibited the cell proliferation of human breast adenocarcinoma (MDA-MB-231), human hepatocellular carcinoma (Hep-G2) and prostate carcinoma (DU-145), between 24% 54%, and 20% to 45%, respectively. In addition, β-vignin protein hydrolysates, derived from the action of pepsin (IC50 = 3.71 µg / mL) and trypsin (IC50 = 3.02 µg / mL) exerted an anti-proliferative action of -95% and -91% , respectively, on the MDA-MB-231 strain. For soybeans, the fraction of peptides between 10-3 kDa of β-conglycinin showed a more significant antiproliferative effect on MDA-MB-231 (IC50 7.4 µg / mL) and DU-145 (IC50 6.0 µg / mL), while the fraction <3 kDa had a better effect against Hep-G2 cells (IC50 5.7 µg / mL), both with dose-dependent effect. In cowpea, the peptide fraction between 10-3 kDa had the best effect against MDA-MB-231 cells (IC50 = 0.62 µg / mL), while the peptide fraction of 30-10 kDa had the best effect inhibitory in Hep-G2 cells (IC50 = 10.63 µg / mL). The results observed in this study indicate the presence of peptides in the fraction between 10-3 kDa, derived from the protein β-conglycinin of soy and β-vignin of cowpea, with antiproliferative action on tumor cell lines, especially for breast adenocarcinoma. However, further studies are needed in order to identify the peptides that exert this effect, and to clarify the mechanism (s) involved in cell death. These issues are currently being studied by our research group.

Keywords*: β-conglycinin, β-vignin, bioactive peptides; cytotoxicity, MDA-MB-231.*

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1 INTRODUÇÃO GERAL

Devido ao envelhecimento populacional e aos hábitos de vida modernos, o câncer é atualmente considerado uma das doenças crônicas mais prevalentes na atualidade. O câncer corresponde a um conjunto de mais de 100 doenças e é decorrente de uma divisão celular descontrolada, com consequente comprometimento funcional tecidual e invasão a outras regiões do organismo. Tal capacidade é viabilizada em razão da propriedade angiogênica do tecido, que sob condições de normalidade é estritamente controlada e na displasia maligna possibilita a disseminação sistêmica das células neoplásicas através do sistema linfático e da corrente sanguínea (BLANCO-MÍGUEZ *et al*., 2016).

As estratégias de tratamento como cirurgia, quimioterapia e radioterapia são frequentemente associadas a efeitos colaterais por causarem danos a tecidos saudáveis (CARRILLO *et al*., 2017), além da resistência a diversos agentes antineoplásicos (KIBRIA; HATAKEYAMA; HARASHIMA, 2014). Por outro lado, resultados de estudos epidemiológicos sustentam a hipótese de que a alimentação desempenha papel importante como forma alternativa (promissora) e auxiliar aos tratamentos, especialmente pela ingestão daqueles alimentos considerados funcionais (CORDEIRO *et al*., 2018).

Diversos estudos apontam que hidrolisados de proteínas de leguminosas são boas fontes para obtenção de peptídeos bioativos que exibem potencial terapêutico para diversas patologias, como hipertensão (CIAU-SOLÍS; ACEVEDO-FERNÁNDEZ; BETANCUR-ANCONA, 2018), diabetes (BECERRA-TOMÁS *et al.,* 2018), aterosclerose (GOMES *et al*., 2020), sindrome metabolica (JAKUBCZYK *et al*., 2017) e sobretudo para o câncer (DIA; DE MEJIA, 2013; LUNA-VITAL; DE MEJÍA; LOARCA-

PIÑA, 2017). Desses fatores, a dieta é responsável por mais de 35% dos casos (RUIZ; HERNANDÉZ, 2014).

Diversos estudos apontam que hidrolisados de proteínas de leguminosas são boas fontes para obtenção de peptídeos bioativos, os quais demonstram exercer potencial terapêutico para diversas patologias, sobretudo para o câncer (GONZÁLEZ-MONTOYA; CANO-SAMPEDRO; MORA-ESCOBEDO, 2017). Dentre estas, as proteínas de soja (Glycine max) têm sido consideravelmente estudadas quanto à presença de peptídeos bioativos derivados da sua hidrólise. Seu consumo tem sido associado a uma menor incidência de câncer de mama (TAKAGI *et al*., 2015; MOUROUTI; PANAGIOTAKOS, 2013), próstata (KOLONEL *et al*., 2000), fígado (ZHOU *et al*., 2016), cólon (YU *et al*., 2016) e endométrio (ZHONG, 2016), principalmente em países orientais, onde seu consumo tem maior abrangência (HE; CHEN, 2013).

As globulinas correspondem a cerca de 70% da fração proteica encontrada em leguminosas, constituídas geralmente de duas proteínas principais, denominadas de globulina do tipo vicilinas e leguminas, usualmente classificadas em proteínas 7S e 11S, respectivamente (SHEVKANI *et al*., 2019). Apesar de serem majoritárias, existem poucos trabalhos na literatura que buscaram investigar se essas proteínas são prováveis candidatas para a geração de peptídeos com propriedades antitumorais (MONTALES *et al*., 2015; WANG *et al*., 2008). Assim, o presente estudo avaliou a atividade antiproliferativa de hidrolisados proteicos e frações peptídicas de proteínas isoladas da soja (*Glycine max*) e do feijão-caupí (*Vigna unguiculata*) – sobre linhagens celulares tumorais, *in vitro*.

2 OBJETIVOS

2.1 Objetivo geral

- Avaliar a citotoxicidade de hidrolisados proteicos e frações peptídicas derivados das proteínas β-conglicinina e glicinina da soja (*Glycine max*) e da β-vignina do feijão-caupí (*Vigna unguiculata*), sobre linhagens celulares tumorais, *in vitro*.

2.2 Objetivos específicos

- Extrair, isolar e purificar as globulinas β-conglicinina e glicinina da soja; e a β-vignina do feijão caupí;
- Hidrolisar a β-conglicinina e glicinina a partir da hidrólise sequencial de pepsina pancreatina; e a β-vignina a partir da ação enzimática individual (pepsina, tripsina) e sequencial (pepsina/pancreatina e alcalase/pepsina), *in vitro*;
- Avaliar a citotoxicidade (IC_{50}) dos hidrolisados da soja frente às linhagens tumorais de adenocarcinoma mamário humano (MDA-MB-231), carcinoma hepatocelular humano (Hep-G2), carcinoma de próstata (DU-145) e na linhagem não tumoral de célula epitelial de cordão umbilical humano (HUVEC), *in vitro*;
- Avaliar a citotoxicidade (IC_{50}) do hidrolisado do feijão-caupí frente às linhagens tumorais de adenocarcinoma mamário humano (MDA-MB-231), carcinoma hepatocelular humano (Hep-G2) e na linhagem não tumoral de célula epitelial de cordão umbilical humano (HUVEC), *in vitro*;
- Fracionar o hidrolisado proteico da soja e do feijão-caupí que causar maior citotoxicidade em peptídeos entre 30 e 10 kDa, peptídeos entre 10 e 3 kDa e peptídeos menores que 3 kDa;
- Avaliar a citotoxicidade (IC_{50}) das frações de peptídeos entre 30 –10 kDa, entre 10 - 3 kDa e menores que 3 kDa.

3 FUNDAMENTAÇÃO TEÓRICA

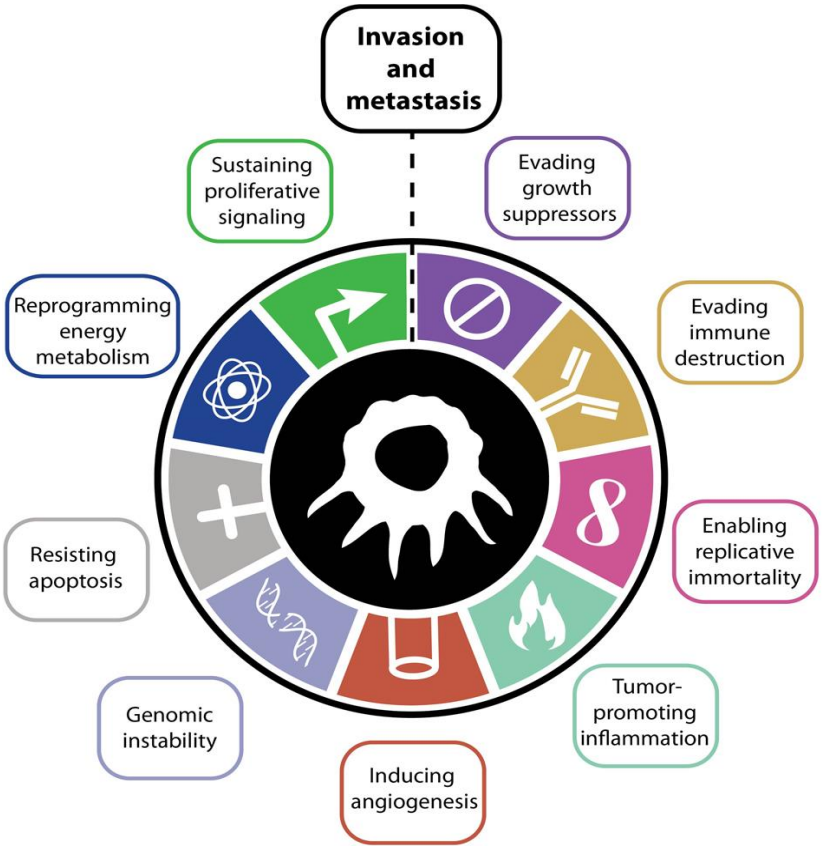
3.1 Câncer: Aspectos gerais

Câncer é o nome dado a um conjunto de mais de 100 doenças que apresenta como característica comum divisão celular descontrolada com consequente comprometimento funcional tecidual e capacidade de disseminação a outras regiões do organismo (BLANCO-MÍGUEZ *et al*., 2016; HANAHAN; WEINBERG, 2000). Em condições de normalidade, as células humanas têm a capacidade de superar alterações em seu material genético devido aos mecanismos de reparo do DNA e apoptose. Sempre que esses mecanismos de proteção celular são alterados constitucionalmente ou o ataque ao DNA ultrapassa as capacidades de uma célula normal, ocorrem mutações permanentes. Essas mutações podem ativar genes envolvidos no crescimento e proliferação celular (oncogenes) ou inativar genes envolvidos na senescência celular e apoptose (genes supressores de tumor) (IMRAN *et al*., 2017). Tais alterações no material genético modificam circuitos regulatórios que mantém a homeostase celular, resultando em uma série de manifestações patológicas a nível sistêmico (HASSANPOUR; DEHGHANI, 2017).

O desenvolvimento das neoplasias pode ser influenciado tanto por fatores de risco intrínsecos não modificáveis quanto por fatores não intrínsecos modificáveis. Os fatores de risco intrínsecos referem-se a erros aleatórios resultantes da replicação do DNA. Os fatores de risco não intrínsecos podem ser subdivididos em endógenos e exógenos. Os endógenos são aqueles parcialmente modificáveis e relacionados às características de um indivíduo, como sistema imunológico, metabolismo, resposta a danos no DNA e níveis hormonais. E os exógenos são aqueles modificáveis, como exposição à radiação, tabagismo, terapia hormonal, dieta, atividade física, entre outros (WU *et al*., 2018).

Hanahan e Weinberg (2000) propuseram no início dos anos 2000 que as mutações genéticas em células cancerígenas resultam em seis alterações fenotípicas que as caracterizam como células tumorais malignas. Essas alterações fisiológicas comuns às células cancerosas ficaram conhecidas como hallmarks do câncer. Posteriormente, com o avanço nas pesquisas sobre a biologia do câncer, foram adicionadas mais quatro alterações (HANAHAN; WEINBERG, 2011), constituindo-se então dez hallmarks: (1) Evasão a supressores de crescimento; (2) Evasão ao sistema imunológico; (3) Imortalidade replicativa; (4) Inflamação promotora de tumor; (5) Indução de angiogênese; (6) Instabilidade genômica; (7) Resistência à apoptose; (8) Reprogramação do metabolismo energético; (9) Sustentação a sinalização proliferativa; e (10) Invasão e metástase (**Figura 1**).

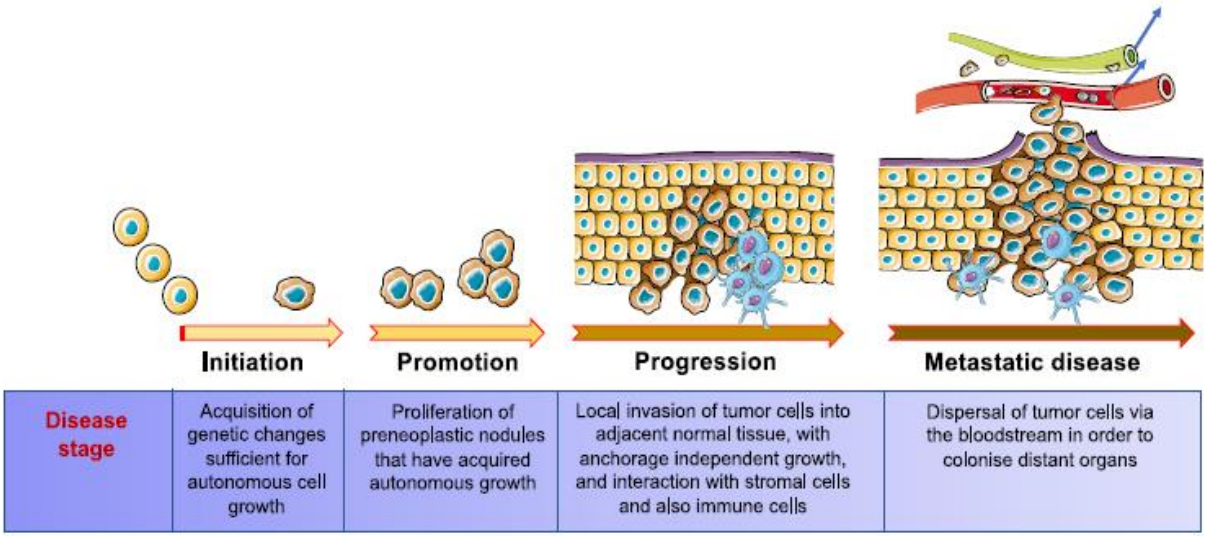
Figura 1 – Os Hallmarks do câncer.



***Fonte**:* Meirson, Gil-Henn e Samson (2020).

O desenvolvimento de uma célula não-tumoral em uma célula maligna ocorre de forma lenta e progressiva, geralmente após anos de exposição a agentes carcinogênicos. Esse processo é dividido em três estágios: iniciação, promoção e progressão (**Figura 2**).

Figura 2 – Desenvolvimento da célula cancerígena durante etapas de iniciação, promoção e progressão.



***Fonte**:* Hayes, Dinkova-Kostova e Tew *(2020).*

A iniciação é a etapa inicial para a formação de células tumorais no organismo. Iniciadores, também conhecidos como agentes carcinogênicos, são compostos capazes de causar mutações no DNA, gerando alterações genéticas permanentes. Quanto maior a exposição a um agente carcinogênico, maior o risco de se iniciar o processo de carcinogênese. Uma vez que uma célula particular foi afetada por um iniciador, ela é suscetível à promoção (LIU *et al*., 2015). A etapa conhecida como promoção é caracterizada pela geração de células-filhas contendo a mutação criada pelo iniciador. Essa proliferação celular é geralmente incitada por agentes conhecidos como promotores, os quais se ligam a receptores na superfície celular afetando as

vias intracelulares que aumentam a proliferação celular. A terceira etapa, denominada de progressão, é irreversível e está relacionada a mudanças cariotípicas, na qual observa-se um aumento da taxa de crescimento, invasividade e metástase devido à instabilidade genética (HAYES; DINKOVA-KOSTOVA; TEW, 2020; LIU *et al*., 2015).

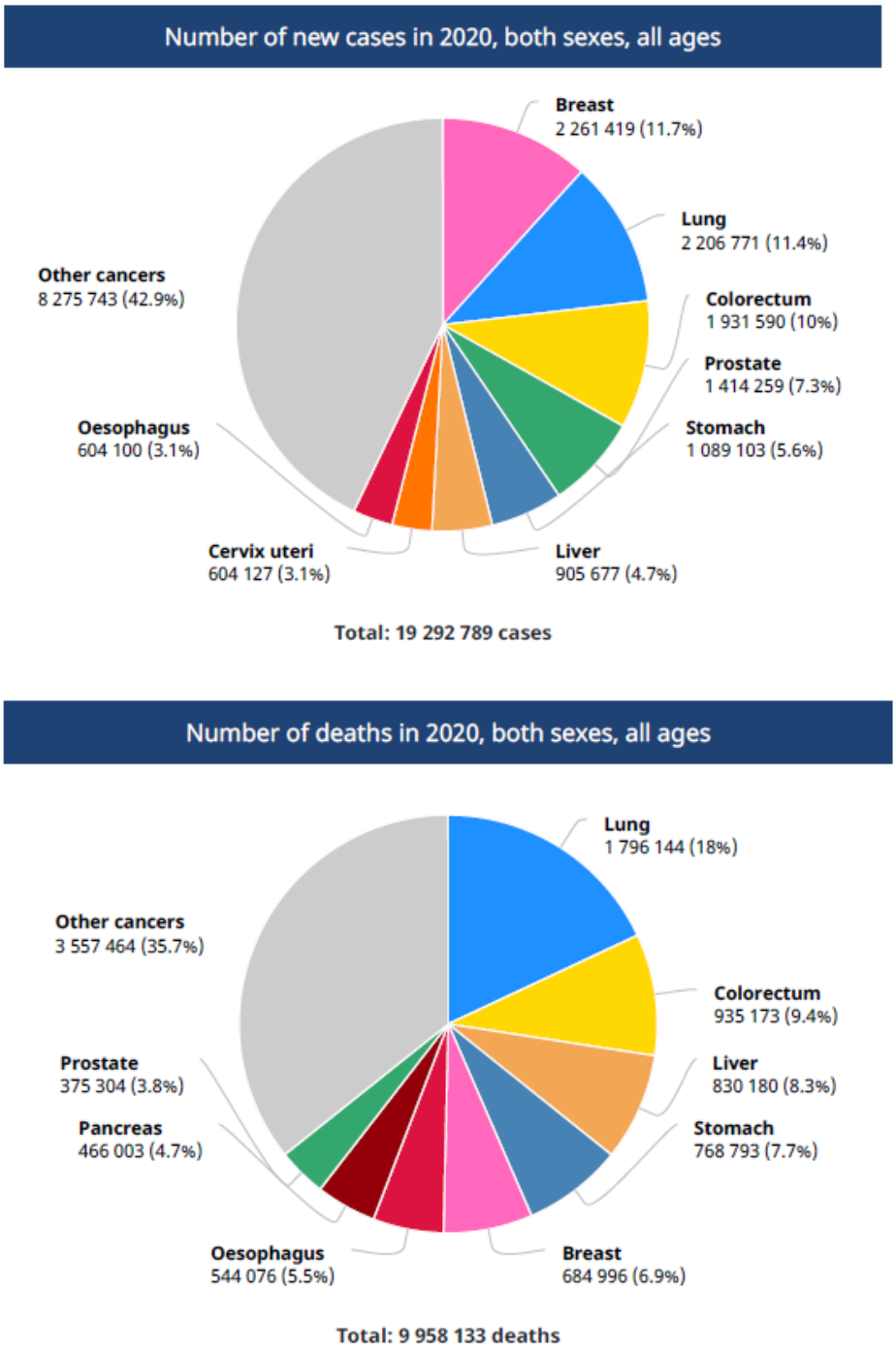
A formação de metástases é um processo complexo e envolve diversas etapas. As células tumorais primeiramente migram e invadem a matriz extracelular circundante para então serem capazes de atingir a vasculatura, sobreviver à corrente sanguínea, invadir outro tecido e se adaptar ao novo microambiente. Sua capacidade de evasão do seu tecido de origem relaciona-se à sua habilidade de sintetizar e secretar fatores pró-angiogênicos, os quais contribuem para a formação de novos vasos sanguíneos e linfáticos a fim de manter sua necessidade nutricional frente à crescente massa tecidual. A disseminação metastática é a principal causa de mortalidade relacionada ao câncer e, por isso, considerado um importante alvo terapêutico (MEIRSON; GIL-HENN; SAMSON, 2020).

3.2 Epidemiologia do câncer

A incidência de câncer e outras doenças crônicas não transmissíveis tem crescido mundialmente e a previsão é de que o número de casos aumente com a melhora da expectativa de vida (CAVAZOS; DE MEJIA, 2013). A incorporação de hábitos relacionados à urbanização, como alimentação inadequada, sedentarismo, alcoolismo, entre outros, têm contribuído para o aumento da incidência (BRAY *et al*., 2018). O câncer foi a causa de morte de 9,9 milhões de pessoas no mundo em 2020. As neoplasias malignas mais incidentes nesse ano foram as de mama (2,2 milhões), pulmão (2,2 milhões), cólon (1,9 milhão), próstata (1,4 milhão) e fígado (0,9 milhão). Aquelas responsáveis pelo maior número de mortes foi o câncer de pulmão (1,7

milhão), seguido de colorretal (0,9 milhão), fígado (0,8 milhão), estômago (0,7 milhão) e mama (0,7 milhão), como demonstrado na **Figura 3**. A previsão é que no ano de 2040 haverá mais de 30 milhões de novos casos (FERLAY *et al.*, 2020).

Figura 3 – Número de novos casos e mortes de cânceres mais incidentes no mundo para ambos os sexos e todas as idades em 2020.



***Fonte**:* Ferlay *et al*. (2020).

Os dados brasileiros parecem acompanhar a tendência mundial em virtude do aumento observado no processo de urbanização (SIEGEL; MILLER; JEMAL, 2018). Tem sido observada uma alteração importante no perfil de morbimortalidade na população brasileira, caracterizada pela diminuição da incidência das doenças infectocontagiosas e um aumento na incidência de doenças crônico degenerativas. Estudos epidemiológicos recentes estimaram a ocorrência de aproximadamente 625 mil novos casos para o triênio de 2020-2022. O câncer de pele não melanoma será o mais incidente (177 mil), seguido pelos cânceres de mama e próstata (66 mil cada), cólon e reto (41 mil), pulmão (30 mil) e estômago (21 mil). A região sudeste concentra mais de 60% da incidência, na qual predominam os cânceres de próstata e mama, bem como o de pulmão e de intestino. A região Nordeste é a segunda mais incidente (27,8%), onde os cânceres de próstata e mama também são os mais importantes, seguido do câncer do colo do útero e de estômago. A região Sul concentra 23,4% dos casos, com padrão da incidência similar ao da região Sudeste (INCA, 2019).

Diante deste contexto, é evidente que o câncer é um problema de saúde pública que gera impacto econômico substancial. Apenas para o câncer de mama no Brasil, os gastos com internações, quimioterapia e benefícios previdenciários aumentaram em mais de 100% comparando-se o ano de 2008 (R$ 302 milhões) e 2015 (R$ 633 milhões) (SIQUEIRA *et al*., 2016). Desse modo, fica evidente a importância do diagnóstico precoce da doença assim como busca por novos tratamentos.

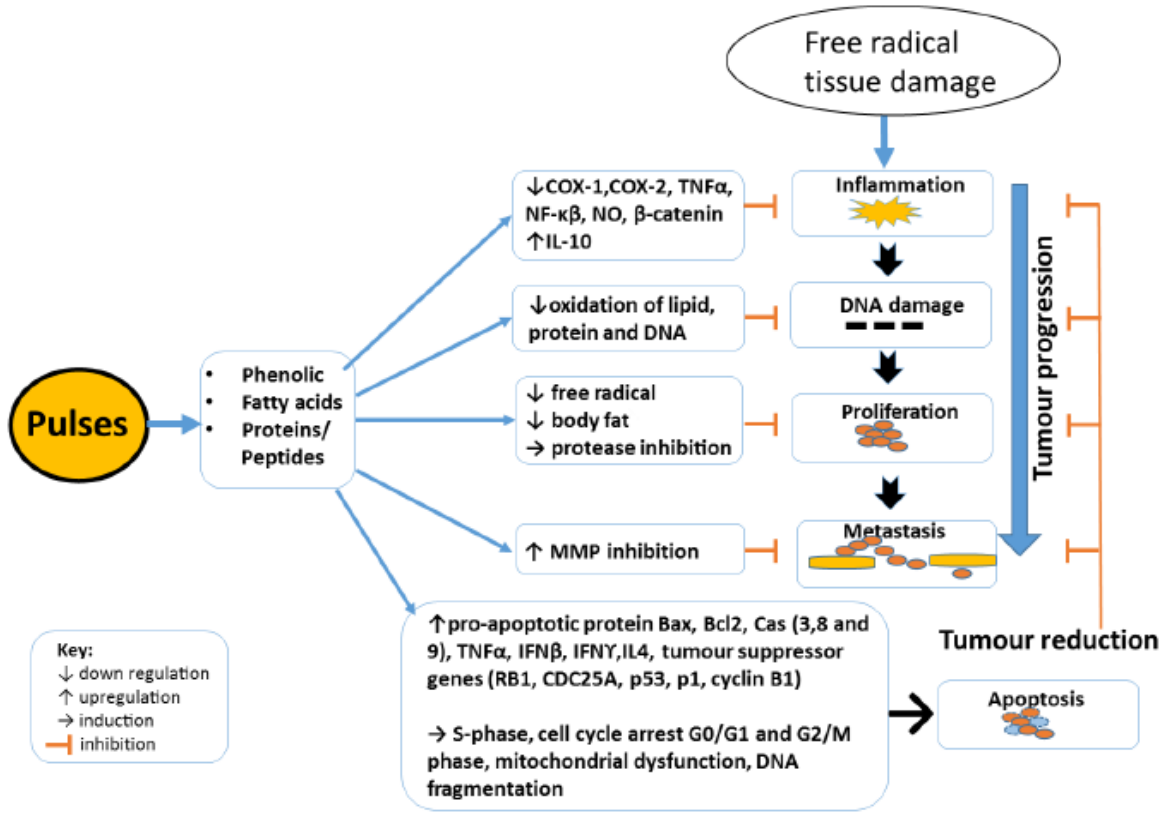
3.3 Grãos de leguminosas x Câncer

A origem do termo leguminosa é derivada da palavra latina “legumen”, que significa “sementes colhidas em vagens”. Os grãos de leguminosas são considerados um importante componente da dieta há milênios, sendo consumida em todo o mundo e representam uma importante fonte de nutrientes (SINGH, 2017). O elevado conteúdo proteico tem sido sem dúvida seu principal aspecto de interesse econômico, uma vez que tem crescido a demanda por proteínas de origem vegetal. Consequentemente, as culturas de leguminosas podem ser exploradas como fontes de proteína sustentáveis e de alta qualidade (BESSADA; BARREIRA; OLIVEIRA, 2019).

Estudos têm demonstrado uma associação entre ingestão de leguminosas e redução do risco de desenvolvimento de alguns tipos de câncer. Uma meta-análise com base em estudos de coorte prospectivos que buscavam investigar a associação entre o consumo de leguminosas na dieta e o risco de câncer colorretal indicou uma associação entre maior ingestão de leguminosas e um risco reduzido deste câncer, principalmente relacionado ao consumo da soja (ZHU *et al*., 2015). Outra meta-análise de estudos de coorte prospectivos apontou que indivíduos com alto consumo de leguminosas experimentaram um risco 3,7% menor de desenvolver câncer de próstata para cada aumento de 20 g/dia de ingestão de leguminosas (LI; MAO, 2017).

Assim, há um interesse considerável em explorar as propriedades quimiopreventivas de compostos presentes nas leguminosas. Algumas das propriedades potencialmente benéficas de compostos presentes nas leguminosas são atribuídas principalmente a suas atividades anti-inflamatória, antiproliferativa, pró-apoptótica e antimetastática (RAO *et al.*, 2018), como ilustrado resumidamente na **Figura 4**.

Figura 4 – Interação potencial de compostos bioativos de leguminosas com vias relacionadas ao desenvolvimento do câncer.



***Fonte**:* Rao *et al.* (2018).

Estudos têm mostrado que compostos fenólicos extraídos de leguminosas apresentam propriedades anti-inflamatórias e antioxidante, as quais podem contribuir para evitar processos celulares que levem à tumorigênese. Foi observado em extratos fenólicos de quatro variedades de feijão comum (*Phaseolus vulgaris*) inibição de proteínas pró-inflamatórias, como ciclooxigenase-2, fator de necrose tumoral e fator nuclear kappa-B, e um aumento da atividade da interleucina 10 (MORENO-JIMENEZ *et al*., 2015). Um extrato com uma variedade de compostos fenólicos do feijão faba (*Vicia faba*) apresentou atividade antioxidante em ensaios de determinação da atividade antioxidante total, de atividade antioxidante no sistema modelo β-caroteno-

linoleato e atividade anti-radicalar contra DPPH, no qual a fração composta por taninos condensados exibiu melhor atividade (AMAROWICZ; SHAHIDI, 2018).

As isoflavonas genisteína, daidzeína e gliciteína têm sido associadas à redução do risco de desenvolvimento de câncer (SPAGNUOLO *et al*., 2015; HUA *et al*., 2018; ZHANG *et al.*, 2015). Dentre estas, a genisteína é a que tem sido mais vastamente investigada, uma vez que a mesma representa cerca de 50% das isoflavonas (MURPHY; BARUA; HAUCK, 2002). Devido à sua semelhança estrutural com o estradiol, ela foi primeiramente descrita como composto antiestrogênico (FOLMAN; POPE, 1966), pois é capaz de se ligar a receptores de estrogênio (ER), principalmente ao ER_{β}, receptor com atividade supressora de crescimento comumente expresso em tumores de mama (AN *et al*., 2001; SAJI; HIROSE; TOI, 2005). Tal seletividade lhe conferiu a classificação de composto modulador seletivo de receptores de estrogênio (RUSSO *et al*., 2016).

Por essa razão, inicialmente sua função quimiopreventiva foi relacionada a seus efeitos antiestrogênicos. Posteriormente foi avaliada a ação da genisteína na inibição de duas linhagens cancerígenas de mama, nas quais uma expressa receptor de estrogênio (MCF-7) e a outra não (MDA-468). Para ambas foi observada inibição do crescimento celular de maneira similar, chegando-se à conclusão de que sua atuação não é receptor-dependente e está relacionada também a outras vias celulares (PETERSON; BARNES, 1991). Outros estudos identificaram que essa isoflavona também é um inibidor de tirosina-quinase do fator de crescimento epidérmico (EGF) em células de carcinoma epidermoide (A431) (AKIYAMA *et al*., 1987) e inibidor de DNA topoisomerase II em células de carcinoma de cólon (MIZUSHINA *et al*., 2013), glioma (SCHMIDT *et al*., 2008) e células leucêmicas (LÓPEZ-LÁZARO; WILLMORE;

AUSTIN, 2007). Além disso, ela age em sinergismo com outros agentes antitumorais (KAUSHIK *et al*., 2016; TANG *et al*., 2018).

3.4 Proteínas e peptídeos antitumorais de leguminosas

Como as leguminosas são uma fonte rica em proteínas (20-40%) (ERBERSDOBLER; BARTH; JAH-REIS, 2017), é notório que sua fração proteica também tenha sido consideravelmente investigada, principalmente pela presença de proteínas e peptídeos derivados da sua hidrólise com atividade antitumoral. Na década de 1940 Bowman (1944) e Kunitz (1945) identificaram frações derivadas da soja capazes de inibir a digestão *in vitro* de proteínas pela ação da tripsina e quimiotripsina, que posteriormente ficaram conhecidas como inibidores de Bowman-Birk (BBI) e do tipo Kunitz (KTI), as quais correspondem a 6% do total das proteínas da soja (RACKIS; ANDERSON, 1964). Esses inibidores de protease foram amplamente estudados quanto a sua estrutura (BIRK, 1961; BIRK, 1985; BIRK; GERTLER; KHALEF, 1963; BOWMAN, 1944; KUNITZ, 1945) assim como sua possível ação biológica (KOBAYASHI *et al*., 2004), principalmente o BBI como agente anticarcinogênico (CHEN *et al*., 2005; KENNEDY, 1998).

Inibidores de protease são fatores antinutricionais de estrutura proteica presentes nas leguminosas que apresentam atividade antiproliferativa em algumas linhagens celulares. Um inibidor de bowman-birk (BBI) isolado da soja foi capaz de inibir a viabilidade de células de câncer de próstata humana (LNCaP), causando indução de conexina 43 (Cx43) e expressão de caspase 3 clivada *in vitro*. Quando administrada *in vivo* em uma dieta contendo 3% de concentrado de BBI (BBIC) em ratos durante 10 semanas, foi observada redução da taxa de crescimento do peso corporal sem causar alterações clínicas ou histopatológicas nos tratados, suprimindo

adenocarcinomas nos lobos laterais da próstata e causando expressão de Cx43 em células mortas de câncer de próstata pós-tratamento (TANG *et al*., 2009). Esse e outros trabalhos (SAITO *et al*., 2007; SAKURAI *et al*., 2008) demonstram que o BBI atua induzindo o gene supressor de tumor Cx, o qual é responsável pela expressão de proteínas transmembranas chamadas de conexinas (Cx), as quais mantêm a homeostase celular via comunicação intercelular juncional, retardando o processo metastático.

Outra proteína já explorada quanto à sua atividade antitumoral são as lectinas, classe de proteínas de origem não-imunológica, as quais apresentam a capacidade de se ligar reversivelmente a carboidratos e que não são consideradas anticorpos, enzimas que usam carboidratos como substratos ou transportadores de sacarídeos livres (MANNING *et al*., 2017). A atividade antitumoral de quatro lectinas vegetais – fitohemaglutinina (PHA) do feijão vermelho (*Phaseolus vulgaris*), o mitógeno de ervilha (PWM) da erva-tintureira (*Phytolacca americana*), a aglutinina (SBA) da soja (*Glycine max*) e aglutinina (WGA) de trigo (*Triticum vulgaris*) foi avaliada em um linfoma ascitico murino. Quando as células foram tratadas *in vitro*, as quatro lectinas causaram redução na contagem de células tumorais e *in vivo* reduziram a progressão de crescimento no hospedeiro. Dentre as lectinas testadas, a WGA foi a que apresentou melhor controle de crescimento do tumor assim como melhor expectativa de vida dos animais (GANGULY; DAS, 1994). O efeito anticâncer da lectina de soja (SBL) também foi avaliada *in vivo* em camundongos portadores de linfoma de Dalton. Foi observado in vitro que a autofagia, apoptose e dano ao DNA mediada por SBL nas células HeLa foram infligidas através da geração de ERO de maneira dose-dependente, o que foi confirmado quando as células foram pré-tratadas com N-acetilcisteína (molécula considerada “limpadora” de ERO). Também foi observada redução da atividade de

autofagia, apoptose e dano ao DNA induzida por SBL, sugerindo que sua atividade citotóxica está intimamente relacionada com a geração de ERO (PANDA *et al*., 2014).

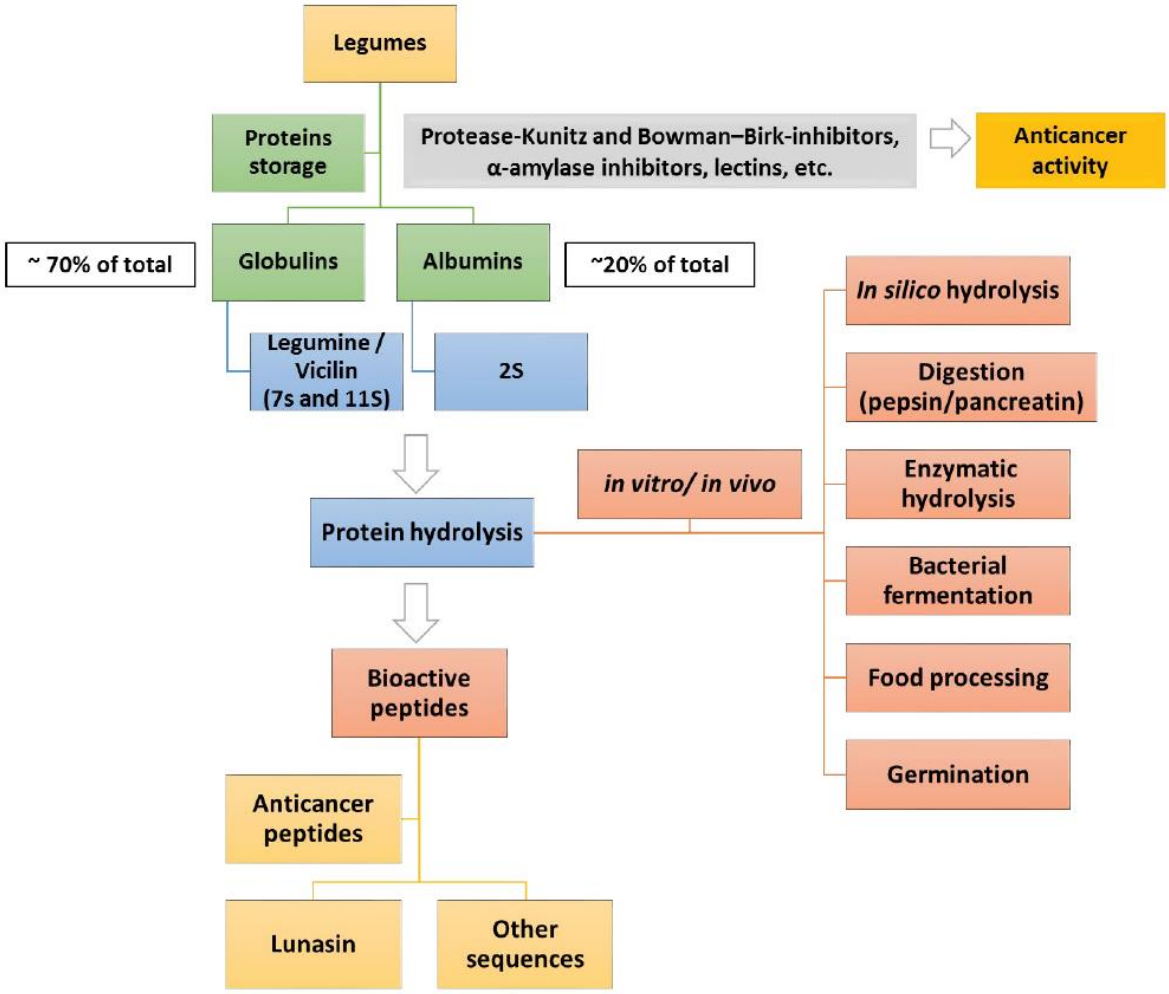
Uma vez demonstrada que algumas proteínas íntegras de leguminosas são capazes de desempenhar atividade antiproliferativa em células cancerígenas, alguns estudos investigam se peptídeos gerados pela hidrólise proteica podem ser de fato as responsáveis pela atividade antiproliferativa das proteínas, pois em sistemas biológicos elas são hidrolisadas no sistema gastrointestinal, sendo absorvidas como peptídeos. Frações de peptídeos entre 50-10 kDa derivados do isolado proteico da soja, pela ação enzimática sequencial da alcalase/pepsina/pancreatina, reduziram de forma intensa a taxa de proliferação celular (-68%) da linhagem tumoral CCRF-CEM (sangue) na concentração de 800 µg/mL (RAYAPROLU *et al*., 2017b). Por isso, ela foi analisada e observada que a mesma é composta por três peptídeos, os quais foram avaliados quanto a sua atividade antiproliferativa em células de câncer hepático (Hep-G2), de sangue (CCRF-CEM) e de cólon (HCT-116). O peptídeo denominado de E67 foi o que apresentou atividade mais proeminente, chegando a inibir 80% das células de CCRF-CEM, o qual foi identificado como um peptídeo de massa molecular de aproximadamente 18 kDa e considerado precursor da albumina 2S da soja (RAYAPROLU *et al*., 2017a).

Essa albumina da soja ficou bastante conhecida no campo de estudo das leguminosas pois foi a partir dela que foi isolado o peptídeo conhecido como lunasina. Esse peptídeo bastante estudado apresenta atividade antitumoral em diversas linhagens celulares, tanto em estudos *in vitro* como *in vivo*. Em células de câncer de mama ele foi capaz de inibir a expressão de duas metaloproteinases de matriz (MMP) relacionadas a metástase, a MMP-2 e principalmente a MMP-9, via sinalização FAK/Akt/ERK e NF-κB (JIANG *et al*., 2016). Em células de câncer de cólon foi capaz

de inibir em 62,8% da proliferação celular na maior concentração testada (100 μM) com IC50 de 61,7 μM, semelhante ao da cisplatina (IC50=76,7 μM), além de causar parada do ciclo celular na fase G2 e alterar a expressão de proteínas relacionadas com a apoptose, como a Bax, Bcl-2 e caspase-3 (DIA; DE MEJIA, 2010). Atividades semelhantes são descritas em células leucêmicas (DE MEJIA; WANG; DIA, 2010), de melanoma (SHIDAL *et al*., 2017) e de pulmão (MCCONNELL *et al*., 2015). Ela também é capaz de reduzir os níveis de ERO e da atividade das enzimas glutationa peroxidase e catalase, além do aumento dos níveis de GSH, desempenhando efeito quimioprotetor em células hepaticas Hep-G2 submetidas ao estresse oxidativo (400 μM de t-BOOH) (FERNÁNDEZ-TOMÉ *et al*., 2014).

Várias estratégias têm sido utilizadas para obter peptídeos com atividade anticâncer, como hidrólise *in vitro* por proteases comerciais, fermentação com cepas bacterianas, digestão gastrointestinal e outros, como ilustrado na **Figura 5**. A hidrólise enzimática é uma das mais comumente utilizadas e pode ser realizada por uma única enzima ou em combinação com diferentes proteinases, como pepsina, tripsina, quimotripsina, bromelina, papaína, alcalase, entre outras. A hidrólise ocorre em condições de pH e temperatura moderadas (pH 5-9; 40-60 °C), condições que devem ser muito bem controladas (GONZÁLEZ-MONTOYA; CANO-SAMPEDRO; MORA-ESCOBEDO, 2017). A hidrólise, ao gerar peptídeos de diferentes tamanhos moleculares, aumenta o número de grupos ionizáveis e pode expor grupos hidrofóbicos. Estudos demonstram que aminoácidos com características hidrofóbicas são comumente encontrados em peptídeos anticâncer derivados de fontes alimentares, como a prolina, leucina, glicina e alanina. Geralmente estes peptídeos apresentam sequências de aminoácidos que variam de 3 a 25 resíduos (CHALAMAIAH; YU; WU, 2018; CHI *et al*., 2015; WANG; ZHANG, 2017).

Figura 5 – Principais proteínas de armazenamento das leguminosas e ferramentas utilizadas para a obtenção de peptídeos com atividade antitumoral.



Fonte: González-Montoya, Cano-Sampedro e Mora-Escobedo (2017).

Os ensaios de citotoxicidade *in vitro* em cultura de células tumorais são uma importante ferramenta para a avaliação de compostos com provável atividade anticâncer, que funcionam como uma espécie de triagem para selecionar aqueles que serão testados em ensaios *in vivo*. Existem diversos métodos para detectar efeitos citotóxicos de compostos e medir sua viabilidade celular, os quais são baseados em diferentes mecanismos de ação – como integridade de membrana, atividade mitocondrial, metabolismo celular, produção de ATP, entre outros. O ensaio de Alamar

Blue causa menor toxicidade comparado a outros métodos, apresenta alta sensibilidade e é adequado para conduzir experimentos de longo prazo sem matar as células (ADAN; KIRAZ; BARAN, 2016).

3.5 Proteínas de reserva de leguminosas

As proteínas de reserva de leguminosas foram classificadas por Osborne (1924) segundo sua solubilidade em: albuminas (solúveis em água), globulinas (solúveis em soluções salinas), prolaminas (solúveis em soluções hidroalcoólicas) e glutelinas (solúveis em soluções ácidas, alcalinas ou na presença de SDS). Elas são constituídas por duas classes principais – as albuminas e globulinas, que apresentam coeficiente de sedimentação entre 1,6S-2S e 7-13S (S, Svedberg), respectivamente. As globulinas correspondem a cerca de 70% do total de proteínas e as albuminas a cerca de 20% (GONZÁLEZ-MONTOYA; CANO-SAMPEDRO; MORA-ESCOBEDO, 2017).

As albuminas correspondem a cerca de 20% das proteínas de reserva do grão de leguminosas (GONZÁLEZ-MONTOYA; CANO-SAMPEDRO; MORA-ESCOBEDO, 2017). São proteínas de geralmente baixo peso molecular (5 – 80 kDa) que apresentam maior teor de cisteína e metionina quando comparada com as globulinas. Em contrapartida, algumas proteínas consideradas como constituintes antinutricionais fazem parte das albuminas, como os inibidores de protease e amilase, assim como as lectinas (SHEVKANI *et al*., 2019).

As globulinas correspondem a cerca de 70% da fração proteica encontrada em leguminosas, constituídas geralmente de duas proteínas principais, denominadas de globulina do tipo vicilinas e leguminas, usualmente classificadas em proteínas 7S e 11S, respectivamente, e uma minoritária, as globulinas 2S. As globulinas 7S

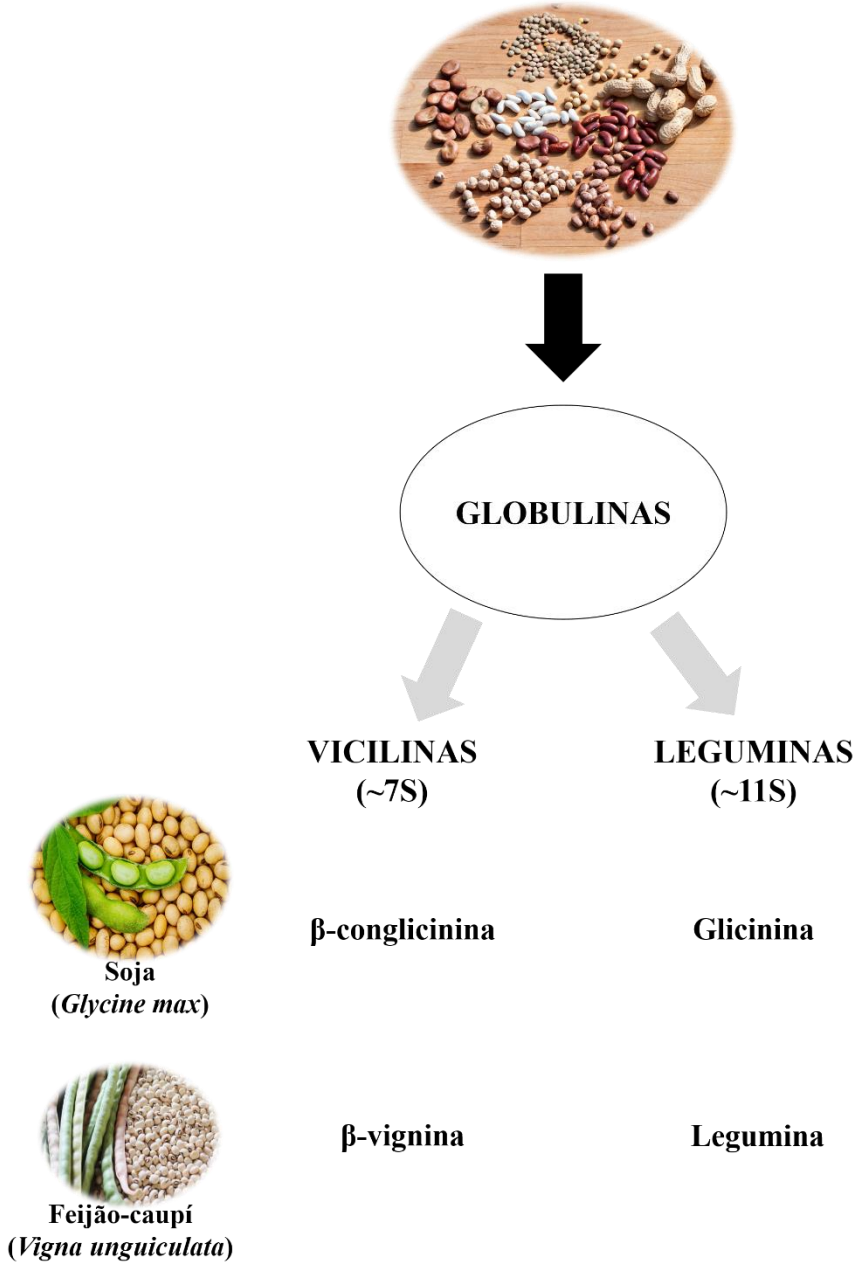
(vicilinas) são proteínas formadas por um trímero de cadeias polipeptídicas unidas por interações hidrofóbicas não covalentes de massa molecular entre 150-190 kDa, que apresentam subunidades glicosiladas entre 50-75 kDa e são a principal proteína de armazenamento em diversas espécies de feijão. As globulinas 11S (leguminas) são complexos oligoméricos com massa molecular entre 270-350 kDa, geralmente formados por 6 pares de subunidades. Cada subunidade de legumina compreende uma subunidade ácida maior de aproximadamente 40 kDa (situadas na superfície) e uma subunidade básica menor de aproximadamente 20 kDa (que formam o núcleo hidrofóbico interno) unidas por ligações dissulfeto. Leguminas geralmente têm maior quantidade de aminoácidos contendo enxofre, como metionina e cisteína, do que as vicilinas (KIMURA *et al*., 2008; SHEVKANI *et al*., 2019).

A depender da leguminosa, as vicilinas e leguminas recebem uma denominação específica relacionada ao gênero a que pertencem. Para leguminosas do gênero Glycine, como a soja, a vicilina e legumina são chamadas de β-conglicinina e glicinina, respectivamente. Para leguminosas do gênero Vigna, como o feijão-caupí, três vicilinas foram identificadas, denominadas de α-, β- e γ-vigninas (FREITAS; TEIXEIRA; FERREIRA, 2004). Destas, a β-vignina tem sido mais explorada por apresentar considerável semelhança com a β-conglicinina da soja (FERREIRA *et al*., 2018) e foi reportado que peptídeos derivados da sua hidrólise apresentam atividade hipocolesterolêmica *in vitro* (SILVA *et al*., 2018). As principais globulinas de leguminosas e suas respectivas nomenclaturas estão ilustradas na **Figura 6**.

Estudos sugerem que proteínas 7S encontradas em distintas sementes de leguminosas apresentam genes ancestrais comuns e que similaridades como sequência de aminoácidos, N-terminal, digestibilidade e atividade biológica são decorrentes de uma evolução genética convergente (GEPTS; BEAVIS; BRUMMER,

2005). Assim, proteínas de outras espécies que apresentem uma semelhança sequencial de aminoácidos a uma outra com ação já estabelecida têm maior probabilidade de exercer a mesma atividade (CAVAZOS; DE MEJIA, 2013).

Figura 6 – Principais globulinas de leguminosas e suas respectivas nomenclaturas na soja (*Glycine max*) e feijão-caupí (*Vigna unguiculata*).



Fonte: Autoria própria.

3.6 Soja (*Glycine max*)

A soja é uma planta da família *Fabaceae* (*Leguminosae*), subfamília *Faboideae* (*Papilionoideae*), gênero *Glycine* e espécie *Glycine max* e forma cultivada Glycine max (L.) Merrill. Em média, a soja é constituída de 40% de proteína, 35% de carboidratos, 20% de lipídios (PENHA *et al*., 2014). As principais proteínas de soja são conhecidas como β-conglicinina e glicinina, que respondem por 65% a 80% das proteínas totais. As proteínas são essenciais na dieta humana e seu valor biológico e nutricional depende da quantidade, digestibilidade, absorção e utilização dos aminoácidos que a compõem em cada alimento. Devido ao elevado teor de proteínas, a soja apresenta grande interesse na busca de peptídeos bioativos (DE MEJIA, LUMEN, 2006).

A glicinina corresponde a 37-45% do conteúdo proteico total da soja e a β-conglicinina (7S) representa 25-30% da proteína total da soja. Esta proteína tem massa molecular de aproximadamente 150 kDa, constituída, geralmente de um trímero de polipeptídios de 71, 67 e 50 kDa, representados pelas subunidades polipeptídicas alfa-prime (α'), alfa (α) e beta (β), respectivamente, onde todas essas apresentam sítios de N-glicosilação (THANH; SHIBASAKI, 1976). Diversos estudos relatam atividade antitumoral de peptídeos derivados de proteínas do soja (DE MEJIA, E.; LUMEN, 2006; RAYAPROLU *et al.,* 2017a), porém poucos estudos exploraram a atividade antitumoral da glicinina e β-conglicinina dessa oleaginosa (MONTALES *et al*., 2015; WANG *et al*., 2008).

3.7 Feijão-caupí (*Vigna unguiculata*)

O feijão-caupí é uma planta Dicotyledonea, da família Fabaceae, tribo Phaseoleae, gênero *Vigna* e espécie *Vigna unguiculata* (L.) (PADULOSI; NG, 1997).

Evidências sugerem que a origem da espécie unguiculata tenha ocorrido no continente Africano e posteriormente se dispersou para outros locais (FAOSTAT, 2019). No Brasil, o feijão caupí é cultivado predominantemente no sertão semiárido da região Nordeste e em pequenas áreas na Amazônia (SILVA *et al*., 2002) e apresenta diferentes denominações a depender da região, como feijão-de-corda e feijão-macassa na região Nordeste, feijão-de-praia, feijão-da-colônia e feijão-de-estrada no Norte e feijão-miúdo no Sul do País, apresentando grande importância tanto na alimentação quanto na geração de emprego e renda (FILHO, 2011).

A semente das espécies do gênero *Vigna* apresentam baixo teor de lipídeos (0,3-3%), considerável teor de carboidratos (50-65%), sendo parte destes compostos por fibras solúveis e insolúveis, assim como minerais importantes como cálcio, ferro e zinco. Dentre seus constituintes, destaca-se por apresentar elevado teor de proteínas, que varia entre 20-39% da sua constituição química, a depender do cultivar (SIVAKANTHAN *et al*., 2020). A fração majoritária da semente do feijão caupí é representada pelas globulinas, constituindo de 51 a 72% das proteínas totais (FERREIRA *et al*., 2018; FREITAS; TEIXEIRA; FERREIRA, 2004). Freitas, Teixeira e Ferreira (2004) isolaram e caracterizaram três globulinas da espécie *V. unguiculata*, e sugeriram a seguinte denominação para essas frações: γ (gama), β (beta) e α (alfa). A fração α apresenta uma cadeia majoritária de 80 kDa, e duas subunidades menores de 58 e 44 kDa. A fração γ, minoritária, apresenta uma única cadeia de 22 kDa, e devido ao baixo peso molecular foi caracterizada como uma proteína 2S. A fração majoritária β, apresenta duas subunidades polipeptídica principais de 60 e 55 kDa glicosiladas. Nenhum estudo foi conduzido até o momento com hidrolisados e frações peptídicas da β-vignina de feijão-caupi com atividade antiproliferativa em células tumorais.

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Capítulo I

*Manuscrito: **β**-conglicinin peptide fractions exerts inhibitor effect on the proliferation of MDA-MB-231, Hep-G2 and DU-145 cancer cells, in vitro.*

β-conglicinin peptide fractions exerts inhibitor effect on the proliferation of MDA-MB-231, Hep-G2 and DU-145 cancer cells, *in vitro*

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ABSTRACT

Peptides derived from soy proteins have received remarkable interest due to its potential antitumor activity.The major storage proteins from this legume are the globulins glycinin and β-conglycinin. Although several studies describe antitumor activity from soy hydrolysates and peptides from total protein, just a few explored the antitumor activity of glycinin and β-conglycinin and its peptides. Therefore, the purpose of this study was to assess the possible antiproliferative effect of glycinin and β-conglycinin hydrolysates and peptide fractions. β-conglycinin and glycinin were isolated, partially purified by chromatographic process and hydrolysed by sequential action (pepsin/pancreatin). The antiproliferative activity was investigated by Alamar Blue assay against human mammary adenocarcinoma (MDA-MB-231), human hepatocellular carcinoma (Hep-G2), prostate carcinoma (DU-145) cell lines. Both glycinin and β-conglycinin hydrolysates were not cytotoxic to non-cancer human cell HUVEC (concentrations 12.5-200 µg/mL), *in vitro*. β-conglycinin hydrolysate exhibited the highest antiproliferation activity (between 24 to 54%), compared glycinin (between 20 to 45%) against MDA-MB-231, Hep-G2 and DU-145 cell lines. The 10-3 kDa peptide fraction from β-conglycinin hydrolysate showed the strongest antiproliferative effect on MDA-MB-231 (between 15 to 63%, IC50 7.4 µg/mL) and DU-145 (between 33 to 60%, IC50 6.0 µg/mL), whereas the < 3 kDa fraction showed better effect against Hep-G2 (between 35 to 63%, IC50 5.7 µg/mL) cells, in vitro. In additional, the antiproliferative activity observed was in a dose-response manner. Future studies should focus especially to identify peptides responsible for its antiproliferative activity. Some of these issues are currently being explored in our laboratory.

Keywords: *Soybean proteins; β-conglicinin hydrolysate; antiproliferative activity; tumor cell lines.*

1 INTRODUCTION

Cancer corresponds to an uncontrolled cell division, causing tissue functional impairment and invasion to other regions of the organism, with about 19.2 million new cases and 9.9 million deaths in 2020 (FERLAY *et al.,* 2020). It is estimated that only 5 to 10% of cancer cases in general can be attributed to genetic inheritance, the other 90 to 95% being related to environmental factors (ANAND *et al.,* 2008). Among these factors, the diet is responsible for more than 35% of the cases (RUIZ; HERNANDÉZ, 2014). More recent studies have described that peptides derived from soy proteins appear to have an antiproliferative effect on tumor lines for colon (GONZÁLEZ-MONTOYA *et al.,* 2018), prostate (RAYAPROLU *et al.,* 2017) and breast (KUERBAN *et al.,* 2017) cancers, among others.

Such evidence gained notable interest after the identification of the lunasin peptide, which has shown to exert remarkable anti-tumor and anti-inflammatory activity (HSIEH *et al*., 2018). Lunasin is able to inhibit the expression of MMP-2 and MMP-9, via FAK/Akt/ERK and NF-κB signaling in breast cancer cells (JIANG *et al.,* 2016) and to inhibit 62.8% of cell proliferation at 100 μM with an IC_{50} of 61.7 μM, similar to cisplatin (IC_{50} = 76.7 μM) (DIA; DE MEJIA, 2010). Similar activities are described in leukemic (MEJIA; WANG; DIA, 2010), melanoma (SHIDAL *et al.,* 2017) and lung (MCCONNELL *et al.,* 2015) cancer cells.

The major storage proteins from soybeans are the globulins glycinin and β-conglycinin, categorized by their sedimentation coefficients as 11S and 7S, respectively, accounting for 70-80% of the total seed proteins (WANG *et al.,* 2014). Although several studies describe antitumor activity from soy hydrolysates and peptide fractions of total protein (CHEN *et al.,* 2019; GONZÁLEZ-MONTOYA *et al.,* 2016;

RAYAPROLU *et al.,* 2013), just a few explored the antitumor activity of glycinin and β-conglycinin proteins and its peptides.

Glycinin and β-conglycinin proteins in their integral form were evaluated for their cytotoxicity against the colon cancer cell HCT-116 at 3 μM, and glycinin showed more prominent cytotoxic activity than β-conglycinin (MONTALES *et al.,* 2015). However, when both were hydrolysed with pepsin/pancreatin and tested in leukemic cells (L1210) at concentrations of 0.3-8 mg/mL, the peptides generated by the hydrolysis of β-conglycinin were more cytotoxic than those generated by glycinin hydrolysis (WANG *et al.,* 2008).

Based on this previous body of highlights, we investigated the effect of glycinin and β-conglycinin hydrolysates by simulated gastrointestinal digestion (pepsin/pancreatin) on the proliferation of breast (MDA-MB-231), liver (Hep-G2) and prostate (DU-145) cancer cells, *in vitro*. The peptides generated by the hydrolysis of β-conglycinin were fractionated in different molecular sizes and were also tested in order to identify in which fraction the peptides responsible for the antiproliferative activity are present. At the best of our knowledge, this is the first report in which peptide fractions of the β-conglycinin hydrolysate were tested for their antiproliferative activity.

2 MATERIAL AND METHODS

2.1 Preparation of defatted soybean flour

The soybean seed (*Glycine max* L. Merr.) was obtained from a local supplier in the city of Salvador (State of Bahia, Brazil). Initially, the grains were selected and immersed in distilled water at 8 °C/12 h. Then, the cotyledon was separated from the epicarp manually, dehydrated in an oven with forced air circulation at 50 °C/12 h; then sprayed and sieved to 60 mesh. The whole soy flour was defatted using n-hexane in the

proportion of 1:8 (m/v), kept stirring for 4 hours at room temperature (25 °C) with repetition of the process in the proportion 1:6 (m/v) for another 4 hours after changing the solvent. Subsequently, filtration and drying were carried out in an oven with forced air circulation at 50 °C/10 h. The defatted flour was stored in a polyethylene container and kept refrigerated at 4 °C.

2.2 Isolation and gel chromatography of the proteins

The proteins glycinin (11S) and β-conglycinin (7S) were obtained according to procedures described by Nagano *et al.* (1992), with some adaptations and modifications (FERREIRA *et al.,* 2011). The isolated protein content was quantified by the Lowry, Rosebrough and Farr (1951) method using bovine serum albumin (Sigma Aldrich® St. Louis, MO, USA) as standard, by measuring absorbance in 750 nm. Aliquots of the isolated proteins (300 mg) were chromatographed on a Sepharose CL-6B column (1.0 x 100 cm), equilibrated with potassium phosphate buffer (10 mmol/L) containing NaCl (0.5 mol/L) and sodium azide (1 g/L). The elution profile was monitored by measuring absorbance in 280 nm. The peak corresponding to glycinin and β-conglycinin was collected, dialyzed and lyophilized for further analysis.

2.3 Simulation of gastrointestinal digestion and ultrafiltration

Samples of the β-conglycinin and glycinin proteins obtained by chromatography process were hydrolysed sequentially using pepsin (1:66 E/S) and pancreatin (1:25 E/S) following the procedures described by Akeson and Stahmann (1964). Briefly, both isolated proteins (200 mg) were hydrolysed by pepsin (enzyme/substrate ratio 1:66, 37 °C for 3 h, pH=2); the pH was neutralized, and then the hydrolysed proteins were further treated with pancreatin (enzyme/substrate ratio 1:25, 37 °C for 3 h, pH=7). The

hydrolysate obtained from soybean β-conglicinin were fractionated through Microcon® Centrifugal Filters (Merck Millipore, Germany) ultrafiltration membrane filters in peptides 30-10 kDa, 10-3 kDa and < 3 kDa.

2.4 Cell proliferation inhibition assay with dose response

The cytotoxicity tests of hydrolysates and peptide fractions was performed on human mammary adenocarcinoma (MDA-MB-231 – ATCC HTB-26), human hepatocellular carcinoma (Hep-G2 – ATCC HB-8065), prostate carcinoma (DU-145 – ATCC HTB-81) as well as normal human umbilical cord epithelial cell (HUVEC). The antiproliferative activity was quantified using the Alamar Blue assay, according to the method reported by Page, Page and Noel (1993). The cells were inserted into 96-well plates for all experiments (1.5 x 10^4 cells/well). After 24 h, the hydrolysates and peptide fractions were dissolved in Milli-Q water, added to each well and incubated at 37 °C in an atmosphere of 5% CO_2 for 24 hours. Complex dilutions were prepared to obtain concentrations ranging from 200 to 12.5 µg/mL. Methyl methanesulfonate at 300 µM was used as the reference cytotoxic drug (positive control). Mili-Q water (0.1% (v/v)) was used to control the vehicle. After 24 h of incubation, 50 µL of Alamar Blue (0.01% w/v resazurin) was added to each well, and the plates were incubated for 1 h at 37 °C in the dark. The fluorescence reading was performed on a CaryEclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA, USA), using excitation and emission filters at wavelengths of 530 and 590 nm, respectively. The cytotoxicity of each treatment was expressed by the percentage of cell viability, calculated in relation to the negative control. The cell viability (%) was expressed as half of the maximum inhibitory concentration (50%) (IC_{50}).

2.5 Statistical analysis

The results were evaluated through one-way analysis of variance (ANOVA) and Tukey's test for multiple comparison, using the software of SigmaStat version 3.5 (Systat software, California, USA). Statistically significance was shown at $p \leq 0.05$. All experiments were performed in triplicates.

3 RESULTS AND DISCUSSION

3.1 Effect of the glycinin and β-conglycinin total hydrolysates on cell lines

Cancer therapy aims to control proliferation of tumor cells without causing damage to healthy tissues. There are reports in the literature that protein hydrolysates from seeds do not show cytotoxicity in non-cancerous cells (CARRILLO *et al.,* 2017; LI *et al.,* 2019). However, Mora-Escobedo *et al.* (2009) reported that soy total protein hydrolysate in concentrations greater than 10 mg/mL were able to inhibit the viability of the HaCaT cells (from a non-cancerous human keratinocytes cell line), which is a much higher concentration than those used in the present study (12.5-200 µg/mL).

Nonetheless, both hydrolysates from glycinin and β-conglycinin proteins were screened through a cytotoxicity assay against the non-cancer human umbilical vein endothelial cells (HUVEC). None of the evaluated hydrolysates presented inhibitory effects on the cell growth of the HUVEC cells as shown in **Figure 1**. There was not a statistically significant difference between the cell viability of glycinin and β-conglycinin hydrolysates at any concentration evaluated and the negative control ($p > 0.05$). Glycinin and β-conglycinin hydrolysates were not cytotoxic to HUVEC cells in the concentrations evaluated, especially when compared to the positive control.

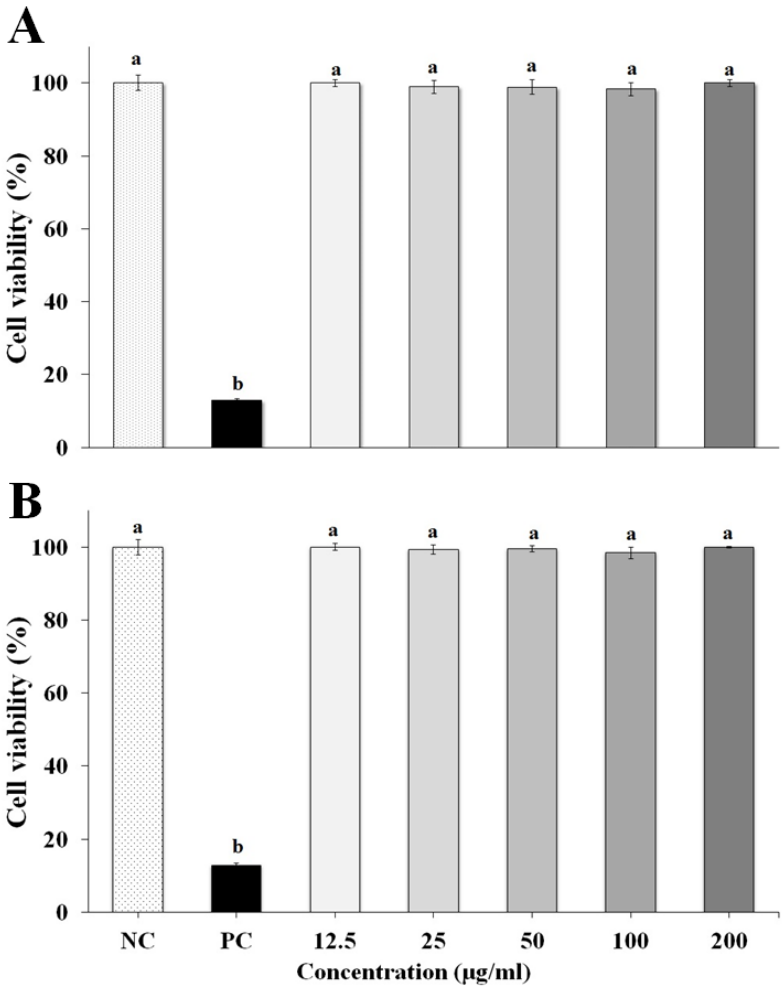


Figure 1 – HUVEC cell line growth treated with the glycinin (A) and β-conglycinin (B) protein hydrolysates. *Mean ± standard deviation (n=3) not connected with the same letters are significantly different (p value ≤ 0.05 by Tukey's multiple-range test)*. *NC: negative control treated with culture media only. PC: positive control treated with 300 µM methyl methanesulfonate.*

The inhibitory activity of glycinin (11S) and β-conglycinin (7S) protein hydrolysates against MDA-MB-231 cancer cells are shown in **Figure 2A**. Both protein hydrolysates and the negative control (culture media only) had a statistical difference ($p < 0.001$) with the positive control (methyl methanesulfonate 300 µM), which inhibited 92.3% of the MDA-MB-231 cells (**Table 1**).

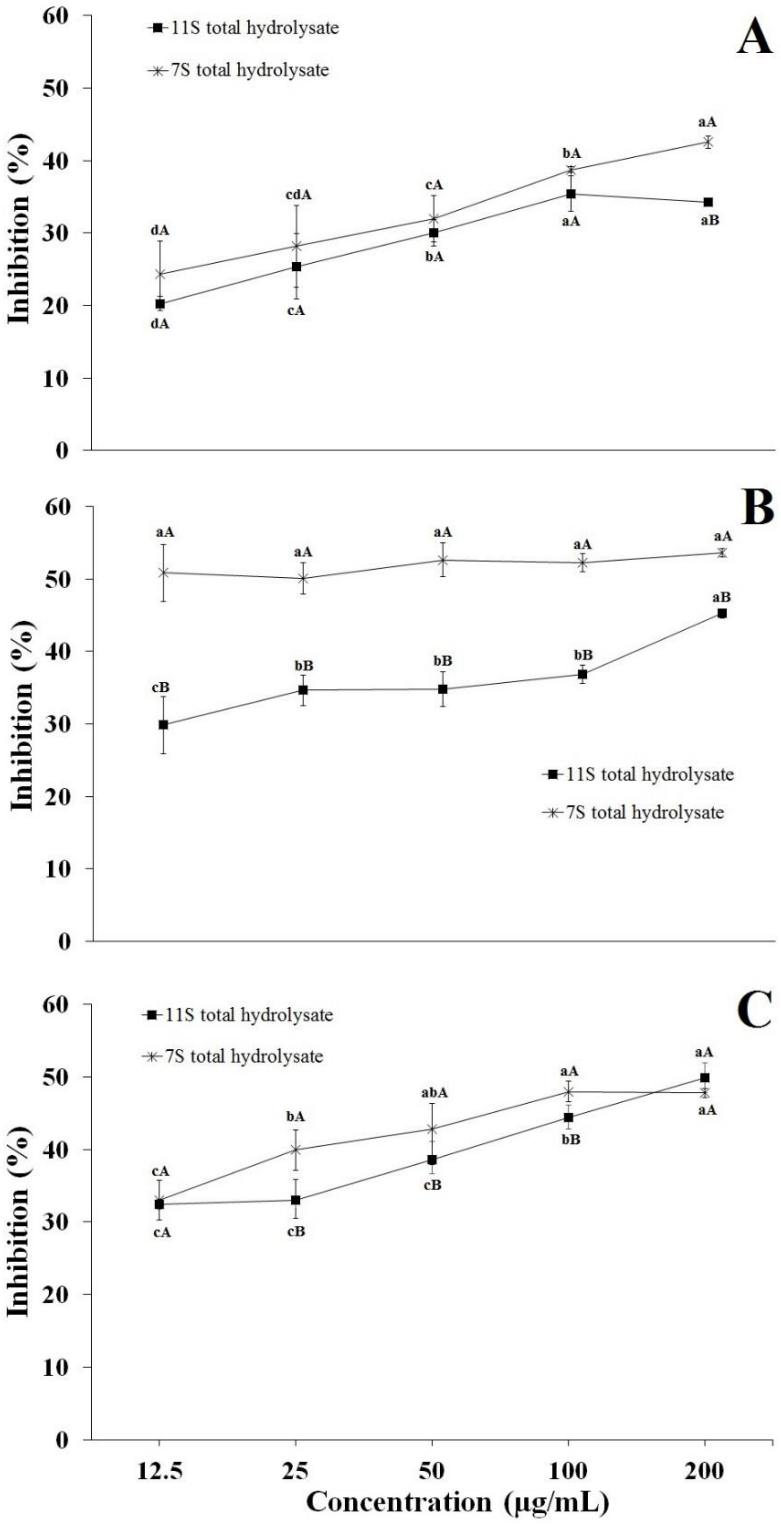


Figure 2 – Antiproliferative effect of glycinin (11S) and β-conglycinin (7S) protein hydrolysates against MDA-MB-231 (A), Hep-G2 (B) and DU-145 (C) cancer cells. *Mean ± standard deviation (n=3) with lower case letters indicate difference between*

192 *concentrations of the same fraction and capital letters indicate difference between*
193 *fractions at the same concentration (p value ≤ 0.05 by Tukey's multiple-range test)*.
194
195 The antiproliferative activity of the 7S hydrolysate was more proeminent in
196 comparison with the 11S hydrolisate at all concentrations, inhibiting 42.5% and 34.23%
197 of MDA-MB-231 cells at 200 µg/mL, respectively. However, there was no statistical
198 difference between the two treatments ($p > 0.05$) in concentrations up to 100 µg/mL. It
199 has been described that at concentrations of 3 mg/mL and superior, 7S hydrolysate
200 shows significantly higher citotoxicity than 11S hydrolysate in L1210 leukemia cells
201 (WANG *et al.,* 2008).
202 The inhibitory activity (%) of soybean protein hydrolysates against Hep-G2
203 cancer cells are illustrated in Figure 2B. Both protein hydrolysates and the negative
204 control had a statistical difference ($p < 0.001$) with the positive control, which inhibited
205 96.8% of Hep-G2 cells (**Table 1**). In Hep-G2 cells, the 7S hydrolysate presented
206 stronger ($p < 0.05$) antiproliferative activity compared to the 11S hydrolysate at all
207 concentrations, corroborating with the findings by Wang *et al.* (2008), that also found
208 that 7S hydrolisates from soybean were more cytotoxic than 11S hydrolysate in
209 leukemia cells (L1210) in concentrations higher than 3 mg/mL. Differences in the
210 cytotoxicity might be explained partially by different amino acid composition – since 7S
211 soybean protein contains more acidic, basic and aromatic amino acids while 11S
212 soybean protein constains more sulfur amino acids (MAHMOUD *et al.,* 2006). It also
213 seems to depend on the tumor cell tested, considering that in the present study no
214 statistical difference ($p > 0.05$) was observed between 7S and 11S hydrolysates in
215 breast cancer cells in concentrations up to 100 µg/mL.

The inhibitory activity (%) of soybean proteins hydrolysate against DU-145 cancer cells are shown in Figure 2C. All protein hydrolysates and the negative control had a statistical difference ($p < 0.001$) with the positive control, which inhibited 95.9% of the DU-145 cells (**Table 1**). There was statistical difference ($p < 0.05$) in the effects observed to 7S and 11S hydrolysates treatments on the DU-145 cancer cells proliferation at 25, 50 and 100 µg/mL, in which the 7S hydrolysate presented stronger inihibitory activity.

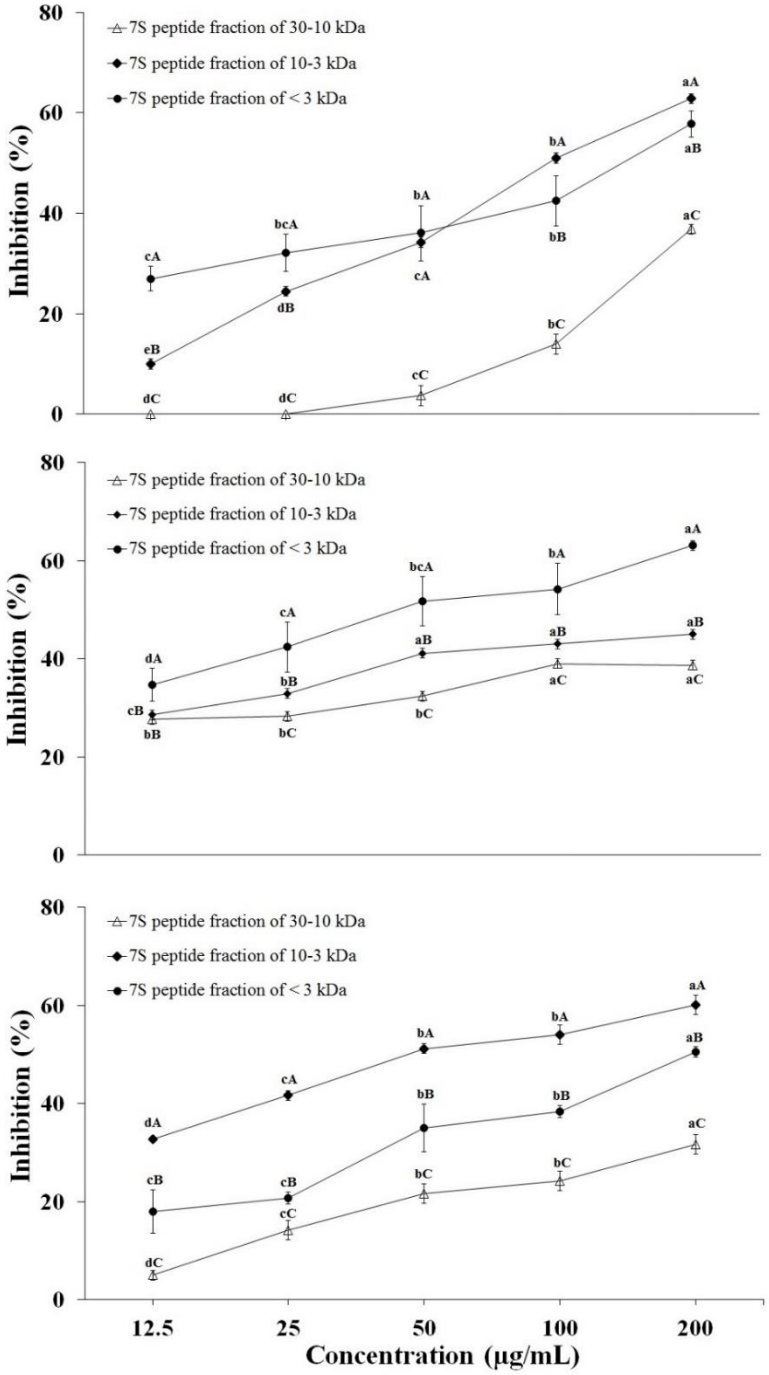
Some legume hydrolysates have been tested against any cancer cell lines, i*n vivo*. Chickpea albumin hydrolysate with flavorzyme were evaluated in mice with H-22 cells (liver carcinoma cell line). It was observed that the tumor volume of the mice treated with the hydrolysate was significantly less ($p < 0.05$) than control group. Furthermore, few scattered tumor cells were seen in the liver sections of the animals treated at a dose of 100 mg/kg (XUE *et al.,* 2012). Total protein of mung beans hydrolysed with papain were also tested on the same cell line (H22). 5-Fluorouracil (positive control) caused liver and kidney damage by exerting its therapeutic effect, while MPH significantly decreased liver impairment caused by tumors in mice ($p < 0.05$). *In vitro*, the maximum rate of Hep-G2 cell inhibition of 92.01% was reached at 16 mg/mL MPH after 72 h of co-culture. The apoptotic rate increased with increasing dose of MPH, as well as blocked the cell cycle in phase S at a low dose and in phase G0/1 at a high dose (8 mg/mL). The fraction of peptides that showed the highest activity (86.35% inhibition) was evaluated for the sequence of its peptides, and four small peptides were identified: VEG, PQG, LAF and EGA (LI *et al.,* 2019).

Further studies are needed in order to evaluate the mechanism of action of hydrolysates as well as identify which individual peptides are responsible for its activity. In the present study, the results showed a more promising effect in peptides generated

241 by 7S hydrolysate, although in some cell lines no statistical differences were observed.
242 Therefore, the 7S hydrolysate was fractionated in peptides 30-10 kDa, 10-3 kDa and
243 < 3 kDa and tested against MDA-MB-231, Hep-G2 and DU-145 cancer cells.
244
245 **3.2 Effect of β-conglycinin peptide fractions on cancer cell lines**
246 The inhibitory activity (%) of soybean peptide fractions against breast cancer
247 cells are shown in **Figure 3A**. The highest inhibitory effect in the present study was
248 found in the 7S 10-3 kDa peptide fraction at the maximum concentration evaluated
249 (200 µg/mL), inhibiting 62.8% of breast cancer cells, which had statistical difference ($p < 0.05$)
250 from the 7S < 3 kDa peptide fraction at the same concentration (57.8%
251 inhibition). The minimum concentration to cause 50% inhibitory activity (IC_{50}) for the
252 10-3 kDa and < 3 kDa fractions was found to be 7.4 µg/mL and 8.6 µg/mL, respectively,
253 which is lower compared to other studies that evaluated citotoxicity of peptide fractions
254 from total protein hydrolysate from soybean. Chen *et al.* (2019) showed that the peptide
255 fraction < 4 kDa of the total soy protein hydrolysate with alcalase inhibited MCF-7
256 breast cancer cells with an IC_{50} of 276 µg/mL. Rayaprolu *et al.* (2017) showed that the
257 10-5 kDa peptide fraction of the total soy protein hydrolysate with
258 pepsin/pancreatin/alcalase inhibited the MCF-7 cells with an IC_{50} of 654 µg/mL.
259 The current results are similar to those showing that shorter peptides from
260 protein hydrolysates usually exert greater anticancer activity than larger peptides.
261 Chen *et al.* (2019) reported that peptide fraction < 4 kDa from black soybean showed
262 significant ($p < 0.05$) antiproliferative effect on breast cancer cell (MCF-7) compared to
263 other high molecular weight peptides (4-6 kDa and > 6 kDa fractions). Zhang and Mu
264 (2018) as well revealed that peptides < 3 kDa from sweet potato protein hydrolysate

265 showed the strongest antiproliferative activity ($p < 0.05$) compared to 3-5 kDa, 5-10
266 kDa and > 10 kDa fractions against colon cancer cells (HT-29).

267



268

269 **Figure 3 –** Antiproliferative effect of peptide fractions from soybean β-conglycinin (7S)
270 against MDA-MB-231 (A), Hep-G2 (B) and DU-145 (C) cancer cells. *Mean ± standard*
271 *deviation (n=3) with lower case letters indicate difference between concentrations of*

the same fraction and capital letters indicate difference between fractions at the same concentration (p value ≤ 0.05 by Tukey's multiple-range test).

Nevertheless, other studies report that peptide fractions with higher molecular weight may exert important cytotoxicity against some cancer cell lines. Peptides between 5-10 kDa derived from soybean total protein isolate, due to the sequential enzymatic action of alcalase/pepsin/pancreatin, showed a significantly high activity among all fractions (50-10 kDa and < 5 kDa) with 63% inhibition of breast cancer cells MCF-7 at 800 µg/mL (RAYAPROLU *et al.,* 2017). González-Montoya *et al.* (2016) found that the > 10 kDa peptide fraction and the total protein hydrolysated with pepsin/pancreatin from soybean germinated for 6 days showed greater cytotoxicity and antioxidant activity compared with 10-5 kDa and < 5 kDa fractions, even suggesting a close relationship between both activities. These reports were not in agreement with the present findings regarding peptides from 7S hydrolysate. Larger peptides (30-10 kDa) showed the lowest inhibitory activity compared to the other treatments, inhibiting 36.8% of the cells at 200 µg/mL without showing any activity at lower concentrations (25 and 12.5 µg/mL).

Since 10-3 kDa and < 3 kDa fractions presented antiproliferative activity superior to 7S hydrolysate, it is possible that shorter peptides are the ones responsible for this biological effect of 7S hydrolysate. Even though they had important inhibitory effect when tested isolated, when together in the total hydrolysate the antiproliferative activity was less proeminent. These results suggest that bioactive peptides with appreciable inhibitory effect on MDA-MB-231 cells might be present in the 7S 10-3 kDa and < 3 kDa peptide fractions from 7S hydrolysate.

All the 7S peptide fractions presented inhibitory effects on cell growth in liver cancer cells (**Figure 3B**). The highest inhibitory effect was found in the < 3 kDa peptide fraction at the 200 µg/mL concentration evaluated, inhibiting 63.1% of liver cancer cells, which had statistical difference ($p < 0.05$) compared to all the other treatments at any concentration evaluated. It has been described that short peptides from soybean total protein hydrolysed by trypsin can inhibit the growth of Hep-G2 cells. QRPR and HCQRPQ peptides individually, and combined had an antiproliferative effect of at least 18%, 39% and 60%, respectively, on liver cancer cells Hep-G2 at 1000 µM. The combination of QRPR and HCQRPQ peptides significantly promoted cell apoptosis, increased the number of cells in phase G1 by 52.44% at 800 µM, caspase-3 and caspase-8 mRNA expression in 4.7-fold and 4-fold compared to the control group at 800 µM (72h), respectively. The study showed that mixed soybean peptides had a higher inhibitory effect on Hep-G2 cells than each peptide alone (PAN *et al.,* 2018).

Again, larger peptides (30-10 kDa) showed weaker inhibitory activity compared to the others peptide fractions (10–3 kDa and < 3 kDa), inhibiting 27.7% at the maximum concentration (200 µg/mL). Peptides of intermediate size (10–3 kDa) did not show antiproliferative activity as intense as the smaller peptides (< 3 kDa). The < 3 kDa fraction was the only one with higher inhibitory effect than the 7S hydrolysate (at 200 µg/mL). Therefore, it is possible to say that < 3 kDa peptides are the ones responsible for this biological activity of 7S hydrolysate. These results suggest that bioactive peptides with appreciable inhibitory effect on Hep-G2 cells might be present in the 7S < 3 kDa peptide fraction.

In the present study, the antiproliferative effect on DU-145 cell line exerted by 7S peptide fractions is presented in **Fig. 3C**. The highest inhibitory effect was in the 7S 10-3 kDa peptide fraction at the 200 µg/mL concentration that inhibited 60.1% of

prostate cancer cells, which had statistical difference ($p < 0.05$) from the 7S < 3 kDa peptide fraction at the same concentration. These results suggest that bioactive peptides with the appreciable inhibitory effect on DU-145 cells might be present in the 7S 10-3 kDa peptides.

Rayaprolu *et al.* (2017) described similar findings on prostate cancer cell PC-3, in which the 5–10 kDa fraction from the S03-543CR soybean line from hydrolysis of total soybean protein with alcalase/pepsin/pancreatin showed the highest reduction on cell counts (63%) compared to 50–10 kDa (approximately 15%) and < 5 kDa fractions (approximately 20%) at 800 µg/mL. González-Montoya *et al.* (2018) found that 5–10 kDa peptides showed greater potency (IC_{50}=11.7 mg/mL) to inhibit colon cancer cells (Caco-2) proliferation compared to > 10 kDa (IC_{50}=13.2 mg/mL) and < 5 kDa peptides (IC_{50} > 15 mg/mL) in concentrations ranging from 2–15 mg/mL. In our study, once again 30–10 kDa peptide fraction was the one with the weakest antiproliferative activity, inhibiting 31.6% of cells at the maximum concentration (200 µg/mL).

Researchers have explored the antiproliferative activity from proteins, hydrolysates and peptides against prostate cancer cells. Glutelin proteins from walnuts inhibited PC-3 prostate cancer cells in a dose-dependent manner with IC_{50} value of 43.9 µg/mL, although globulins were not effective to inhibit the growth against any cancer cell tested (CARRILLO *et al.,* 2017). Peptides from soybean total protein hydrolysed with trypsin was cytotoxic to PC-3 cells as well, with IC_{50} of 3.0 mg/mL (KUERBAN *et al.,* 2017). The peptide ILYMP, isolated from the protein hydrolysate of *Cyclina sinen* (a bivalve mollusk) exhibited cytotoxicity against DU-145 cells in a dose-dependent manner, with an inhibition rate of 84.1% at 22.5 mM at the 72 h time interval (IC_{50} of 11.2 mM). It also enhanced the expression of cleaved caspase-3 and caspase-9 and suppressed B-cell lymphoma 2 expression (YU *et al.,* 2018).

346 Further studies are needed to explore the antiproliferative activity of peptides
347 in prostate cancer cells, especially those derived from legumes.
348 The overall results from the study show that intermediate (10–3 kDa) and
349 smaller (< 3 kDa) peptides from β-conglicinin hydrolysate had better inhibitory activity
350 against breast, liver and prostate cancer cells (**Figure 3**). 7S 10–3 kDa peptide fraction
351 treatment presented higher antiproliferative activity in concentrations above 100 µg/mL
352 against MDA-MB-231 cells and in all concentrations for DU-145 cells. For Hep-G2
353 cells, however, the 7S < 3 kDa fraction showed better activity in all concentrations
354 evaluated.
355 The antiproliferative effect of the β-conglycinin peptides fractions were
356 assessed *in vitro* by determining the percentage inhibition of growth of the cell lines,
357 which is recognized as a mechanism for findinding new antitumoral agents. The results
358 of the inhibition assays are shown in **Table 1**. The peptide fractions exerted a dose-
359 dependent inhibitory effect on cancer cells line *in vitro*, with a 50% inhibitory
360 concentration (IC_{50}) of 5.47 µg/mL, 7.4 µg/mL and 6.0 µg/mL, to < 3 kDa fraction on
361 Hep-G2, and 10-3 kDa fraction on MDA-MB-231 and DU-145, respectively (**Figure 4**).
362 It was observed a dose-response correlation in breast (R^2 = 0.9884) and prostate (R^2
363 = 0.9654) cells when subjected to peptides 10-3 kDa. And for liver (R^2 = 0.9789) cancer
364 line when subjected to peptides < 3 kDa. Dose-response correlation from soybean
365 peptides has been described but usually in higher concentrations than those used in
366 this study. Rayaprolu *et al.* (2013) identified a dose-response effect in 10–50 kDa
367 peptide fraction of N98-4445A soy line on HCT-116 colon cancer cell line in
368 concentrations from 100–1000 µg/mL. Chen *et al.* (2017) showed a dose-response
369 effect especially in the range of 200–600 µg/mL of isolated proteins from soybean,
370 black soybean, adzuki bean and mung bean in ovarian (SKOV3) and liver (SMMC-
371 7721) cancer cells.

372 **Table 1** – Inhibition of cancer cell lines growth treated with the β-conglycinin peptide fractions.

Peptide fraction/cell lines	[ ] of inhibitor (µg/mL)	**MDA-MB-231**		**Hep-G2**		**DU-145**	
		Inhibition (%)	IC_{50} (µg/mL)	Inhibition (%)	IC_{50} (µg/mL)	Inhibition (%)	IC_{50} (µg/mL)
Control	-	0.0	-	0.0	-	0.0	-
methyl methanesulfonate	300 µM	92.3	-	96.8	-	95.9	-
30-10 kDa	12.5–200	0–34.4	20.9	27.7–38.7	25.5	8.0–31.6	28.1
10-3 kDa	12.5–200	15.0–62.8	7.4	28.6–45.3	10.4	32.7–60.1	6.0
< 3 kDa	12.5–200	27.0–57.7	8.6	34.7–63.1	5.7	18.0–50.5	10.3

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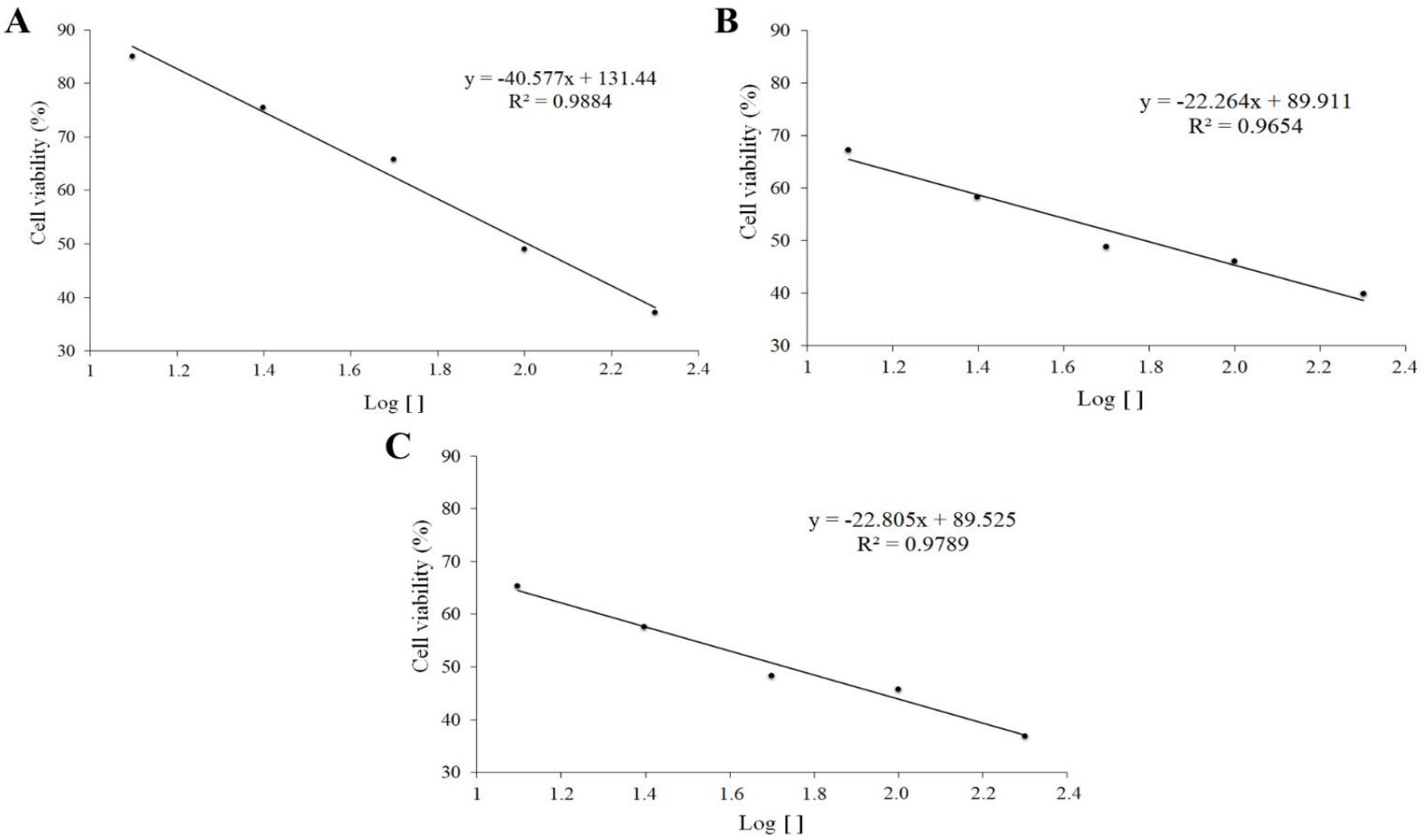

374

375 **Figure 4 –** Concentration-response effect of 10-3 kDa fraction on MDA-MB-231 (A) and DU-145 (B), and < 3 kDa fraction on Hep-G2

376 (C) from β-conglycinin hydrolysate (*n* = 3).

4 CONCLUSION

In the presente study, β-conglycinin peptide fractions prepared by pepsin/pancreatin hydrolysis showed certain inhibition effect on the proliferation of MDA-MB-231, Hep-G2 and DU-145 cells, without affecting the growth of normal cells (HUVEC). β-conglycinin hydrolysate exhibited the highest antiproliferation activity (between 24 to 54%) to MDA-MB-231 (breast), Hep-G2 (hepatocellular), DU-145 (prostate) tumor cell lines, from which peptide 10-3 kDa fraction showed the strongest antiproliferative effect on MDA-MB-231 (between 15 to 63%, IC_{50} 7.4 µg/mL) and DU-145 (between 33 to 60%, IC_{50} 6.0 µg/mL), whereas the < 3 kDa fraction showed better effect against Hep-G2 (between 35 to 63%, IC_{50} 5.7 µg/mL) cells. In additional, the antiproliferative activity observed was in a dose-response manner. Future studies should focus especially to identify peptides responsible for its antiproliferative activity. Some of these issues are currently being explored in our laboratory.

ACKNOWLEDGMENTS

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STATEMENT OF CONFLICT OF INTEREST

All the authors declare no conflict of interest about the described research, the publication of the result and financial issues.

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Capítulo II

*Manuscrito: Cowpea **β**-vignin (7S globulin) hydrolysates and peptide fractions inhibit human breast and liver cancer cell proliferation, in vitro*

Cowpea β-vignin (7S globulin) hydrolysates and peptide fractions inhibit human breast and liver cancer cell proliferation, *in vitro*

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ABSTRACT

Several studies indicate that legume protein hydrolysates are good sources for obtaining antitumor peptides, but not many have sought to investigate the biological activity of peptides derived from bean vicilins with antiproliferative activity in cancer cells. Hence, the purpose of this work was to investigate the possible antiproliferative effect of β-vignin hydrolysates and peptide fractions from cowpea bean. β-vignin was isolated, purified by size exclusion chromatographic process and hydrolysed using different enzymatic sistems: (i) pepsin; (ii) trypsin; (iii) pepsin/pancreatin and (iv) alcalase/pepsin. The trypsin hydrolysate (IC_{50}=3.02 µg/mL) exhibited the highest antiproliferation activity (90.73%) at 200 µg/mL in breast cancer cells, which had no statistical difference from the other hydrolysates at the same concentration. And the pepsin hydrolysate (IC_{50}=3.71 µg/mL) exhibited the stronger antiproliferation activity (94.55%) at 200 µg/mL, and again had no statistical difference from the other hydrolysates at the same concentration. Taking into account results from studies that used gastrointestinal simulation to generate peptides with anticancer properties, we decided to investigate the cytotoxic effect of peptide fractions from pepsin/pancreatin hydrolysate. The 10-3 kDa peptide fraction (60.45-67.68%, IC_{50}=0.62 µg/mL) presented better effect against breast cancer cells, while 30-10 kDa peptide fraction (23.73-48.44%, IC_{50}=10.63 µg/mL) had the best inhibitory effect on Hep-G2 cells. Further work is needed to characterize these anticancer peptides, which has been currently explored by our research group.

Keywords: *Vigna unguiculata*; pepsin/pancreatin enzymes; bioactive peptides; antiproliferative activity; tumor cell lines.

1 INTRODUCTION

Globally, the odds of developing cancer during a lifetime (ages 0-79 years) is 1 in 3 for men and 1 in 4 for women (FITZMAURICE *et al*., 2019). This disease was responsible for about 9.9 million deaths worldwide in 2020 (FERLAY *et al*., 2020) and has been characterised by mutations of somatic genes that alters the cellular function (MARQUS; PIROGOVA; PIVA, 2017). Inhibition of deregulated cell proliferation is a common strategy for treating malignant tumors and among the different options in cancer therapy; chemotherapy is still the most common method. However, it causes several side effects since it also affects healthy tissues (BUKOWSKI; KCIUK; KONTEK, 2020). Therefore, new antineoplastic agents are sought from natural sources, such as food peptides, described to have better selectivity and, consequently, less side effects (HERNÁNDEZ-LEDESMA; HSIEH, 2017).

Several studies indicate that legume protein hydrolysates are good sources for obtaining bioactive peptides that exhibit therapeutic potential for several pathologies (BECERRA-TOMÁS *et al*., 2018; SILVA *et al*., 2018; JAKUBCZYK *et al*., 2017), including cancer (DIA; DE MEJIA, 2013; LUNA-VITAL; DE MEJÍA; LOARCA-PIÑA, 2016; GUPTA; SRIVASTAVA; BHAGYAWANT, 2018). Among legumes, soybean proteins (*Glycine max*) have been considerably studied for the presence of antitumor peptides derived from their hydrolysis (PAN *et al*., 2018). Soybean lunasin, a peptide of 43 amino acid residues originally isolated from the 2S albumin, has been shown to exert remarkable anti-tumor and anti-inflammatory activity (HSIEH *et al*., 2018), as well as protective activity against oxidative stress (FERNÁNDEZ-TOMÉ *et al*., 2014).

Proteins and peptides from other legumes such as chickpeas (XUE *et al*., 2015) and from various bean species, such as common bean (LUNA-VITAL; DE MEJÍA; LOARCA-PIÑA, 2016), mungbean (GUPTA; SRIVASTAVA; BHAGYAWANT,

2018), ayocote bean (TENIENTE-MARTÍNEZ *et al*., 2019) and cowpea bean (THUMBRAIN *et al*., 2020) have also shown to exert anti-tumor activity.

Previous studies with the total protein extract and β-vignin from cowpea beans have been performed to investigate its biological activity. Recently, the Gln-Asp-Phe peptide, derived from the cowpea β-vignin protein, has shown to exert remarkable hypocholesterolemic activity, inhibiting the enzyme HMG-CoA reductase, a key enzyme in the production of endogenous cholesterol (SILVA *et al*., 2018). Nevertheless, no study has been conducted so far on cowpea β-vignin hydrolysates and peptide fractions with antiproliferative activity in tumor cells. In the present study, we showed the antiproliferative effect exerted by hydrolysates and peptide fractions obtained from cowpea β-vignin, against breast (MDA-MB-231) and liver (Hep-G2) cancer cell, *in vitro*.

2 MATERIAL AND METHODS

2.1 Material

The seed of cowpea (*Vigna unguiculata* L.) was obtained from the Northeast region of the State of Bahia, kindly provided by the Bahiana Agricultural Development Company. The grains were selected and immersed in distilled water at 8 °C/12 h. Then, the cotyledon was separated from the epicarp manually, dehydrated in an oven with forced air circulation at 50 °C/10 h; then sprayed and sieved to 60 mesh.

2.2 Isolation and chromatography procedure

The β-vignin protein were obtained according to previously established separation and isolation procedures (FERREIRA *et al.,* 2015). The cowpea flour was homogenized in NaCl 0.1 mol/L (1:20 m/v), pH 7.5. Then, the material was centrifuged. The supernatant

was diluted, homogenized, pH adjusted to 5.0 and left overnight. Subsequently, it was centrifuged and the precipitate was solubilized in water (1:20 m/v), homogenized, pH adjusted to 7.0, kept under stirring for 10 minutes. Soon after, the material was centrifuged. The precipitate was solubilized in NaCl 0.1 mol/L (1:20 m/v), pH 7.5, homogenized, kept under stirring for 20 minutes and centrifuged. The supernatant was diluted (1x), homogenized, pH adjusted to 5.0 and left to stand overnight. Subsequently, it was centrifuged and the precipitate (β-vignin) was solubilized in potassium phosphate buffer (10 mmol/L). Samples of the isolated β-vignin were purified by size exclusion chromatography through Sepharose CL-6B column (1.0 x 100 cm), equilibrated with potassium phosphate buffer (10 mmol/L) containing NaCl (0.5 mol/L) and sodium azide (1 g/L). The elution profile were monitored by measuring the absorbance at 280 nm. The peak corresponding to the β-vignin were collected, dialysed and lyophilized. The protein was quantified by the method of Lowry, Rosebrough and Farr (1951), using bovine serum albumin as a standard, through the measurement of absorbance at 750 nm.

2.3 Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The total protein isolate, total globulin, β-vignin isolated and β-vignin obtained by exclusion chromatography were analyzed by SDS-PAGE according to the method described by Laemmli (1970) in polyacrylamide gel (12 g/100 g) with sodium dodecyl sulfate (0.1 g/100 g). The pepsin/pancreatin hydrolysate and its peptide fractions were analyzed according to the same method methodology in polyacrylamide gel (20 g/100 g). The gels were stained in a Coomassie brilliant blue solution (R-250), bleached with methanol/acetic acid/water (1:1:8 v/v/v). The images weree digitized and analyzed using the AlphaEase software (Alpha Innotech®, San Leandro, USA).

2.4 Simulation of gastrointestinal digestion and hydrolysate fractionation

The β-vignin protein obtained by chromatographic process were hydrolysed using (a) pepsin (1:66 E/S); (b) trypsin (1:10 E/S), (c) pepsin (1:66 E/S)/pancreatin (1:25 E/S) and (d) alcalase (1:10 E/S)/pepsin (1:66 E/S) following the procedures described by Akeson and Stahmann (1964). The pepsin/pancreatin hydrolysate obtained from cowpea β-vignin were fractionated through Microcon® Centrifugal Filters ultrafiltration membrane filters (Merck Millipore, Germany) in peptides 30-10 kDa, 10-3 kDa and < 3 kDa.

2.5 Cytotoxicity assay

The cytotoxicity tests of hydrolysates and peptide fractions was performed on human mammary adenocarcinoma (MDA-MB-231 – ATCC HTB-26), human hepatocellular carcinoma (Hep-G2 – ATCC HB-8065), prostate carcinoma (DU-145 – ATCC HTB-81) as well as normal human umbilical cord epithelial cell (HUVEC). The antiproliferative activity was quantified using the Alamar Blue assay, according to the method reported by Page, Page and Noel (1993). The cells were inserted into 96-well plates for all experiments (1.5 x 10^4 cells/well). After 24 h, the hydrolysates and peptide fractions were dissolved in Milli-Q water, added to each well and incubated at 37 °C in an atmosphere of 5% CO_2 for 24 hours. Complex dilutions were prepared to obtain concentrations ranging from 200 to 12.5 µg/mL. Methyl methanesulfonate at 300 µM was used as the reference cytotoxic drug (positive control). Mili-Q water (0.1% (v/v)) was used to control the vehicle. After 24 h of incubation, 50 µL of Alamar Blue (0.01% w/v resazurin) was added to each well, and the plates were incubated for 1 h at 37 °C in the dark. The fluorescence reading was performed on a CaryEclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA, USA), using excitation and

emission filters at wavelengths of 530 and 590 nm, respectively. The cytotoxicity of each treatment was expressed by the percentage of cell viability, calculated in relation to the negative control. The cell viability (%) was expressed as half of the maximum inhibitory concentration (50%) (IC_{50}).

2.6 Statistical analysis

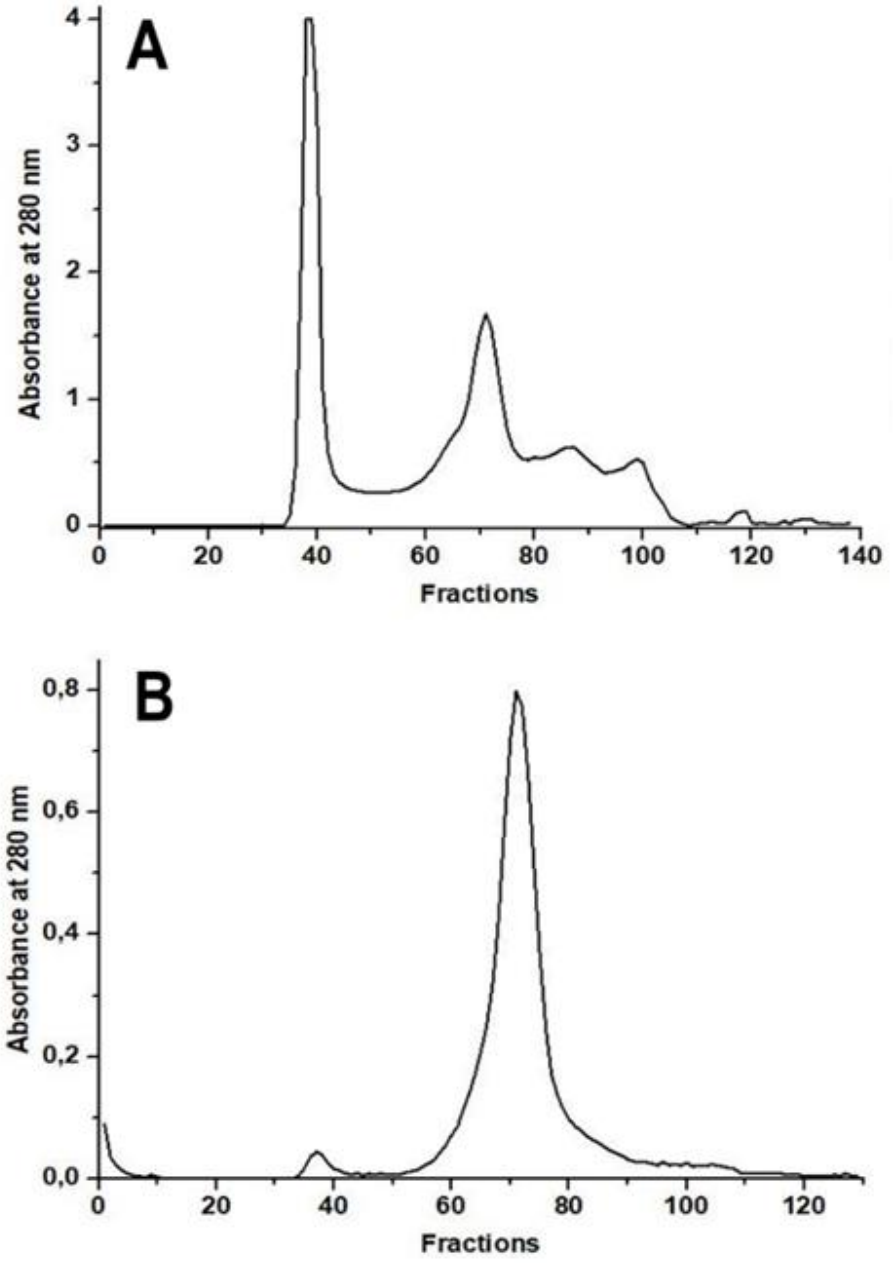
The results were evaluated through one-way analysis of variance (ANOVA) and Tukey's test for multiple comparison, using the software of SigmaStat version 3.5 (Systat software, California, USA). Statistically significance was shown at $p \leq 0.05$. All experiment was performed in triplicates.

3 RESULTS AND DISCUSSION

3.1 Isolation and purification of the β-vignin protein

Cowpea bean (*Vigna unguiculata* (L.)) is a legume seed consisting of 20-39% of protein (SIVAKANTHAN *et al*., 2020). Globulins are considered the major protein fraction in cowpea seeds, representing 51-72% of the total protein (FERREIRA *et al*., 2018). Vicilin-type globulins are called vignins, term used by several authors to designate the 7S globulin from seeds of the genus *Vigna*. The β-vignin subunit is a major individual globulin (FREITAS; TEIXEIRA; FERREIRA, 2004). In addition, the β-vignin protein is a major cowpea storage protein.

The flour obtained from cowpea bean seed was used to obtain the protein of interest and its isolation was according to the methodology proposed by Ferreira *et al.* (2015). Afterwards, the isolated β-vignin was purified by size exclusion chromatography through Sepharose CL-6B column and its chromatographic profile is illustrated in **Figure 1A.**



178

179 **Figure 1 –** Size exclusion chromatography profile of β-vignin protein isolated (A) and
180 the peak corresponding to β-vignin protein (B) from cowpea bean.

181

182 It is possible to observe the presence of two major peaks. Protein quantification
183 and SDS-PAGE analysis of peak 1 showed that this first peak corresponds to a low
184 molecular weight non-protein chemical compound (data not shown). Studies carried
185 out by other authors have shown that the component in question would be related to
186 phenolic compounds that also present detection at 280 nm (NEVES *et al*., 2009). The

second peak corresponds to cowpea β-vignin protein, which was collected, homogenized, concentrated and purified by size exclusion chromatography. Its chromatographic profile is illustrated in **Figure 1B**, where it is possible to observe the presence of only one peak related to β-vignin protein. This result was confirmed by SDS-PAGE under denaturing conditions, as shown in **Figure 2**.

As seen in the SDS-PAGE profile (**Figure 2**), lane 1 shows the total protein isolate of cowpea and it is possible to perceive, even at low resolution, the presence of several bands, ranging from 20 kDa to 97 kDa. Lane 2 illustrates the total globulin fraction, in which the 56 kDa, 60 kDa and 35 kDa bands are evident, and represent β-vignin (7S) (FERREIRA *et al*., 2018).

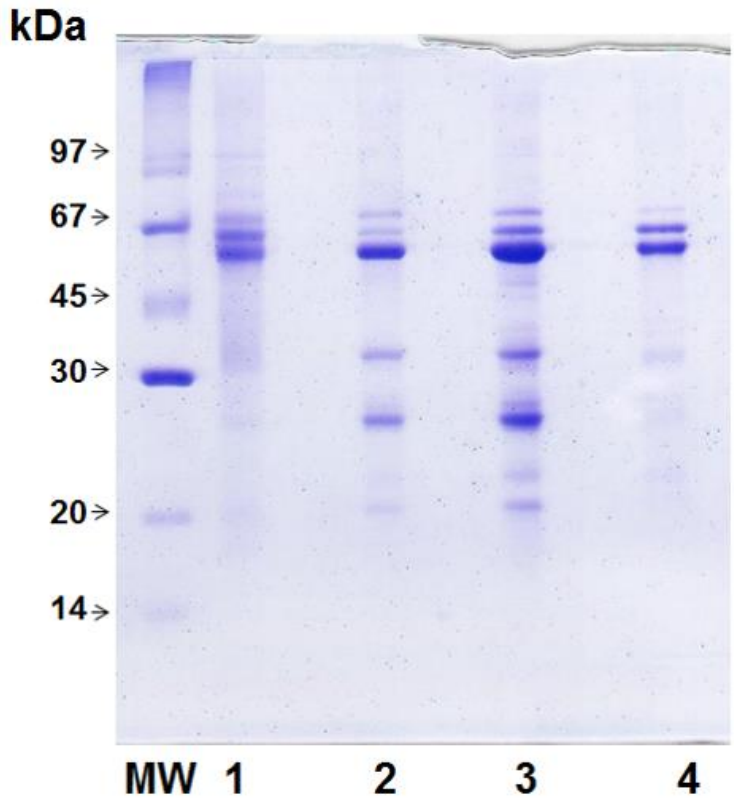


Figure 2 – SDS-PAGE under reducing conditions of proteins from cowpea. *MW column represents the molecular marker proteins; Lane 1 - total protein isolate; Lane 2 - total globulin fraction; Lane 3 - β-vignin protein isolated and Lane 4 - β-vignin protein obtained by size exclusion chromatography.*

Lower bands, between 20 and 30 kDa, are also evident, revealing acidic and basic subunits of 11S globulin (FERREIRA *et al*., 2018). Lane 3 corresponds to isolated β-vignin and it is possible to observe that there are still other globulins, such as 11S, after extraction, which is expected since it is difficult to separate them only due to differences in isoelectric point and solubility. Therefore, the extracted β-vignin was purified by size exclusion chromatography and its product is illustrated in lane 4, showing there was an efficient isolation and the presence of three bands. It has been described that the β-vignin protein is a trimer, in which the two major bands correspond to the major polypeptides of 60 and 56 kDa, typical of the vicilin family polypeptides. Therefore, the analysis of the polyacrylamide gel demonstrates that the protein isolation step, followed by the size exclusion chromatography process, was efficient for obtaining the β-vignin protein.

3.2 Antiproliferative effect of the β-vignin hydrolysates on cancer cells

Studies that describe anti-tumor properties from cowpea are related to its phenolic extracts (LIYANAGE, 2018). Regarding the antitumor effect of proteins from this legume, a study isolated a 36 kDa protein similar to a polygalactorunase inhibitor that showed cytotoxic activity in lymphoma (MBL2) and leukemic (L1210) cells with an IC_{50} of 7.4 μM and 5.4 μM, respectively (TIAN *et al*., 2013). A cowpea seed extract has been shown to reduce the viability of different colon cancer cells (E705, DiFi, SW480) in a dose-dependent manner and to reduce the phosphorylation level of the epidermal growth factor receptor (EGFR). It was also able to act synergistically with cetuximab, an antineoplasic with therapy directed to EGFR. When the profile of the extract was evaluated, a bowman-birk inhibitor was identified, which was considered the main responsible for its antitumor activity (PANZERI *et al*., 2020).

Recently, a study evaluated the antioxidant and antiproliferative activity of the total protein isolate (IPT) from five cultivars of cowpea bean. The IPT of the cultivar *Glenda* showed considerable inhibition of proliferation in lung cancer cells A549 (IC_{50} = 30 µg/mL) and the IPT of the cultivar *Veg cowpea 2* in breast cancer cells MCF-7 (IC_{50} = 15 µg/mL) (THUMBRAIN *et al*., 2020).

In the present study, the purified β-vignin was subjected to hydrolysis using different enzymatic sistems: (i) pepsin; (ii) trypsin; (iii) pepsin/pancreatin and (iv) alcalase/pepsin. Enzymatic hydrolysis is usually used to generate protein hydrolysates and peptides of different food proteins that act as anticancer agents in *in vitro* and *in vivo* studies (CHALAMAIAH; YU; WU, 2018). Each enzyme has its own specificity and selectivity, therefore, the final product (peptides) of protein hydrolysis varies depending on the enzyme/enzymes applied (TACIAS-PASCACIO *et al*., 2020). Pepsin is an aspartic acid protease that usually hydrolyses hydrophobic amino acids, especially aromatic residues such as phenylalanine, tryptophan and tyrosine (LUO *et al*., 2018); trypsin is a serine protease that preferably favors basic residues like lysine and arginine (MA; TANG; LAI, 2005); pancreatin is a ferment preparation containing a plurality of proteins, starch and fat splitting enzymes (SERGE, 1965); and alcalase is a serine endopeptidase that cleaves proteins in the middle of the amino acid chain and can be used to obtain small peptides with hydrophobic characteristics (TACIAS-PASCACIO *et al*., 2020).

All four β-vignin hydrolysates from *Vigna unguiculata* were screened through a cytotoxicity assay against breast (MDA-MB-231) and liver (Hep-G2) cancer cells, as illustrated in **Figure 3**. To generate the IC_{50} values (**Table 1**), all samples were assayed at different concentrations (12.5–200 µg/mL) against both cancer cell lines over the 24 h treatment period.

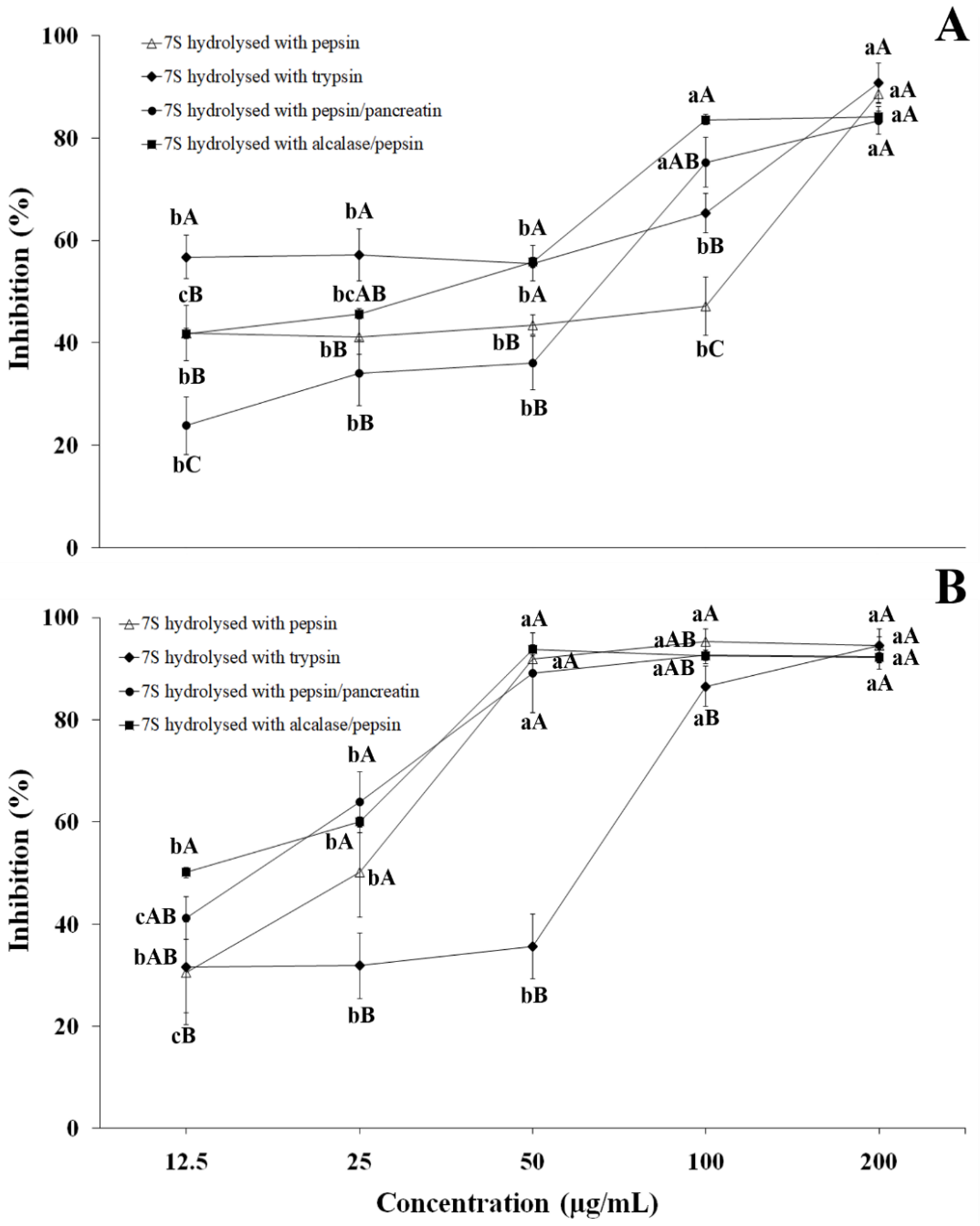


Figure 3 – Antiproliferative effect of β-vignin (7S) hydrolysed with pepsin, trypsin, pepsin/pancreatin and alcalase/pepsin against MDA-MB-231 (A) and Hep-G2 (B) cancer cells. *Mean ± standard deviation (n=3) with lower case letters indicate difference between concentrations of the same hydrolisate and capital letters indicate difference between hydrolisates at the same concentration (p value ≤ 0.05 by Tukey's multiple-range test).*

Table 1 – Antiproliferation effect of β-vignin hydrolysates on MDA-MB-231 and Hep-G2 cells.

Hydrolysates/cell lines	MDA-MB-231		Hep-G2	
	Inhibition (%)	**IC_{50} (µg/mL)**	**Inhibition (%)**	**IC_{50} (µg/mL)**
H-pep	41.89 - 88.55	5.07	30.55 - 94.55	3.71
H-tryp	56.76 - 90.73	3.02	31.52 - 94.52	4.95
H-pep/pan	23.8 - 83.42	5.42	41.18 - 92.38	3.03
H-alc/pep	41.79 - 84.15	4.06	50.08 - 92.18	2.69

H-pep - hydrolysate with pepsin enzyme; H-tryp - hydrolysate with trypsin enzyme; H-pep/pan - hydrolysate with pepsin/pancreatin enzymes; H-alc/pep - hydrolysate with alcalase/pepsin enzymes.

The present study found that all β-vignin hydrolysates released peptides with antiproliferative activity against breast and liver cancer cells in concentrations between 12.5–200 µg/mL. Proliferation inhibition (%) of the hydrolysates was in the range of 23.8–56.8% at 12.5 µg/mL, which reached 83.4–90.7% at 200 µg/mL in breast cancer cells. The trypsin hydrolysate exhibited the highest antiproliferation activity (90.7%) at 200 µg/mL, which had no statistical difference from the other hydrolysates at the same concentration. In liver cells, inhibition was 30.6–50.1% at 12.5 µg/mL and 92.2–94.6% at 200 µg/mL. The pepsin hydrolysate exhibited the highest antiproliferation activity (94.6%) at 200 µg/mL, and again as seen in the breast cancer cells, had no statistical difference from the other hydrolysates at the same concentration. The IC_{50} value of the hydrolysates was in the range of 3.02–5.42 µg/mL in both cell lines.

Other studies have investigated the effect of different food hydrolysates in several cancer cells *in vitro*. Carrillo *et al.* (2017) evaluated the antiproliferative activity of several protein hydrolysates (with pepsin; pepsin + corolase pp; protamex; flavor; and neutrasa enzymes) from walnut (*Juglans regia* L.) against 10 human cancer cell lines at different concentrations (0.25–250 µg/mL). The hydrolysate obtained with pepsin + corolase pp enzymes showed a stronger cytotoxic effect with an IC_{50} value of 0.25 µg/mL against UACC-62 cells (melanoma), followed by the hydrolysate obtained with neutrase (IC_{50}=25 µg/mL) and pepsin (IC_{50}=71 µg/mL) in the same cell line. In U251 cells (central system nervous cancer cell), the hydrolysate obtained with flavor enzyme showed a cytotoxic effect with an IC_{50} value of 167.4 µg/mL. The other hydrolysates showed no significant antiproliferative activity against the cancer cells tested.

Hydrolysates obtained with alcalase and with trypsin from mungbean vicilin protein were tested against breast cancer cells (MCF-7 and MDA-MB-231) at

concentrations between 0.2–1.0 mg/mL in a citotoxicity assay. After 48 h of treatment, the hydrolysate with alcalase demonstrated cytotoxic activity similar to the hydrolysate with trypsin, with IC_{50} value of approximately 0.73 and 0.45 mg/mL in MCF-7 cells and 0.48 and 0.54 mg/mL in MDA-MB-231 cells, respectively (GUPTA; SRIVASTAVA; BHAGYAWANT, 2018). Hydrolysates from sweet potato protein (*Ipomoea batatas* (L.) Lam) obtained by different proteases (with alcalase; proleather FG-F; AS1.398; neutrase; papain; and pepsin enzymes) showed dose-dependent antiproliferation effects on colon cancer cells (HT-29). Among the six hydrolysates, alcalase hydrolysate exhibited the highest proliferation inhibition effect with the lowest IC_{50} value of 119.72 µg/mL (ZHANG; MU, 2018).

Amaranth (*Amaranthus cruentus*) protein hydrolysates obtained with three different enzymes (alcalase, trypsin, and pepsin) in a cytotoxicity assay conducted on MCF-7 (breast cancer), A549 (human lung cancer) and HEK 293 (human embryonic kidney) cell lines showed that trypsin hydrolysate exhibited a preeminent anti-cancer effect with na IC_{50} value of 3.87 µg/mL and 14.10 µg/mL in breast and human lung cancer cells, respectively. It also induced apoptosis in all cell lines by increasing the expression of caspase-3/7 (RAMKISSON *et al*., 2020). Heat denatured proteins from amaranth seeds were also subjected to simulated gastrointestinal digestion (with pepsin/pancreatin enzymes) and showed concentration dependent effects on growth inhibition of human breast cancer cells (MDA-MB-231) with na IC_{50} value of 48.3 µg/mL, when tested in concentrations that ranged between 20-500 µg/mL. It also lead to a significant change in membrane breakage, decreased in cell number and blebbing similar to that of curcumin (positive control) treated cells, induced apoptosis by increasing caspase-3 activity and inhibited cellular migration across an artificial wound (TANIYA *et al*., 2020).

The bioactivity of generated peptides in protein hydrolysis depends on the ratio enzyme/substrate, time of hydrolysis and enzymes combination. The combination of proteases has been used in common bean (*Phaseolus vulgaris*) protein and has been reported to have a synergistic effect, specially gastrointestinal simulation by using pepsin/pancreatin enzymes, which has a broad specificity on proteins in the generation of bioactive peptides (LUNA-VITAL *et al*., 2015). In the present study, for convenience and taking into account results from studies that used gastrointestinal simulation to generate peptides with anticancer properties (GONZÁLEZ-MONTOYA *et al*., 2018; LUNA-VITAL *et al*., 2014; VILCACUNDO *et al*., 2018). In this sense, we decided to investigate the cytotoxic effect of peptide fractions from pepsin/pancreatin hydrolysate.

Before fractionating the pepsin/pancreatin hydrolysate into different molecular sizes, we evaluated the cell viability (%) of the hydrolysate in non-tumor human umbilical cord epithelial cells (HUVEC). In concentrations that ranged between 12.5–200 µg/mL used in our study, pepsin/pancreatin hydrolysate was able to inhibit cell viability of breast and liver cancer cells (**Figure 3**), but not the cell viability of non-tumor cells (HUVEC) (**Figure 4**). There was not a significant difference between the cell viability (%) of the negative control (cells with culture media only) and cells treated with pepsin/pancreatin hydrolysate at any concentration evaluated. Therefore, peptides present in the pepsin/pancreatin hydrolysate from β-vignin cowpea protein might be a good candidate to find a new peptide or peptides with antitumoral capacity, to selectively act on tumor cells. The positive control (methyl methanesulfonate, 300 µM) inhibited 87.15% of HUVEC cells.

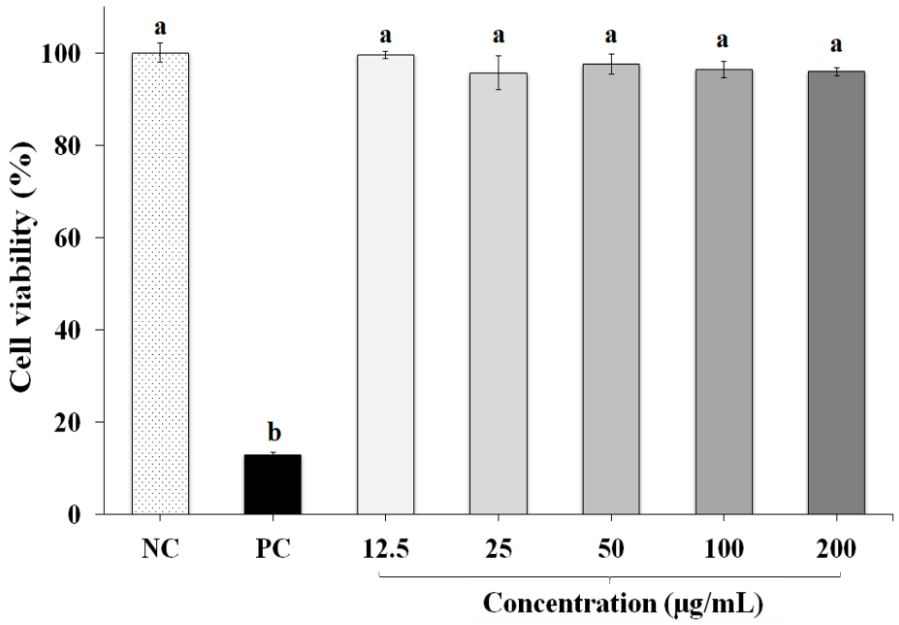


Figure 4 – HUVEC cell line growth treated with the β-vignin hydrolysed with pepsin/pancreatin. *Mean ± standard deviation (n=3) not connected with the same letters are significantly different (p value ≤ 0.05 by Tukey's multiple-range test). NC: negative control treated with culture media only. PC: positive control treated with 300 µM methyl methanesulfonate.*

3.3 Effect of β-vignin peptide fractions on cancer cell lines

The hydrolysate derived from the β-vignin protein, by the sequential action of the enzymes pepsin and pancreatin, was fractionated by different molecular weight in peptides greater than 30 kDa, peptides 30-10 kDa, peptides 10-3 kDa and peptides < 3 kDa. These fractions as well as pepsin/pancreatin total hydrolysate were subjected to analysis by SDS-PAGE under denaturing conditions, as shown in **Figure 5**.

Lane 1 and 7 shows 14 kDa and 45 kDa molecular weight standard, respectively. Lane 2 corresponds to the pepsin/pancreatin hydrolysate and it is possible to see the presence o several bands corresponding to peptides with molecular weight superior to 14 kDa. Lane 3 shows the profile of the fraction that corresponds to

peptides with molecular weight with 30 kDa and superior. However, we see a molecular profile similar to the pepsin/pancreatin hydrolysate. Lane 4 show the profile of peptides between 30 and 10 kDa, in which it is possible to see a band of peptides of around 20 kDa; however, it was not possible to observe the presence of peptides with lower molecular masses. There were also no bands in the lanes 5 and 6, that corresponds to peptides 10-3 kDa and < 3 kDa, respectively. This may be due to the low molecular weight peptides (10 kDa or less) running out from the gel easily or its lower ability to interact with the developer solution (coomassie blue).

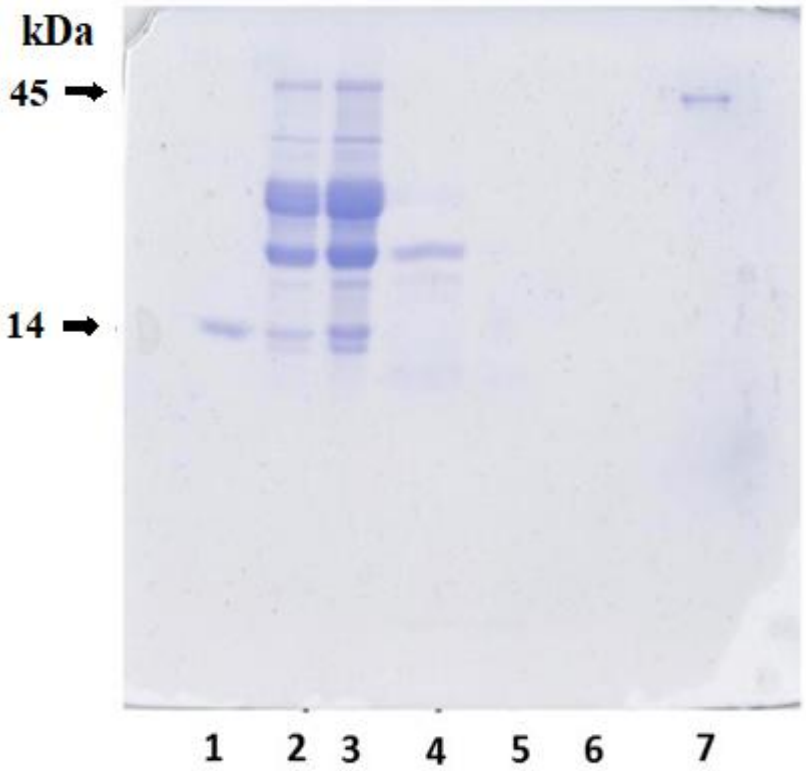
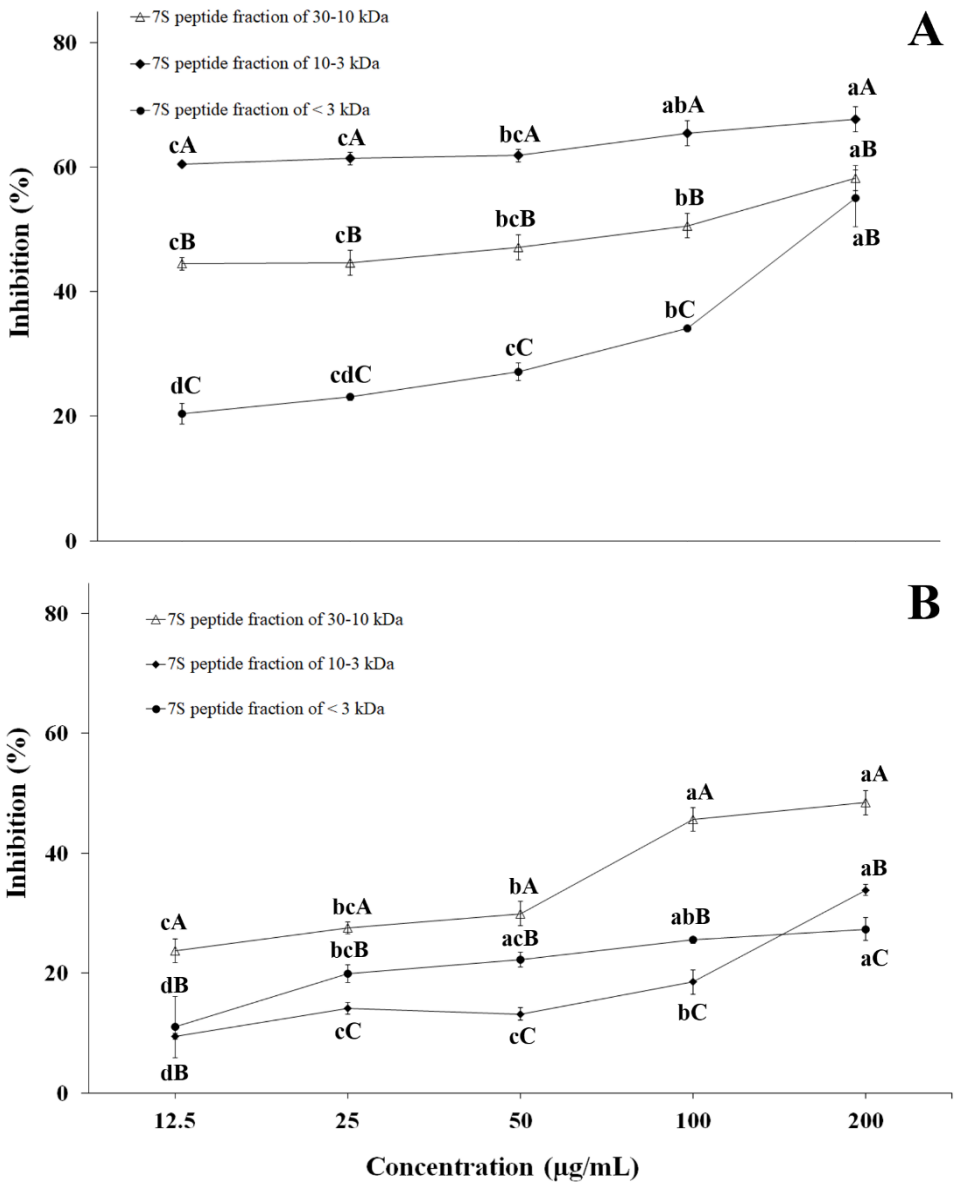


Figure 5 – SDS-PAGE under reducing conditions of total β-vignin hydrolysate and peptide fractions from sequential hydrolysis of pepsin/pancreatin from cowpea bean. *Lane 1 - 14 kDa Molecular Weight Standard; Lane 2 - Pepsin/pancreatin hydrolysate; Lane 3 - Peptides greater than 30 kD;, Lane 4 - Peptides between 30 and 10 kDa; Lane 5 - Peptides between 10 and 3 kDa; Lane 6 - Peptides less than 3 kDa; Lane 7 - Molecular mass standard 45 kDa.*

Hence, we evaluated the inhibitory activity (%) of pepsin/pancreatin peptide fractions (30–10 kDa, 10–3 kDa and peptides < 3 kDa) from β-vignin against breast and liver cancer cells. Methyl methanesulfonate was used as a positive control. In MDA-MB-231 cells (**Figure 6A**). The highest inhibitory effect was found in the 10–3 kDa peptide fraction (60.6–67.7%; IC_{50}=0.62 µg/mL), followed by 30–10 kDa (44.5–58.3%; IC_{50}=0.85 µg/mL) and peptides fraction < 3 kDa (20.5–55%; IC_{50}=1901.15 µg/mL) peptide fractions (**Table 2**). As expected, the positive control (methyl methanesulfonate) inhibited 92.3% of MDA-MB-231 cells. The current results are similar to those found by Rayaprolu *et al.* (2017a) in breast cancer cells (MCF-7), in which peptide fraction of intermediate size (10–5 kDa) from alcalase/pepsin/pancreatin hydrolysate from total protein of R95-1705 soybean line showed better cytotoxic activity (63%) compared to 50-10 kDa (~45%) and < 5 kDa (~38%) peptide fractions at 800 µg/mL.

In **Figure 6B** is illustrated the antiproliferative effect of peptide fractions (30–10 kDa, 10–3 kDa and < 3 kDa) from β-vignin hydrolysed with pepsin/pancreatin against Hep-G2 cells. Peptides 30–10 kDa presented the highest inhibitory effect (23.7–48.4%; IC_{50}=10.63 µg/mL), while 10–3 kDa and peptides fraction < 3 kDa had similar inhibition of 9.5–34% (IC_{50}=33.44 µg/mL) and 11–27.3% (IC_{50}=52.21 µg/mL), respectively. Methyl methanesulfonate inhibited 96.8% of Hep-G2 cells. Other studies with soybean hydrolysates found similar results. The 50–10 kDa fractions of N98-4445A and S03-543CR soybean lines inhibited approximately 70% of HepG-2 cells at 800 µg/mL, which was not significantly different from the positive control genistein at 200 µg/mL (RAYAPROLU *et al*., 2013). HPLC analysis of this peptide fraction of N98-4445A soybean line revealed three peaks at varying elution times, in which was identified three peptides named E58, E67, and E79. E67 had significant anti-

393 proliferative activity against colon and blood cancer cells with 74% and 80% inhibition
394 at 700 µg/mL. This peptide was identified as an 18 kDa peptide and recognized as the
395 precursor of 2S albumin from soybean (RAYAPROLU *et al*., 2017).



396

397 **Figure 6 –** Antiproliferative effect of peptide fractions (30-10 kDa, 10-3 kDa and < 3
398 kDa) from β-vignin (7S) hydrolysed with pepsin/pancreatin against MDA-MB-231 (A)
399 and Hep-G2 (B) cancer cells. *Mean ± standard deviation (n=3) with lower case letters*
400 *indicate difference between concentrations of the same fraction and capital letters*
401 *indicate difference between fractions at the same concentration (p value ≤ 0.05 by*
402 *Tukey's multiple-range test).*

403

404

405 **Table 2** – Inhibition of cancer cell lines growth treated with the peptide fractions of β-vignin pepsin/pancreatin hydrolysates.

Peptides fraction/cell lines	[ ] of inhibitor (µg/mL)	MDA-MB-231		Hep-G2	
		Inhibition (%)	IC_{50} (µg/mL)	Inhibition (%)	IC_{50} (µg/mL)
Control	-	0.0	-	0.0	-
Methyl methanesulfonate	300 µM	92.28	-	96.82	-
Peptides 30–10 kDa	12.5–200	44.48-58.27	0.85	23.73-48.44	10.63
Peptides 10–3 kDa	12.5–200	60.45-67.68	0.62	9.46-33.9	33.44
Peptides < 3 kDa	12.5–200	20.45-55.00	1901.15	11.01-27.34	52.21

406

Although fractions of intermediate (10–3 kDa) and larger (30–10 kDa) peptides from pepsin/pancreatin β-vignin hydrolysate have shown stronger antiproliferative activity in MDA-MB-231 and Hep-G2 cells, respectively, it has been reported that small peptides play an important role in the anticancer activities of protein hydrolysates from food sources (CHALAMAIAH; YU; WU, 2018).

The non-digestible fraction hydrolysed with pepsin/pancreatin of five cultivars from common bean (*Phaseolus vulgaris* L.) presented antiproliferative activity in colorectal cancer cells (HCT-116 and RKO) in concentrations that ranged between 0.125–1 mg/mL, specially the extract from Bayo Madero cultivar (IC_{50}=0.51 mg/mL). Five peptides (GLTSK, LSGNK, GEGSGA, MPACGSS and MTEEY) with small molecular masses (505 to 1019 Da) were found to be the most abundant in all peptide extracts (LUNA-VITAL *et al*., 2014). GLTSK (IC_{50}=134.6 µM) and GEGSGA (IC_{50}=156.7 µM) were able to inhibit colon cancer (HCT-116) cell growth in a dose-response manner, while the other three peptides had no inhibitory effect at the concentrations tested (0–200 µM). They were also able to interacted synergistically with oxaliplatin. The peptide GLTSK triggered cell cycle arrest and apoptosis by causing loss of mitochondrial membrane potential, releasing pro-apoptotic signals and increasing the intracellular ROS concentration. GEGSGA peptide had a similar pattern of loss of mitochondrial membrane potential, intracellular ROS and cell cycle arrest than oxaliplatin (LUNA-VITAL; DE MEJÍA; LOARCA-PIÑA, 2016).

Hydrolysates of wheat germ protein prepared separately with alcalase, pepsin and proteinase K decreased lung cancer cell (A549) viability in a concentration-dependent manner in concentrations of 0.195–25 mg/mL, with IC_{50} values of 12.94 mg/mL, 11.17 mg/mL and 11.27 mg/mL, respectively. Three peptides of the alcalase hydrolysate, two peptides of the pepsin hydrolysate, and two peptides of the proteinase

K hydrolysate were identified as the main components. Both pepsin-derived peptides (SSDEEVREEKELDLSSNE and KELPPSDADW) showed the highest effect (IC_{50}=2.34 and 7.25 μM, respectively) compared to alcalase-derived peptides TVGGAPAGRIVME and VGGIDEVIAK (IC_{50}=11.2 and 8.2 μM, respectively) and proteinase K-derived peptides SGGSYADELVSTAK and MDATALHYENQK (IC_{50}=10.7 and 9.7 μM, respectively) (KARAMI *et al*., 2019).

The anticancer activity of peptides from food proteins depend not only on peptide length, but also in its amino acid composition, sequence, and charge/hydrophobicity. A review published by Chalamaiah, Yu and Wu (2018) concluded that hydrophobic amino acids such as proline, leucine, glycine, alanine, and residues of lysine, arginine, serine, glutamic acid, threonine and tyrosine are frequently present in the sequence of anticancer peptides of food proteins. Future studies should focus on identifying the peptides present on pepsin/pancreatin hydrolysates from cowpea β-vignin, specially the 30-10 kDa and 10-3 kDa peptide fractions.

4 CONCLUSION

Cowpea is a rich source of proteins, and therefore an interesting legume for the search of bioactive peptides. This work assessed the antiproliferative activity of four β-vignin hydrolysates and peptide fractions from pepsin/pancreatin hydrolysate. The trypsin hydrolysate (IC_{50}=3.02 μg/mL) exhibited the highest antiproliferation activity (90.7%) at 200 μg/mL in breast cancer cells, which had no statistical difference from the other hydrolysates at the same concentration. And the pepsin hydrolysate (IC_{50}=3.71 μg/mL) exhibited the stronger antiproliferation activity (94.6%) at 200 μg/mL, and again had no statistical difference from the other hydrolysates at the same concentration. The 10-3 kDa peptide fraction (60.5–67.7%, IC_{50}=0.62 μg/mL) from pepsin/pancreatin

hydrolysate presented better effect against breast cancer cells, while 30–10 kDa peptide fraction (23.7–48.4%, IC_{50}=10.63 µg/mL) had the best inhibitory effect on Hep-G2 cells. Further work is needed to characterize these anticancer peptides, which has been currently explored by our research group.

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STATEMENT OF CONFLICT OF INTEREST

All the authors declare no conflict of interest about the described research, the publication of the result and financial issues.

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4 CONCLUSÃO GERAL

A partir dos resultados observados no presente estudo foi possível evidenciar que o processo de hidrólise das proteínas β-conglicinina (7S) da soja, e a β-vignina (7S) do feijão-caupí originou frações peptídicas capazes de causar um efeito antiproliferativo (citotóxico) sobre as linhagens tumorais de câncer de mama (MDA-MB-231), próstata (DU-145) e hepático (Hep-G2), sendo observado um efeito dose-dependente. No entanto, os hidrolisados não mostram ação citotóxica sobre as células humanas não-tumorais (HUVEC).

Além disso, a fração constituída de peptídeos entre 10-3 kDa, de ambas as proteínas, β-conglicinina e β-vignina, mostraram um efeito antiproliferativo mais significativo sobre as linhagens MDA-MB-231 e DU-145. Isto indica que o(s) peptídeo(s) com potencial antitumoral apresente massa molecular nesse intervalo. Por fim, estudos adicionais são necessários a fim de identificar os peptídeos presentes nessas frações assim como investigar por quais mecanismos de ação eles atuam. Atualmente, essas questões estão sendo investigadas pelo nosso grupo de pesquisa.