UADY

POSGRADO INSTITUCIONAL EN CIENCIAS QUÍMICAS Y BIOQUÍMICAS

EVALUACIÓN DE LA ACTIVIDAD ANTICANCERÍGENA DE DERIVADOS PROTEÍNICOS DE *S. hispanica*

TESIS

PRESENTADA POR

I.B. NIDIA DEL CARMEN QUINTAL BOJÓRQUEZ

EN OPCIÓN AL GRADO DE

MAESTRA EN CIENCIAS QUÍMICAS Y BIOQUÍMICAS

MÉRIDA, YUCATÁN, MÉXICO
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La tesis "Evaluación de la actividad anticancerígena de derivados proteínicos de *Salvia hispanica*" presentada por la I.B. Nidia del Carmen Quintal Bojórquez, en cumplimiento parcial de los requisitos para optar por el grado de Maestra en Ciencias Químicas y Bioquímicas, ha sido aprobada en cuanto a su contenido científico y en cuanto a lo establecido en el Manual de Procedimientos del Posgrado Institucional en Ciencias Químicas y Bioquímicas, por lo que se le autoriza la digitalización de los ejemplares correspondientes.

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RESUMEN

El cáncer es la segunda causa de muerte a nivel mundial y la tercera en México. Los tratamientos de primera opción para esta enfermedad son de alto costo y ocasionan serios efectos secundarios en los pacientes, por lo que actualmente se buscan tratamientos, particularmente de origen natural. Entre la amplia gama de tratamientos complementarios, los derivados proteínicos han ganado gran interés ya que han reportado potencial uso en la prevención y tratamiento de enfermedades como cáncer. Las semillas de *Salvia hispanica* poseen un contenido de proteína de hasta el 26% por lo que representan una fuente potencial para la obtención de derivados proteínicos. Por tal motivo, el objetivo del presente trabajo fue evaluar la actividad anticancerígena de derivados proteínicos de *S. hispanica.* Para alcanzar tal objetivo, las semillas de *S. hispanica* fueron desgomadas, desgrasadas, molidas y tamizadas para posteriormente ser sometidas a una hidrólisis enzimática con el sistema Pepsina-Pancreatina®. El hidrolizado resultante fue sometido a ultrafiltración, generando así los siguientes derivados proteínicos: <1, 1-3 y 3-5 kDa. Se evaluó el efecto de tales derivados, a diferentes concentraciones (0.25, 0.5, 0.75 y 1 mg/mL), en la viabilidad celular de las líneas de cáncer de mama (MCF-7), colon (Caco2), próstata (PC-3) e hígado (HepG2) con el ensayo MTT. El efecto de cada factor (derivados proteínicos y concentraciones) se analizó con ANOVA de una vía, y el efecto resultante de la interacción de ambos factores se analizó a través de ANOVA multifactorial con un posthoc de Tukey ($p<0.05$). El derivado proteínico <1 kDa, a una concentración de 1 mg/mL, tuvo el mayor efecto estadístico en las líneas celulares de cáncer evaluadas. Se observó que, la actividad anticancerígena fue mayor al disminuir el peso molecular y aumentar la concentración de los derivados proteínicos. Se determinó la secuencia aminoacídica del derivado <1 kDa y, a través de un análisis de decision multicriterio, el péptido KLKKNL resultó en la primera posición del ranking, por lo que tiene mayor probabilidad de presentar actividad anticacerígena. En conclusión, la secuencia aminoacídica KLNKKNL, presente en la fracción proteínica <1 kDa, podría representar una opción terapéutica para el tratamiento de cuatro de los tipos de cáncer con mayor prevalencia a nivel mundial. Los resultados obtenidos refieren a continuar con estudios *in vivo* y clínicos para el establecimiento de esta opción terapéutica.

ABSTRACT

Cancer is the second leading cause of death worldwide and the third in Mexico. The first option treatments for this disease are very expensive and cause serious side effects on patients, which is why there is current search for treatments, particularly of natural origin. Among the wide range of complementary treatments, proteinic derivatives have gained great interest since they have reported potential use in the prevention and treatment of diseases such as cancer. *Salvia hispanica* seeds have up to 26% content of protein, which has made it a potential source for the obtention of proteinic derivatives. Thus, the objective of this study was to evaluate the anticancer activity of proteinic derivatives from *S. hispanica.* To achieve the objective, *S. hispanica* seeds were degummed, defatted, ground and sieved to be then submitted to enzymatic hydrolysis with the system Pepsin-Pancreatin®. The hydrolysate obtained from the latter was submitted to ultrafiltration in order to separate the proteinic derivatives by different molecular weights: <1, 1-3 y 3-5 kDa. The effect of such derivatives on the cellular viability of the breast (MCF-7), colon (Caco2), prostate (PC-3) and liver (HepG2) cancer lines was evaluated, at different concentrations (0.25, 0.5, 0.75 y 1 mg/mL), with the MTT assay. The effect of each factor (proteinic derivatives and concentrations) was analyzed with one-way ANOVA, and the resulting effect of the interaction of both factors was analyzed through multifactorial ANOVA with a Tukey post hoc ($p<0.05$). The proteinic derivative <1 kDa at a concentration of 1 mg/mL, had the highest statistical effect on the cellular viability of all evaluated cancer lines. It was observed that the anticancer activity of the proteinic derivatives was lower when the molecular weight was increased, and the concentration decreased. The amino acid sequence of the proteinic derivative <1 kDa was determined and through a multicriteria analysis, the peptide KLKKNL was identified, since it was listed as number one in the resulting rank, it has a higher possibility to present anticancer activity. In conclusion, the aminoacidic sequence KLKKNL, within the <1 kDa proteinic derivative, may represent a therapeutic option for the treatment of the four main types of cancer with the highest prevalence worldwide. The results obtained indicate that further *in vivo* and clinic trials are necessary for the confirmation of this therapeutic option.

ÍNDICE

INTRODUCCIÓN

En el 2018, el cáncer fue responsable de 9.6 millones de muertes a nivel mundial y de 83,476 en México convirtiéndose así en uno de los principales problemas de salud. Entre los principales tipos de cáncer que afectan a la población mexicana se encuentran el de mama, próstata, colon e hígado (Bray *et al.,* 2018). Los tratamientos convencionales para dicha enfermedad incluyen la quimioterapia, radioterapia y cirugía. Sin embargo, estos ocasionan serios efectos secundarios que disminuyen significativamente la calidad de vida del paciente (American Cancer Society, 2016). Así, las cifras de mortalidad y los efectos colaterales de los tratamientos convencionales, ponen de manifiesto la necesidad de encontrar tratamientos complementarios.

El uso de los tratamientos complementarios ha reportado beneficios en los pacientes, como la reducción de dolor y síntomas relacionados a la enfermedad, además de la mitigación de efectos secundarios de los tratamientos convencionales (Witt *et al.,* 2017). Dentro de la amplia gama existente de tratamientos complementarios, los derivados proteinicos han ganado gran interés toda vez que, han reportado beneficios a la salud humana al tener actividad antioxidante, antihipertensiva, reductora de riesgos de enfermedades cardiacas (Mohar-Betancourt *et al.*, 2017; De Souza-Ferreira *et al.*, 2013) y de diabetes (Ali *et al.*, 2012). En los últimos años se ha reportado que los derivados proteínicos de bajo peso molecular obtenidos a partir de fuentes con alto contenido proteico, presentan actividad anticancerígena al inhibir la migración y proliferación celular, la angiogénesis y al inducir apoptosis en diversas líneas celulares de cáncer (Quintal-Bojórquez & Segura-Campos, 2020). Entre las ventajas particulares del uso de los derivados proteínicos para el tratamiento del cáncer, se encuentra su alta selectividad, baja toxicidad y alta difusión entre membranas (Oelkrug *et al.,* 2015).

La búsqueda de fuentes con alto contenido proteico para la obtención de derivados proteinicos con posible actividad anticancer ha resultado en el estudio de *S. hispanica.* Esta planta, también conocida como chía, es actualmente sugerida como un alimento funcional ya que no solo cumple con la funcion de nutrir, si no que también aporta beneficios a la salud. Además, sus semillas poseen hasta un 26% de proteína, lo cual la hace una potencial fuente para la obtención de derivados proteinicos con actividad biológica (Segura-Campos *et al.,* 2014; Cárdenas *et al.,* 2018). Entre la amplia gama de beneficios que aportan las semillas de *S. hispanica* a la salud humana, se destaca la actividad anticancerígena de sus compuestos activos. Por ejemplo, se ha reportado el perfil fitoquímico de las semillas de chia, donde se demuestra gran abundancia de compuestos fenólicos y de flavonoides los cuales han demostrado actividad anticancerígena al disminuir la viabilidad de líneas celulares de cáncer (Oliveira-Alves *et al.,* 2017; Knez Hrnčič *et al.,* 2019). Además, Martín-Ortega y Segura-Campos (2020) reportaron la extracción del aceite de las semillas de chía, el cual demostró capacidad inhibitoria de la viabilidad celular de una linea celular de cáncer de mama (MCF-7).

Sin embargo, aún no existen reportes de la evaluación de la actividad anticancerígena de los derivados proteínicos de las semillas de chía. Así, por el alto contenido proteínico de las semillas de *S. hispanica* y los beneficios a la salud que ésta reporta, resultó de interés plantear la obtención de derivados proteínicos de chía para evaluar su potencial anticancerígeno en líneas celulares de cáncer de mama (MCF-7), colon (Caco2), próstata (PC-3) e hígado (HepG2).

HIPÓTESIS

Los derivados proteínicos de *S. hispanica* presentan actividad anticancerígena al exhibir una reducción de la viabilidad de las líneas celulares de cáncer de mama, colon, próstata e hígado.

JUSTIFICACIÓN

El cáncer es una de las principales causas de muerte a nivel mundial y presenta un panorama de prevalencia negativo, toda vez que se estima un aumento del 60% en los fallecimientos causados por esta enfermedad. Una posible causa de dicho aumento es que las terapias convencionales para su tratamiento, generalmente, producen alta toxicidad, ocasionando en los pacientes una amplia gama de efectos secundarios los cuales disminuyen significativamente su calidad de vida (Bray et al., 2018; Ortiz-Martinez et al., 2014). Estos efectos negativos vuelven indispensable el desarrollo de nuevas terapias complementarias no tóxicas para las células sanas. En este contexto, los derivados proteínicos han ganado gran relevancia (Shoombuatong et al., 2018).

Los derivados proteínicos, particularmente aquellos con actividad anticancerígena, son mayormente obtenidos de fuentes naturales que poseen un alto contenido proteico (Quintal-Bojórquez & Segura-Campos, 2020), como es el caso de las semillas de la *Salvia hispanica*. Esta planta, al ser endémica de México y actualmente sugerida como un alimento funcional, resulta de interés para la obtención de dichos derivados, debido a que sus semillas contienen hasta un 26% de proteína (Cárdenas et al., 2018). En el contexto de su funcionalidad, se ha reportado que ciertos compuestos activos extraídos de las semillas de *S. hispanica* poseen actividad anticancerígena. Sin embargo, hasta ahora, no existen reportes acerca de la actividad anticancerígena de los derivados proteínicos obtenidos a partir de las semillas de *S. hispanica,* ni se han identificado péptidos que pudieran presentar esta actividad.

Por lo anterior, la evaluación del efecto de los derivados proteínicos en la viabilidad de las líneas celulares de cáncer de mama, colon, próstata e hígado, permitirá conocer su potencial anticancerígeno, brindando así soporte a la funcionalidad de los péptidos de *S. hispanica,* contribuyendo al planteamiento de estrategias que coadyuven al tratamiento del cáncer.

OBJETIVOS

Objetivo general

Evaluar la actividad anticancerígena de derivados proteínicos de *S. hispanica* obtenidos por hidrólisis enzimática secuencial y ultrafiltración.

Objetivos específicos

- Determinar el grado de hidrólisis del hidrolizado enzimático de *S. hispanica* conseguido con el sistema secuencial Pepsina-Pancreatina®, así como el contenido proteico de los derivados obtenidos por ultrafiltración.
- Analizar las secuencias aminoacídicas de los derivados proteínicos obtenidos por ultrafiltración del hidrolizado enzimático a través de una nano LC-MS/MS y analizar las secuencias resultantes mediante un análisis multicriterio utilizando las herramientas AHP y TOPSIS.
- Analizar el efecto de los derivados proteínicos de *S. hispanica* en la viabilidad de líneas celulares de cáncer de mama, colon, próstata e hígado, así como su toxicidad en la línea celular de fibroblastos humanos.

CRONOGRAMA

	SEMESTRE I				SEMESTRE II							SEMESTRE III					SEMESTRE IV							
ACTIVIDAD	**SEP**	**OCT**	**NOV**	**DIC**	**ENE**	**FEB**	**MAR**	**ABR**	**MAY**	**JUN**	**JUL**	**AGO**	**SEP**	**OCT**	**NOV**	**DIC**	**ENE**	**FEB**	**MAR**	**ABR**	**MAY**	**JUN**	**JUL**	**AGO**
Revisión bibliográfica (Review)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Redacción de artículo científico en inglés	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Obtención de la harina desgomada y desgrasada			✓	✓																				
Determinación de la humedad					✓																			
Hidrólisis de la harina con el sistema enzimático Pepsina-Pancreatina						✓																		
Determinación del grado de hidrólisis obtenido a partir de la hidrólisis enzimática						✓																		
Obtención de fracciones peptídicas por ultrafiltración						✓	✓																	
Cuantificación de la proteína a través del método de Lowry							✓	✓																
Determinación de la secuencia aminoacídica de los derivados proteínicos									✓	✓														
Cultivo celular de las líneas cancerosas y fibroblastos										✓	✓	✓	✓											
Evaluación del efecto de los derivados proteicos de *S. hispanica* sobre la viabilidad celular de líneas													✓	✓	✓	✓	✓							
Secuenciación peptídica de los derivados de *S. hispanica*																	✓	✓	✓					
Redacción de tesis																			✓	✓	✓	✓		

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ARTÍCULO: ACTIVIDAD ANTICANCERÍGENA DE LOS DERIVADOS PROTEÍNICOS DE *Salvia hispanica*

Salvia hispanica as a source of proteinic derivatives with anticancer activity

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Abstract

Salvia hispanica demonstrated to be a source of proteinic derivatives with anticancer activity. Three proteinic derivatives (<1, 1-3 and 3-5 kDa) were obtained by ultrafiltration of the *S. hispanica* seeds hydrolysate. Moreover, the effect on the cellular viability against four cancer cell lines (MCF-7, Caco2, PC-3 and HepG2) and human fibroblasts (hFB) as normal cells, at different concentrations (0.25, 0.5, 0.75, 1 mg/mL), was evaluated. The proteinic derivatives did not show cytotoxic effects on hFB. The proteinic derivative <1 kDa at 1 mg/mL showed the highest statistical effect on the cellular viability of all evaluated cancer lines; thus, its amino acid sequence was analyzed. From the multicriteria decision analysis of the peptide sequences, the peptide KLKKNL was considered since it was ranked as number one, meaning a higher possibility of presenting anticancer activities. In conclusion, proteinic derivatives could represent a therapeutic option for cancer treatment. However, further investigations are necessary to stablish conclusive arguments.

Keywords: proteinic derivatives, anticancer activity, *S. hispanica, amino acid sequence.*

Practical application: The work of this article is based on the background of the increasing potential of peptides for the treatment of chronic diseases. The results of this study present peptides of low molecular weight, obtained from chia seeds, as a potential adjuvant option for cancer treatment.

Enviado a la revista “Journal of Food Science”. Elaborado acorde con las normas editoriales de dicha revista. En el Anexo 2 de la tesis se adjunta el comprobante de envío.

1. Introduction

The World Health Organization (2018) ranks cancer as one of the leading causes of death worldwide; in Mexico, the four most frequent types of cancers are breast, prostate, colorectal and liver, in that order (Bray *et al.,* 2018). Due to all the conventional therapies' consequences to health, patients seek complementary treatments to their therapy, such as diet therapy.

The use of dietary modifications to complement conventional cancer therapies is a practical approach that has gained great relevance. Bioactive compounds are present in nature, and also in dietary food, representing a fundamental link to health promotion, prevention and treatment of chronic diseases. The identification of bioactive compounds, also known as proteinic derivatives, is a current and innovative strategy for the complementary treatment of cancer (Hernández-Ledesma & Hsie, 2015). There are several advantages to the use of proteinic derivatives in cancer therapy such as high specificity and affinity, low toxicity, high diffusion between membranes and very low accumulation in organs (Blanco-Míguez *et al.,* 2016).

Remarkably, several studies have reported that proteinic derivatives from natural sources possess anticancer activities in cultured cancer cells via different mechanisms including induction of apoptosis and necrosis, cell cycle arrest and inhibition of angiogenesis. Wang & Zhang (2016) described a derivative (HVLSRAPR) obtained from *Spirulina platensis* that demonstrated antiproliferative activity on MCF-7, HepG-2 and SGC-7901 cancer cell lines; such activity is probably attributed to the derivative's low molecular weight. Wang *et al.,* (2016) reported the separation and purification of a proteinic derivative of 0.408 kDa from *Brassica campestris* which induced apoptosis on the HepG-2 cancer cell line. Luna-Vital *et al.,* (2014) characterized five derivatives present in *Phaseolus vulgaris,* all with a molecular weight lower to 1 kDa, that modified the expression of molecules involved in the cell cycle arrest and apoptosis in human colorectal cancer cell lines. Most of the proteinic derivatives that have exerted anticancer activities exhibit similar characteristics among them a cationic nature, amphipathicity and low molecular weight (Hoskin and Ramamoorthy, 2008; Oelkrug *et al.,* 2015).

Currently, and as mentioned before, plants and seeds have become promising sources of proteinic derivatives with anticancer activity, particularly those with high protein content.

Salvia hispanica, commonly known as "chia" is a native plant of Mexico and Central America that is now recognized as functional food since, in addition to its high nutritional value, it causes beneficial effects on human health like improvement of the digestive system, stronger bones and muscles, and reduction of risk of heart diseases. Chia seeds contain up to 26% of protein, making it a potential source of proteinic derivatives with possible anticancer activity (Cárdenas *et al* 2018).

Considering *S. hispanica*'s high protein content, its known benefits to health and the serious threat that cancer represents to society, the present study aims to evaluate the anticancer potential of proteinic derivatives from this seed in four cancer cell lines: MCF-

7 (breast cancer), Caco2 (colorectal cancer), PC3 (prostate cancer) and HepG2 (liver cancer), and its toxicity to normal human cells (fibroblasts).

2. Materials and methods

2.1 Plant material

The *Salvia hispanica* L., seeds were obtained from producers located in Guadalajara, Mexico; these seeds belong to the harvest of January 2017. The biologist José Luis Tapia Muñoz identified the seeds and one sample was stored in the Center of Scientific Investigation of Yucatan (CICY) for future references (identification number 69494).

2.2 Biological material

To evaluate the anticancer effect of the proteinic derivatives, the following four cancer cell lines were evaluated: MCF-7 (breast cancer; ATCC: CLR-1435), Caco2 (colorectal cancer; ATCC: HTB-22), PC3 (prostate cancer; ATCC: HTB-37) and HepG2 (liver cancer; ATCC: HB-8065); the toxicity of the proteinic derivatives was evaluated on human fibroblasts (hFB). The Faculty of Chemical Engineering of the Autonomous University of Yucatan provided the cancer cell lines and human fibroblasts.

2.3 Obtention of degummed and defatted flour from S*. hispanica* L.

The method reported by Salazar *et al*. (2020) was followed with some adjustments. The mucilage was extracted by preparing a suspension with whole seeds and distilled water in a 1:40 (w/v) proportion. The degummed seeds were crushed and ground in a Tomas-Wiley® model A grinder. Subsequently, the oil was extracted by pressing the thick flour with a TRUPER® hydraulic jack. To extract the remaining oil, the Soxhlet method was followed twice using hexane High Purity® as solvent. Finally, thick flour was ground with a 0.5 mm mesh and then sieved with a 140 µm mesh in a Ro-Tap® model E sieve shaker. From the degummed and defatted flour obtained from the latter process, the percentage of humidity was determined by the AOAC gravimetric method (925.09) on a Fisher-Scientific® stove. The percentage of humidity (%H) was calculated with the following equation:

$$\%H = \left(\frac{W_1 - W_2}{W_s}\right) x\ 100$$

Where W_1 is the constant weight of the crucible with the sample, W_2 is the crucible's weight with dry sample and W_s is the sample's exact weight.

Additionally, the protein content of the degummed and defatted flour was determined by the Kjeldahl method (954.01) reported by the AOAC. The percentage of proteins (%P) was calculated on wet basis with the following formula:

$$\%P = \left(\frac{(V_{HCl})\ (N_{HCl})\ (6.25)\ (0.014)}{P_m}\right) x\ 100$$

Where V_{HCl} is the volume of HCl consumed, N_{HCl} is the normality of HCl, 6.25 is the protein factor, 0.014 is the molecular weight of nitrogen divided by 1000 and P_m is the exact weight of the sample.

2.4 Enzymatic hydrolysis

The method reported by Martinez-Leo *et al*., (2019) was followed, were the enzymatic system Pepsin-Pancreatin® was used sequentially for 90 min. The parameters used were: 4% substrate concentration, 1/10 enzyme-substrate, 37°C, pH 2 for Pepsin and pH 7.5 for Pancreatin®. The total time of reaction (90 min) was divided in two stages: first, hydrolysis with Pepsin (45 min) and then, hydrolysis with Pancreatin (45 min). The hydrolysis was stopped with a water bath at 80°C for 20 min.

2.5 Degree of hydrolysis (DH)

The ortho-phthalaldehyde (OPA) technique proposed by Nielsen et al., (2001) was followed with some adjustments. For the calibration curve, L-serine solution (1:10 v/v) was used as standard at different volumes. Aliquots of the hydrolysates were mixed with the OPA reactant, and the mixture was read at 340 nm. Once the absorbance was obtained, the number of equivalents of amino free groups was determined by comparing it to the L-serine standard curve.

Finally, the following equation by Adler- Niessen was used to determine the DH:

$$\% \, DH = \left(\frac{h}{h_{tot}}\right) \, x \, 100$$

Where the *h* is the number of hydrolyzed peptide bonds (milliequivalents/g protein) and h_{tot} is the total number of hydrolysable peptide bonds. The h_{tot} depends on the seed's aminoacid composition.

2.6 Fractionation by ultrafiltration

The proteinic hydrolysate was fractioned by ultrafiltration according to the methodology proposed by Martinez-Leo & Segura-Campos (2020). Three membranes of different molecular weight cut-off (1, 3 and 5 kDa) were used. The hydrolysate's soluble fraction was passed through each membrane, starting with the lowest molecular weight (1 kDa). Three proteinic derivatives with different ranges of molecular weight: <1 kDa, 1-3 kDa, and 3-5 kDa, were obtained. Subsequently, protein content was determined by means of Lowry (1951). The absorbance of the solution was detected at 580 nm wavelength in a UV-Vis Thermo Scientific® spectrophotometer.

2.7 Cell culture

MCF-7 (ATCC: CLR-1435), Caco2 (ATCC: HTB-22), PC3 (ATCC: HTB-37), HepG2 (ATCC: HB-8065) and hFB cell lines were cultured in Dubelco's Modified Eagle Medium (DMEM) without phenol red, supplemented with 1.2 g/L of $NaHCO_3$ and 10% of FBS (fetal bovine serum) in sterile 75-cm^2 flasks. The cell cultures were incubated at 37°C with 5% of CO_2 and a humidified atmosphere in a Lab-Line® incubator.

For the subcultures, the cells were washed with 5 mL of PBS 1X. Then, 5 mL of trypsin 0.025% were added and incubated for 5 min. Afterwards, 5 mL of medium were added to stop the reaction and then, the resulting 10 mL were centrifuged at 1500 rpm for 5 min.

Finally, the pellet was re-suspended with fresh medium and inoculated in sterile 75-cm^2 flasks.

For cell count, the following method was followed. To a 200 µL sample of the cell suspension, 20 µL of 0.4%trypan blue were added, placed in a Neubauer chamber and observed with a microscope. The blue cells were counted as dead. Cell concentration and viability percentage were calculated. Cultures with viability greater than 95% were used for the assays. Viable cells by mL (NoVC/mL) were calculated with the following formula:

$$\frac{NoVC}{mL} = \frac{(V)(1x10^4)(FD)}{NoQ}$$

Where *V* is the number of viable cells, *1x10⁴* is the Neubauer chamber factor, *FD* is trypan blue's dilution factor and *NoQ* is the number of quadrants used for the counting (Oliveira *et al*. 2011).

2.8 Proteinic derivatives' effect on the cellular viability of cancer cell lines

The effect of the proteinic derivatives on the cellular viability of MCF-7 (breast cancer), Caco2 (colorectal cancer), PC3 (prostate cancer) and HepG2 (liver cancer) lines was evaluated. Cells were sub-cultured into 96-well plates with 100 µL of medium ($1x10^4$ cells/well) for 48 h and then exposed for 48 h to 100 µL of the proteinic derivatives at different concentrations (0.25, 0.5, 0.75, 1 mg/mL). After incubation, the cellular viability was evaluated by exposure of the plates to MTT (0.5 mg/mL) following the procedure proposed by Mosmann (1983) with some adjustments. Then, the medium was removed and DMSO was added to determine the absorbance at 570 nm. The toxicity of the proteinic derivatives was evaluated on human fibroblasts (hFB). The positive control was Taxol at a concentration of 30 ng/mL and the negative control was medium. Cell viability was calculated with the following formula:

$$\%\ P = \left(\frac{OD_{w/t}}{OD_{ctrl}}\right)\ x\ 100$$

Where $OD_{w/t}$ is the optic density of cells with treatment, OD_{ctrl} is the optic density of the negative control.

2.9 Amino acid sequence identification

The procedure for the amino acid sequence identification was followed as proposed by Han *et al.* (2011). To prepare the sample for sequence identification, it was purified with a Sep-Pak® column C18. The prepared sample was injected onto an in house-packed 20 cm capillary column (inner diameter 75 µm, 3.5 µm Kromasil C18 media). An EasyLC nano liquid chromatography system was used to apply a gradient of 4–40% ACN in 0.1% formic acid over 30 min at a flow rate of 250 nl/min; total acquisition time was 60 min. Separated peptides were eluted directly from the column and sprayed into an Orbitrap Velos Mass Spectrometer (ThermoFisher Scientific, Hemel Hempstead, UK) using an electrospray capillary voltage of 2.7 kV. Precursor ion scans were acquired in the Orbitrap with resolution of 60000. Dynamic exclusion of 30 s was used. Peptide

MS/MS data were processed with PEAKS Studio X+ (Bioinformatic Solutions Inc, Waterloo, Ontario, Canada) and searched against the Uniprot databases (release 2020_03).

2.10 Multicriteria decision analysis for peptide selection with anticancer potential

The amino acid sequences, identified by MS/MS of the proteinic derivative with the highest statistical inhibitory effect on the viability of the cancer cell lines, were analyzed through a multicriteria decision analysis by the AHP (Analytic Hierarchy Process) and TOPSIS (Technique for Order of Preference by Similarity to Ideal Solution) methods. The criteria stablished to select a peptide with anticancer potential were the following: net charge on a range from +2 to +9, amino acid sequence from 5 to 20 residues, hydrophobicity from 30% to 50% and an α-helix conformation (Hoskin and Ramamoorthy, 2008; Oelkrug *et al.,* 2015; Felício *et al.,* 2017). From the resulting ranking, the peptide that best met all the criteria was selected.

3. Statistical analysis

The results are presented as mean $\pm$ SD of triplicates (n=3). The protein content of the hydrolysate and its derivatives was analyzed through one-way ANOVA ($p<0.05$) The effect of each factor (proteinic derivatives and concentrations) on the cellular viability of the cancer cell lines and the effect of the interaction of both factors was analyzed through multifactorial ANOVA with a post hoc Tukey test ($p<0.05$). The results were analyzed with the statistical software Statgraphics Centurion and the figures made with GraphPad Prism version 8.

4. Results and discussion

4.1 Obtention of the degummed and defatted flour

The mill yield of the degummed and defatted flour was 70.8%, which was lower than the 84.33% reported by Segura-Campos *et al.,* (2013); these variations could be attributed to the different methods used for mucilage and oil extraction and the different screen's sizes used during the milling process. The moisture content of the protein rich flour determined by the gravimetric method was 8.4 $\pm$ 0.04%; which was slightly higher than the 7.9 $\pm$ 0.0.16 % reported by Coelho (2018). The difference between both moisture contents is mainly attributed to the different drying techniques, since Coelho (2018) followed the AOAC gravimetric method 935.29; which is recommended for the determination of humidity in beverages and beverage materials. However, the percentage of humidity obtained in this study is acceptable by the Official Mexican Standards (NOM-247-SSA1-2008), which establishes that the humidity content for flours should be lower than 15%. The protein content of the degummed and defatted flour obtained from *S. hispanica* seeds was 75.28 ± 1.08%. The percentage obtained in the

present study is higher than the reported by Salazar *et al.* (2020), who in spite of obtaining a protein concentrate had a lower protein content (40.5 ± 0.5%), probably attributed to differences in the degumming procedure and the additional defatting steps performed in the present study.

4.2 Enzymatic hydrolysis of protein rich flour

Among the methods for the extraction of proteinic derivatives, enzymatic hydrolysis is the most accepted since it is Generally Recognized as Safe (GRAS) by the FDA (Marciniak *et al.,* 2018); also, the hydrolysis products are not toxic, and enzymatic conditions can be easily controlled (Kim & Wijesekara, 2010). The use of Pepsin and Pancreatin® in enzymatic hydrolysis is recommended since they have reported the release of proteinic derivatives with short amino acid sequences (Shahidi & Zhong, 2008), and they simulate protein digestion in the human body. From the enzymatic hydrolysis, the degree of hydrolysis (DH) obtained was, which according to Benítez *et al.,* (2008) a DH above 10% is categorized as an extensive hydrolysate with potential biological activity. The 33.79 ± 2.14% was higher than the 15% reported by Cotabarren *et al.,* (2018) who used papain on *S. hispanica* for 120 min showing that the use of an enzymatic system instead of a single enzyme results in a higher DH in less time.

4.3 Low molecular weight proteinic derivatives

Ultrafiltration has been generally used to concentrate fractions of a certain molecular weight before proceeding to peptide amino acid identification. From the ultrafiltration of the proteinic hydrolysate of chia, three proteinic derivatives with different molecular weight ranges were obtained: <1, 1-3, and 3-5 kDa. The anticancer activity of the proteinic derivatives is, among other things, attributed to their low molecular weight (Chalamaiah *et al.,* 2018). Roy *et al.,* (2002) reported a proteinic derivative with a molecular weight <2 kDa, obtained from bovine lactoferrin by hydrolysis with Pepsin that showed antiproliferative activity on the HL-60 cell line by induction of apoptosis. Also, Song *et al.,* (2014) reported that a proteinic derivative with a molecular weight of <1 kDa obtained from *Setipinna taty* (anchovy) by enzymatic hydrolysis with Pepsin, induced apoptosis of PC-3 cell line.

4.4 Protein content of proteinic hydrolysate and its derivatives

The protein content of the proteinic hydrolysate and its derivatives was determined by the Lowry method. The results indicate that the protein content of the proteinic hydrolysate and its derivatives <1, 1-3 y 3-5 kDa was of 0.507 ± 0.02, 0.074 ± 0.02, 0.069 ± 0.01 and 0.059 ± 0.02 mg/mL, respectively. According to the Tukey statistical test, there was statistical difference between the homogeneous group, formed by the <1 and 1-3 kDa derivatives, and the 3-5 kDa derivative. The protein content obtained was higher than the 0.04 ± 0.03 and 0.06 ± 0.01 mg/mL reported by Chan-Zapata *et al.,* (2018) for the <1 and 1-3 kDa proteinic derivative, respectively. Since the starting material of Chan-Zapata *et al.,* (2018) was also chia, and the same enzymes where employed for the hydrolysis, this

variation could be attributed to the fact that they obtained a protein isolate or to the order in which the membranes where used in the ultrafiltration. In the present study the smallest membrane (1 kDa) was used first in order to concentrate the protein with low molecular weight as much as possible since these proteinic derivatives are more bioavailable and display greater bioactivity than the parent protein.

4.5 Evaluation of cellular viability on cancer cell lines

A large number of proteinic derivatives from plant origin have been evaluated for anticancer activities and for further use in cancer treatment. However, no reports are available on the anticancer activity of proteinic derivatives obtained from *S. hispanica.* The effect of the proteinic derivatives (<1, 1-3, 3-5 kDa), at different concentrations (0.25, 0.5, 0.75 and 1 mg/mL), on the cellular viability of cancer cell lines was evaluated through the MTT assay. The toxicity was evaluated on normal fibroblast cells (hFB) and as shown in Figure 1. The hFB cells showed a viability over 85% at all evaluated concentrations from all the proteinic derivatives. According to the International Organization for Standardization (ISO-10993-5:2009) a reduction of the cell viability by more than 30% is considered a cytotoxic effect, and since the viability of hFB was reduced by less than 15%, a cytotoxic effect is not considered.

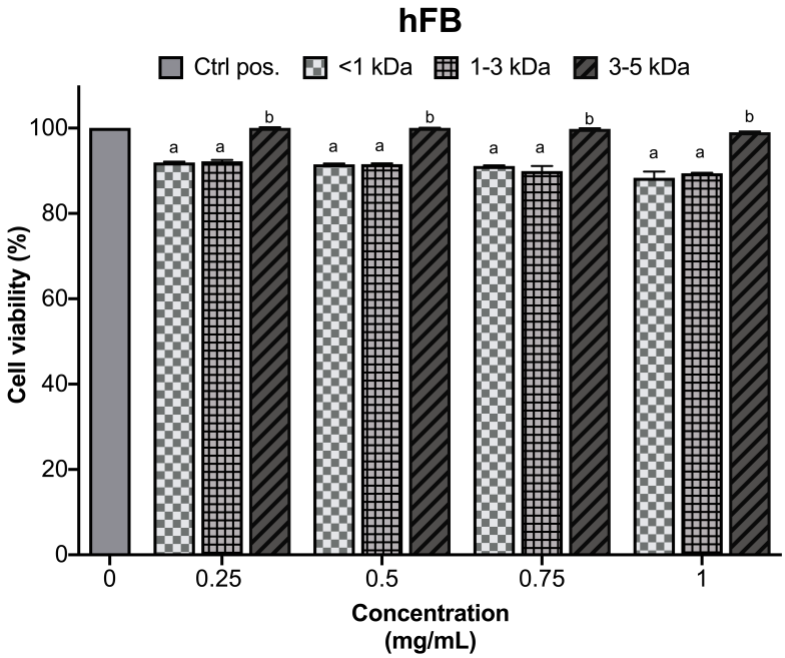

Figure 1. Viability of hFB cell line treated with the proteinic derivatives using the colorimetric MTT assay. Values expressed as mean ± SD. a-c indicate statistical difference only between samples in each concentration ($p<0.05$). A-D indicate statistical difference only between each proteinic derivative at different concentrations ($p<0.05$). Positive control, medium.

The individual effect of each factor (proteinic derivatives and concentrations) on the cellular viability of the cancer lines, and the effect of the interaction of both factors was analyzed through multifactorial ANOVA with a post hoc Tukey test ($p<0.05$); the results are discussed in that same order, respectively, by cell line. On MCF-7, the positive control (Taxol) showed the highest inhibitory effect of the viability by reducing it a $75.62 \pm 0.26\%$. Also, according to the multifactorial ANOVA analysis of the individual effect of the factors, the <1 kDa derivative from *S. hispanica* showed the highest statistical effect on the viability of the cell line, since it was inhibited by $23.35 \pm 0.08\%$. The 3-5 kDa derivative showed $7.94 \pm 0.08\%$ of cell viability inhibition. which was significantly lower ($p<0.05$). In addition to the individual analysis of factors, the concentration dependent response (0.25, 0.5, 0.75 and 1 mg/mL) showed significant increase of the effect at 1 mg/mL by reducing a $24.54 \pm 0.10\%$ the cellular viability ($p<0.05$). According to the results of the interaction of both factors analyzed by multifactorial ANOVA presented in Figure 2, on the MCF-7 breast cell line, the <1 kDa derivative showed the highest significant reduction of viability at 1 mg/mL reporting $30.91 \pm 0.17\%$ of inhibition. In contrast, derivative 3-5 kDa derivative at a concentration of 0.25 mg/mL had the lowest statistical effect by significantly decreasing the viability of the MCF-7 cell line by $4.2445 \pm 0.10\%$ ($p<0.05$).

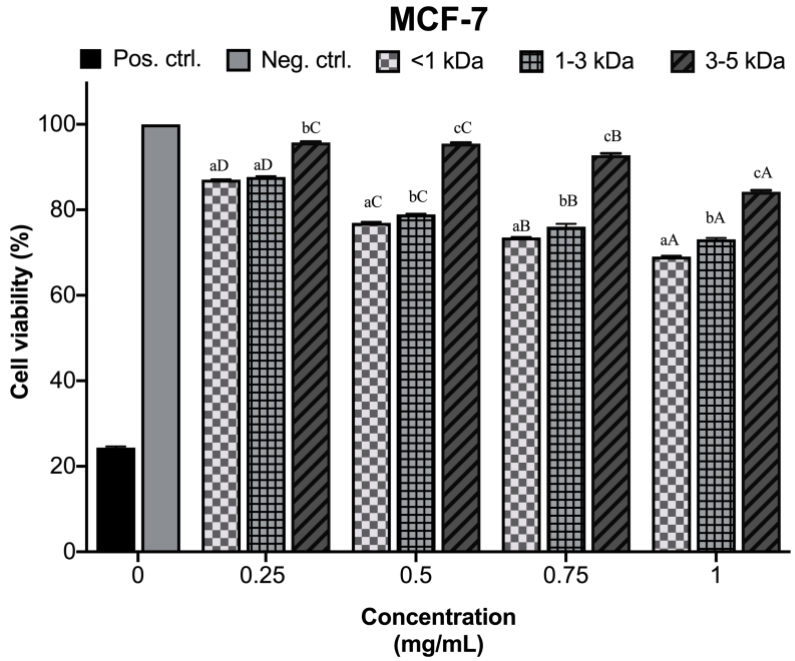

Figure 2. Cell viability of MCF-7 cancer cell line treated with the proteinic derivatives using the colorimetric MTT assay. Values expressed as mean ± SD. a-c indicate statistical difference only between samples in each concentration ($p<0.05$). A-D indicate statistical difference only between each proteinic derivative at different concentrations ($p<0.05$). Positive control, Taxol 30 ng/mL; negative control, medium.

Anticancer peptides have been proposed as a promising complementary cancer treatment due to its wide range of advantages over the traditional therapies (Zheng et al.,

2015). For instance, Xue et al. (2015) reported that evaluation of the peptide CPe-III (RQSHFANAQP, 1.155 kDa), obtained from *Cicer arietinum* L., on the MCF-7 breast cancer cell line resulted in a positive inhibitory effect on the cellular viability. In another study, Zheng et al. (2015) evaluated the anticancer effect of three synthetic peptides (RHPFDGPLLPPGD, RCGVNAFLPKSYLVHFGWKLLFHFD and KPEEVGGAGDRWTC), identified from *Dendrobium catenatum*, and the results showed that they were capable of inhibiting 30-41.8% of the cell viability on the MCF-7 cell line at a concentration of 0.5 mg/mL, respectively. The isolated peptide HVLSRAPR (935.1 Da), from *Spirulina platensis*, showed inhibition activity on the MCF-7's viability of approximately 5% at a concentration of 0.5 mg/mL (Wang & Zhang, 2016).

The results of the proteinic derivatives' anticancer activity on the Caco2 cancer cell line was analyzed as above. The positive control (Taxol) showed the highest inhibitory effect of the viability by reducing it an $82.77 \pm 0.31\%$. According to the to the multifactorial ANOVA analysis of the individual effect of the factors, the <1 kDa derivative demonstrated the highest anticancer activity by inhibiting $35.33 \pm 0.17\%$ the viability. The derivative with the lowest significant anticancer activity was 3-5 kDa, since it reduced the viability a $23.81 \pm 0.17\%$ ($p<0.05$). In addition to the individual analysis of factors, a concentration dependent behavior was observed, the highest significant effect was demonstrated at 1 mg/mL ($38.39 \pm 0.2\%$, $p<0.05$). According to the results of the interaction of both factors analyzed by multifactorial ANOVA, presented in Figure 3, the highest statistical effect on the viability was by the <1 kDa derivative at a concentration of 1 mg/mL with an inhibition of $46.34 \pm 0.35\%$. The lowest statistical effect was demonstrated by the 3-5 kDa derivative at a concentration of 0.25 mg/mL, where the viability was decreased by $17.1 \pm 0.35\%$ ($p<0.05$).

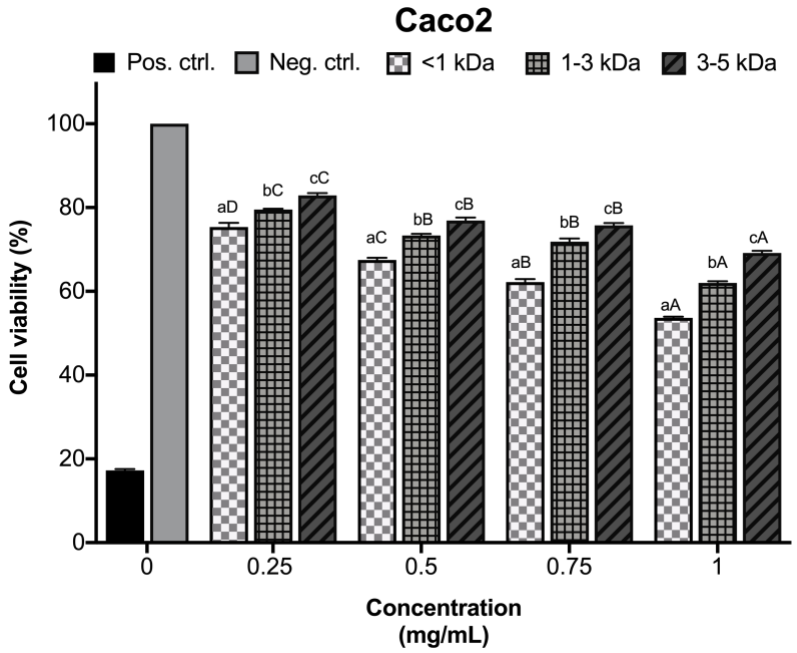

Figure 3. Cell viability of Caco2 cancer cell line treated with the proteinic derivatives using the colorimetric MTT assay. Values expressed as mean ± SD. a-c indicate statistical difference only between samples in each concentration ($p<0.05$). A-D indicate statistical difference only between each proteinic derivative at different concentrations ($p<0.05$). Positive control, Taxol 30 ng/mL; negative control, medium.

There is suggesting evidence that proteinic derivatives may have potential for human health promotion and disease risk reduction (Wang & Zhang, 2016). For instance, Luna-Vital et al., (2014) identified five peptides from *Phaseolus vulgaris*, all with molecular weight lower than 1 kDa, that showed anticancer activity by inhibiting the viability of colon cancer cell lines in a concentration dependent manner. However, the effectiveness to inhibit cell proliferation were different depending on the cell line. These derivatives stimulated the activation of the tumor suppressor protein p53 and p21 in the HTC116 colon cancer line; these increases could be a potential mechanism of action by causing cell cycle arrest. In another study, Wang & Zhang, (2016) reported that derivatives with molecular weight <3 kDa from *Spirulina platensis* showed the strongest inhibition on five cancer cell lines, among them, a colon cancer cell line. Also, they identified a peptide, HVLSRAPR (935.1 Da), that inhibited cellular viability of the colon cancer line up to 60% when tested alone.

On the PC-3 cancer cell line, the positive control (Taxol) showed the highest inhibitory effect of the viability by reducing it a 72.85 ± 0.82%. According to the multifactorial ANOVA analysis of the individual effect of the factors, the <1 kDa derivative reported the highest statistical effect by reducing the viability 20.15 ± 0.60% ($p<0.05$). In addition to the individual analysis of factors, as for the concentrations evaluated, the highest statistical effect was reported at 1 mg/mL where the cellular viability was decreased 25.83

± 0.69% (p<0.05). According to the results of the interaction of both factors analyzed by multifactorial ANOVA, as shown in Figure 4, the overall highest statistical effect on the PC-3 line, was reported on the <1 kDa proteinic derivative at a concentration of 1 mg/mL where the cellular viability was decreased 32.35 ± 1.20% (p<0.05). Contrarily, the lowest statistical effect was by the 1-3 kDa proteinic derivative at a concentration of 0.5 mg/mL where the viability reduction was of 5.62 ± 1.20% (p<0.05). Proteinic derivatives with low molecular weight have demonstrated anticancer effect on the PC-3 cancer cell line. Kim *et al.* (2013), from *Ruditaphes philippinarum,* reported the identification of the peptide AVLVDKQCPD that at 1.29 mg/mL reduced 50% of the cellular viability. Also, Song *et al.* (2014) identified the peptide YALPAH (670.77 kDa) from *Setipinna taty,* that reduced 50% of the cellular viability on PC-3 at a concentration of 11.3 mg/mL.

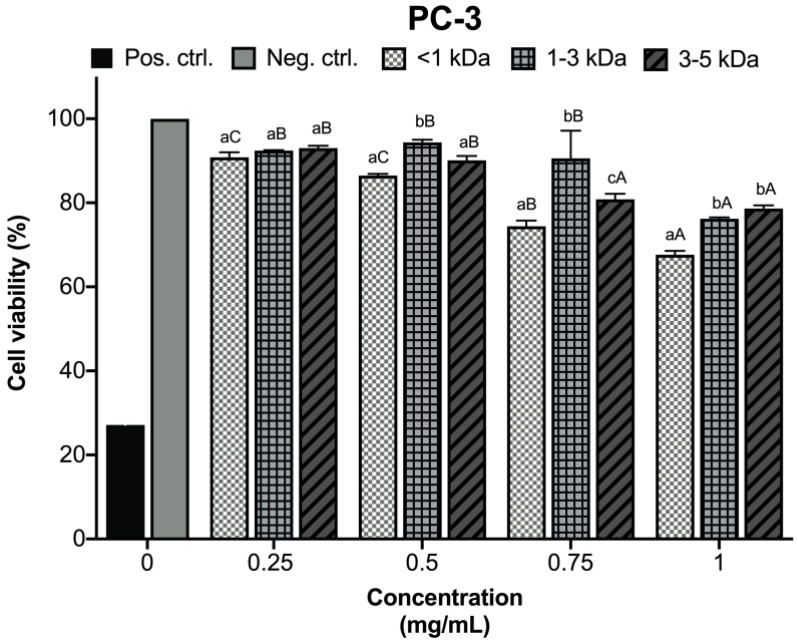

Figure 4. Cell viability of PC-3 cancer cell line treated with the proteinic derivatives using the colorimetric MTT assay. Values expressed as mean ± SD. a-c indicate statistical difference only between samples in each concentration (p<0.05). A-D indicate statistical difference only between each proteinic derivative at different concentrations (p<0.05). Positive control, Taxol 30 ng/mL; negative control, medium.

The results of the MTT assay on the HepG2 cell line, were analyzed as the other cancer cell lines. The positive control (Taxol) showed the highest inhibitory effect of the viability by reducing it a 32.96 ± 0.57%. According to the multifactorial ANOVA analysis of the individual effect of the factors, the proteinic derivative that reported the highest statistical inhibition was the <1 kDa derivative, since it decreased the viability 29.18 ± 0.20% (p<0.05). As for the results of the concentrations evaluated through the individual analysis of factors, the highest statistical effect was at 1 mg/mL since the viability was inhibited a 38.07 ± 0.23% (p<0.05). According to the results of the interaction of both factors analyzed by multifactorial ANOVA and as shown in Figure 5, the <1 kDa proteinic

derivative at 1 mg/mL resulted in the overall highest statistical effect on the HepG2 line, where the viability was reduced a 38.07 ± 0.41% ($p<0.05$); conversely, the lowest overall statistical effect was reported on the 3-5 kDa proteinic derivative at 0.5 mg/mL since the inhibition of viability was 8.44 ± 0.41% ($p<0.05$).

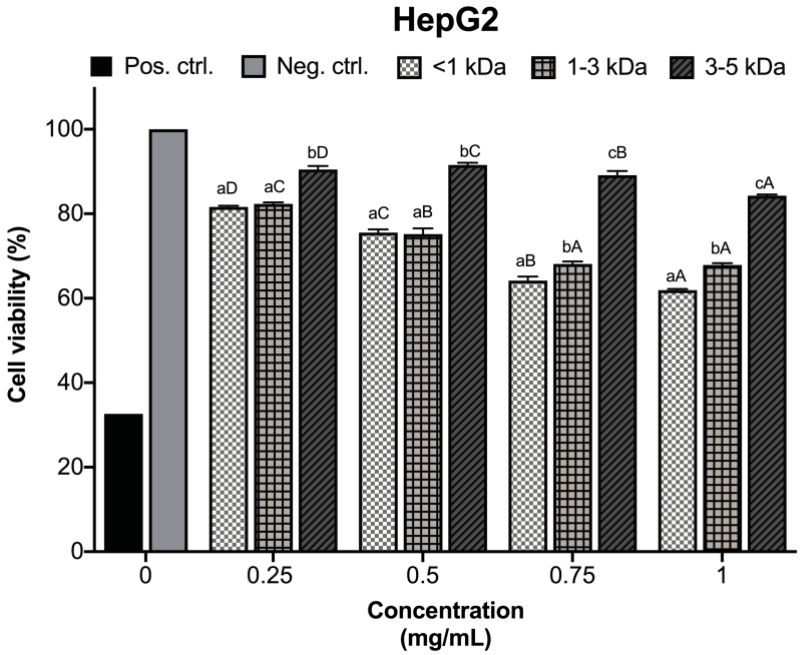

Figure 5. Cell viability of HepG2 cancer cell line treated with the proteinic derivatives using the colorimetric MTT assay. Values expressed as mean ± SD. a-c indicate statistical difference only between samples in each concentration ($p<0.05$). A-D indicate statistical difference only between each proteinic derivative at different concentrations ($p<0.05$). Positive control, Taxol 30 ng/mL; negative control, medium.

Several proteinic derivatives have demonstrated anticancer effects on HepG2 cancer line. Li *et al.* (2013) reported that peptides from corn with a molecular weight lower than 5 kDa, inhibited cellular viability up to 25% after the cells were treated for 48h at a peptide concentration of 1.28 mg/mL; in further studies, they were able to identify that these peptides caused apoptotic death. Also, Wang *et al.* (2016) from *Brassica campestris* identified a peptide (WTP), of 408.2 Da that at a concentration of 1.6 mg/mL and 72 h of treatment, was able to reduce cellular viability of HepG2 by 36.97 ± 4.06%. On the HepG2 cell line, the percentage of inhibition reported by the <1 kDa derivative is higher than those reported by the single identified peptides discussed above; these differences in activity could be attributed to the characteristics of the peptides that compose the proteinic derivative, such as their amino acid composition, length, net charge and structural conformation.

The highest statistical inhibitory effect on cell viability was reported by the <1 kDa proteinic derivative at a concentration of 1 mg/mL. According to the International Organization for Standardization (ISO-10993-5:2009), the interaction of both factors

(proteinic derivative and concentration), showed a cytotoxic effect on all of the evaluated cancer lines, since it reduced the cell viability over 30% in all cases (Table 1). This is the first study to report that proteinic derivatives obtained from *S. hispanica* possess anticancer activity. In spite of cancer being a complex disease involving numerous biological processes, there are altered processes of cancer cells, such as resistance to cell death, immortality or accelerated proliferation, that are directly related to the differences in lipid composition of the cancer cell membrane (Zalba & ten Hagen, 2017). One of the main differences is a de-regulation in the lipid asymmetry resulting in the negative charge of the cancer cell outer membrane, which is known to be a target for the anticancer proteinic derivatives (Bernardes & Fialho, 2018). Due to the alterations in cancer cell metabolism and structure it is not possible to determine the exact reason why the proteinic derivatives are more toxic in some cancer lines than others. However, it could be attributed to the different lipids that generate the negative surface, that increase or decrease the attraction of the proteinic derivatives to the cancer cell membrane and cause further inhibition.

Table 1. Cytotoxic effect of the proteinic derivatives <1 and 1-3 kDa at 1 mg/mL on evaluated cancer cell lines according to the ISO-10993-5:2009.

Cancer cell line	Proteinic derivative	Cell viability (%)	Cytotoxic effect
MCF-7	<1 kDa	69.09 ± 0.17^{a}	Yes
	1-3 kDa	73.09 ± 0.17^{b}	No
Caco2	< 1 kDa	53.66 ± 0.35^{a}	Yes
	1-3 kDa	61.99 ± 0.35^{b}	Yes
PC-3	<1 kDa	68.64 ± 1.20^{a}	Yes
	1-3 kDa	76.20 ± 1.20^{b}	No
HepG2	<1 kDa	61.93 ± 0.41^{a}	Yes
	1-3 kDa	67.85 ± 0.41^{b}	Yes

$^{a\text{-}c}$ indicate statistical difference only between proteinic derivatives in each cancer cell line ($p<0.05$).

The anticancer activity reported by the <1, 1-3, 3-5 kDa derivatives, could be attributed to the experimental strategy followed during the present study. For the obtention of the protein rich fraction, the defatting process was repeated twice, which could have had a direct influence in the resulting substantial protein content. As for the hydrolysis, low molecular weight proteinic derivatives released by use of the enzymes As mentioned by Quintal-Bojórquez & Segura-Campos (2020), Pepsin and/or Pancreatin have exerted antiproliferative effects on cancer cell lines like Caco-2, PC-3, MCF-7 and HepG2, results that are consistent with the ones obtained in this study. Furthermore, the degree of hydrolysis is directly related to the size of the peptides that conform the proteinic derivatives, making it crucial for its following biological activities (Cotabarren *et al.,* 2019).

When comparing the proteinic derivatives, the highest protein content was reported in the <1 kDa derivative, result that could be attributed to the order in which the membranes were used during the ultrafiltration, given that when used contrarily, the higher protein content is found in the larger derivatives (Chan Zapata *et al.,* 2018). This result could also be due to the fact that the enzymatic system Pepsin-Pancreatin is known to release a higher rate of short amino acid sequences from proteins (Shahidi & Zhong, 2008).
The highest statistical inhibitory effect on the viability of the evaluated cancer cells, was reported by the <1 kDa proteinic derivative at a concentration of 1 mg/mL. Low molecular weight peptides could display a higher cytotoxic effect since they show greater motility and diffusivity, which are related to increased molecular interactions with the components of the cancer cell membrane (Jumeri & Kim, 2011). Most of the proteinic derivatives and peptides that show anticancer activity have a molecular weight lower than <3 kDa (Chalamaiah *et al.,* 2018), which is consistent with the results reported in the present study. In addition, the highest anticancer effects, *i.e.,* over 50% inhibition of proliferation, are shown by isolated peptides (Quintal-Bojórquez & Segura-Campos, 2020), making clear the importance of the amino acid sequence identification of the peptides present in the proteinic derivatives.

4.6 Multicriteria decision analysis for peptides with anticancer potential

Between the three proteinic derivatives obtained from *S. hispanica* seeds, the <1 kDa derivative reported the highest statistical inhibitory effect on the viability of the cancer cell lines evaluated, for that reason, its amino acid sequence was analyzed. The different beneficial effects of the proteinic derivatives could depend on their amino acid composition, sequence and low molecular weight. However, there are particular characteristics identified for proteinic derivatives that exert anticancer properties and such characteristics are: a cationic nature (net charge from +2 to +9), amphipathic structure, amino acid sequence from 5 to 20 residues with presence of lysine and arginine, hydrophobicity of approximately 30-50% and, in terms of secondary structure, an α-helix conformation (Hoskin and Ramamoorthy, 2008; Oelkrug *et al.,* 2015; Felício *et al.,* 2017). Also, the amino acid sequence of the proteinic derivative with anticancer activity is mainly composed by nonpolar and hydrophobic amino acids such as glycine, proline, leucine, phenylalanine and alanine; followed by positive and negative hydrophilic amino acids such as lysine, arginine and histidine, hydrophilic and polar amino acids such as serine, asparagine, glutamine and cysteine. Chia seeds contain all amino acids for human nutrition, among them are glutamine, aspartate, arginine, leucine, and phenylalanine are present in higher concentrations; in lower concentrations are histidine and threonine (Grancieri *et al.,* 2019).
All of the characteristics mentioned above are important for the interaction of the peptide(s) with the cellular membrane of the cancer cell and where taken into consideration for the proposal of peptides with possible anticancer activities. From sequencing of the <1 kDa proteinic derivative, 1201 peptide sequences were recovered. The latter sequences were submitted to a multicriteria analysis and a rank was obtained

as a result; the peptide that best met all the characteristics was listed as number one since it is more likely to present anticancer activities. The peptide ranked as number one was KLKKNL, which is composed by 6 amino acid residues, has a molecular weight of 742.4 Da, a net charge of +3 and hydrophobicity of 33%; in addition, its structure is an α-helix conformation. The positive net charge of the peptide favors its union to a negative charged cellular membrane (cancer cell membrane) through electrostatic interactions between the lateral chains of the cationic amino acids and the anionic heads of phospholipids (Sánchez-Acosta, 2016), such as phosphatidylserine (PS) and phosphatidylethanolamine (PE). In spite of the elucidation of several mechanisms of action of peptides, the exact mechanisms through which peptides destroy cancer cells are yet to be described (Wang *et al.,* 2017). For peptides that are amphipathic, present a short amino acid sequence and an α-helix conformation, such as KLKKNL, the mechanism of action proposed is the in-plane diffusion model. As shown in Figure 6, this mechanism consists on the diffusion of the peptide into the plasmatic membrane of cancer cells, which leads to its thinning, pore formation and necrosis of the cancer cell (Oelkrug, 2015). On the contrary, when the net positive charge exceeds the proposed limits, the insertion of the peptide into the membrane could be blocked (Sánchez Acosta, 2016). The amphipathic α-helix conformation facilitates both electrostatic and hydrophobic interactions of the peptide with the target membrane, in fact, it is considered essential for the following insertion and disruption of the membrane (Zelezetsky & Tossi, 2006). Also, according to Lau & Dunn (2017), most peptides entering clinical development, tend to have an amino acid length of 2 to 10 residues.

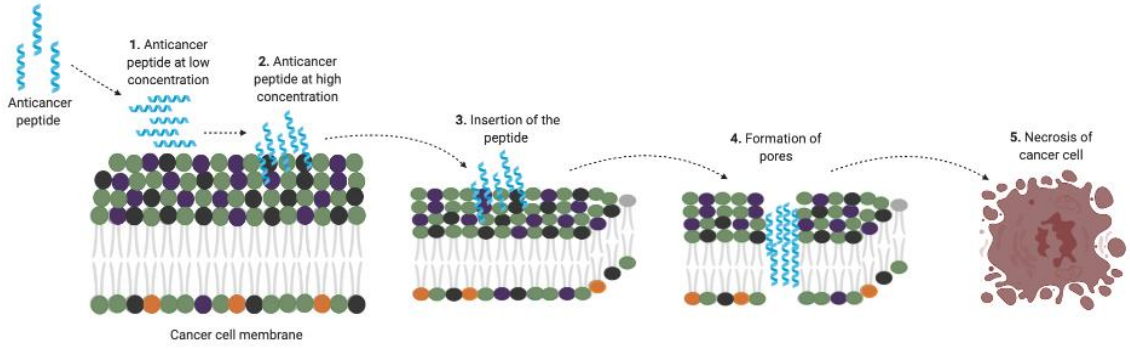

Figure 6. In-plane diffusion model. (1) The anticancer peptide binds parallel to the cancer cell membrane at low concentration. (2) As the concentration increases, the anticancer peptide acquires a perpendicular orientation. (3) At high peptide/lipid ratio, the anticancer peptide inserts into the membrane causing membrane thinning. (4) Trans-membrane pores are formed leading to (5) necrosis of the cancer cell.

5. Conclusion

In conclusion, the proteinic derivatives from *S. hispanica* reported significant inhibition of the viability on cancer cell lines; additionally, the proteinic derivatives did not show cytotoxicity on normal human cells (hFB). The <1 kDa proteinic derivative at a concentration of 1 mg/mL reported the highest statistical anticancer effect on all four cancer cell lines. Since the <1 kDa had the highest anticancer effect, its amino acid sequence was identified. The 1201 peptides sequences obtained from the sequencing were evaluated through a multicriteria decision analysis, and the peptide ranked as number one was KLKKNL. In spite of KLKKNL being ranked as number one, other peptides present in the <1 kDa derivative could exert anticancer activity. Further *in silico* and *in vivo* evaluations of the peptides present in the <1 kDa derivative obtained from chia are necessary prior to their use as complementary treatment for cancer. However, there is potential for the application of proteinic derivatives as functional food and nutraceutical ingredients, particularly as complementary treatments for cancer therapy.

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Author's contributions

Maira Rubí Segura-Campos directed the investigation, conceived the original idea, designed the study and revised the manuscript. Leydi Maribel Carrillo-Cocom supervised the cell evaluation methodology and revised the manuscript. Alan Javier Hernández-Álvarez supervised the methodology for the amino acid sequencing and analysis and revised the manuscript. Nidia del Carmen Quintal-Bojórquez conducted the experiments, collected and analyzed the data and drafted the manuscript.

Declaration of interests

The authors declare no conflict of interest.

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ARTÍCULO DE REVISIÓN: PÉPTIDOS BIOACTIVOS COMO TERAPEÚTICOS ADYUVANTES PARA CÁNCER

Bioactive peptides as Therapeutic Adjuvants for Cancer

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Abstract

Conventional cancer treatments such as chemotherapy, radiotherapy and surgery cause serious side effects on cancer patients which decrease their quality of life. In the past few years, cancer patients have been interested in the use of complementary medicine to improve the efficacy of conventional cancer treatments and decrease the side effects. Among the broad spectrum of complementary medicine, bioactive peptides from natural sources have gained great interest due to their potential use in the treatment of chronical diseases such as cancer. This review reports an updated survey of bioactive peptides, from natural sources, with anticancer and immunomodulatory activities obtained by enzymatic hydrolysis. Several peptides have demonstrated anticancer effects on *in vitro* and *in vivo* essays, such as: selective cytotoxicity, inhibition of growth, tumor size reduction and immunomodulation. However, there is absence of formal pharmacokinetic profiles and standardized extraction procedures of bioactive peptides. Further clinical trials are necessary to verify these anticancer effects and, facilitate the use of peptides in the treatment of cancer.

Keywords: Cancer prevention, dietary intervention, diet

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Introduction

According to the World Health Organization (WHO) cancer is the second cause of death worldwide; the estimated number of deaths was approximately 9.5 million in 2018. Currently, first option cancer treatments include surgery, chemotherapy and radiotherapy. However, these treatments are expensive and cause serious side effects in cancer patients because they are not specific to cancer cells (1). In the last decade, patients with cancer have been interested in the use of complementary medicine (CM) to improve their quality of life by increasing the efficacy of conventional cancer treatments, their chance of survival and to decrease the side effects caused by the therapies and the disease (2). One category of CM includes the use of natural products, particularly of bioactive peptides, which are defined as amino acids short sequences (3-20 AAs) within a protein that exert beneficial effects in the modulation and regulation of metabolic processes (3) and have potential use in the treatment and prevention of diseases. Bioactive peptides are inactive when encrypted in a parent protein and need to be released by enzymatic hydrolysis, food processing or microbial fermentations to exert their beneficial effects. Most of the body's natural processes are signaled or regulated by the interaction of specific amino acid sequences in the form of peptides or fragments of proteins, meaning they could be used in a wide range of therapeutic applications (4).

Based on their amino acid sequence and molecular weight, bioactive peptides can modify how the human body responds to a disease like cancer, or they can enhance a specific system like the immune. However, the relationship between the structure and activities of bioactive peptides are not well stablished. In this context, the scope of this review includes the latest studies on the anticancer and immunomodulatory activities of bioactive peptides from natural sources extracted by enzymatic hydrolysis.

1. Anticancer activity of bioactive peptides

1.1 Therapies for cancer

Cancer is defined as a collection of diseases in which the body's cells begin to divide without control and spread to surrounding and distant tissues (5). This disease develops through a multistep process, called carcinogenesis (6). In 2018, the countries with the highest incidence rate were Asia with a 48.4%, followed by Europe with a 23.4%, North America with 23% and Latin America and the Caribbean with 7.8% (7). The most common cancer treatments are chemotherapy, surgery and radiotherapy, which are also known as conventional therapies or western medicine. However, the main disadvantages on the use of the conventional treatments are their elevated cost and the serious side effects they cause in patients, who usually develop symptoms such as pain, fatigue, insomnia and infertility, thus, decreasing their quality of life and their probability of survival. To reduce or alleviate these side effects cancer patients are turning to the use of complementary medicine (CM), which is defined as the use of unconventional medicine combined with

Western medicine, rather than as alternative (8). The National Center for Complementary and Integrative Health Medicine (NNCIH) classified CM into 5 categories (Figure 1).

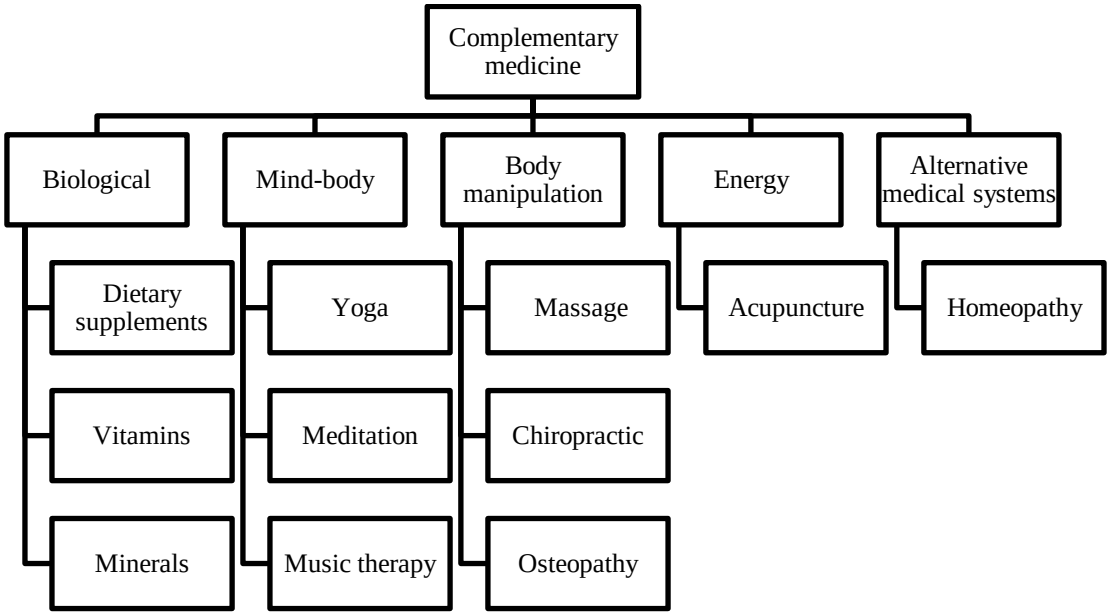

Figure 1. Categorization of complementary medicine (9).

Among the biological category, dietary supplements from natural sources have gained great importance. Generally, human beings consume carbs, lipids, proteins and other nutrients that once ingested, are metabolized into monosaccharides, amino acids or peptides that can be absorbed by the intestine and used by the body (10). Particularly, bioactive peptides have gained great importance due to their benefits to human health. The advantages in the use of bioactive peptides as CM include low toxicity, high tissue penetration due to their small size and cell diffusion, and high permeability (11).

1.2 Production of anticancer peptides

Bioactive peptides are generally obtained from plant or animal protein sources and its beneficial effects have been reported in a wide range of studies. To obtain a bioactive peptide, it needs to be extracted from the parent protein, which can be achieved by several strategies like enzymatic hydrolysis, microbial fermentation or gastrointestinal digestion. However, enzymatic hydrolysis is the most common method for the extraction of bioactive peptide since it doesn't produce toxic secondary metabolites, it can simulate gastrointestinal digestion and the time of reaction is shorter. Among the commercial enzymes successfully used for the extraction of bioactive peptides with anticancer activity are pepsin, pancreatin, alcalase, Flavorzyme, trypsin, chymotrypsin and papain (12). Figure 2 shows a general flowchart of the obtention of bioactive peptides.

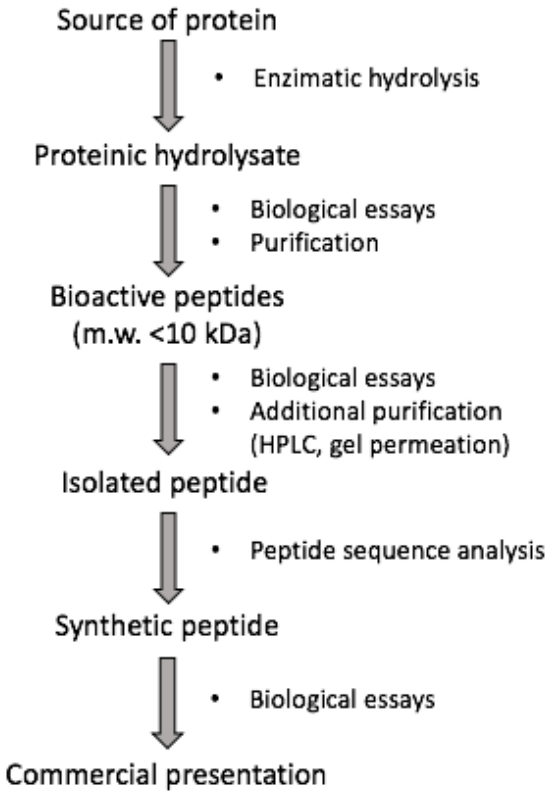

Figure 2. General process to obtain bioactive peptides from natural sources. Modified from Chalamaiah et al. (12).

1.3 Peptides with anticancer activities

Bioactive peptides have exhibited a wide range of anticancer activities in established cell lines such as inhibition of cell migration, inhibition of tumor angiogenesis, antioxidant activity, inhibition of cell proliferation, induction of apoptosis and cytotoxicity (13) which are summarized in Table 1.

Table 1. Bioactive peptides from natural sources with anticancer activity.

Source	Enzyme used to produce hydrolysate	Peptide sequence and/or molecular weight	Animals or cancer cell lines used in tests	Anticancer activity	Reference
Phaseolus vulgaris (common bean)	Pepsin and pancreatin	GLTSK (505.48 Da), LSGNK (518.29 Da), GEGSGA (521.22 Da), MPACGSS (656.01 Da) and MTEEY (671.98 Da)	HCT-116, RKO and KM12L4 (human colon cancer cell lines)	Inhibition of cell growth and modification of expression of cell cycle regulatory proteins p53, p21, cyclin B1, BAD, cytC, c-casp3, Survivin and BIRC7	(14)
Setipinna taty (half-fin anchovy)	Pepsin	YALPAH (670.35 Da)	PC-3 (human prostate cancer cell line)	Induced apoptosis	(15)
Saccostrea cucullata (oyster)	Proteases	LANAK (515.29 Da), PSLVGAPPVGKLTL (1.432 kDa) and VLVLLEHPVL (1.145 kDa)	HT-29 (human colon carcinoma) cell line	Inhibition of cell growth, morphological changes and DNA damage	(16)
Labeo rohita egg (rohu)	Pepsin	-	Caco-2 (human colon cancer cell line)	Antiproliferative activity	(17)
Telligarca granosa (blood clam)	Neutrase	WPP (398.44 Da)	PC-3, DU-145, H-1299 and HeLa cell lines	Cytotoxicity and change of PC-3 cells morphology	(18)
Capra aegagrus hircus (goat)	-	≈8 kDa	HCT-116 (human colon cancer cell line)	Antiproliferative activity	(19)
Cicer arietinum (chickpea)	Flavorzyme	RQSHFANAQP (1.155 kDa)	MCF-7 and MDA-MB-231 (breast cancer cell lines)	Antiproliferative activity by increasing p53 concentration.	(20)
Dendrobium catenatum (orchid)	Alcalase and trypsin	RHPFDGPLLPPGD (1.416 kDa), RCGVNAFLPKSYLVHFGWKLLFHFD (2.994 kDa) and KPEEVGGAGDRWTC (1.504 kDa)	HepG-2 (liver), SGC-7901 (gastric), MCF-7 (breast) cancer cell lines	Antiproliferative activity	(21)
Pachymedusa dacnicolor (leaf frog)	-	GMWSKIKETAMAAAKEAAKAAGKTISDMIKQ	H157, PC-3, U251MG cancer cell lines	Antiproliferative activity	(22)
Crocodylus siamensis (freshwater crocodile)	-	NGVQPKYK WWKWWKKWW (2. 433 kDa) and NGVQPKYRWWRWWRRWW (2.545 kDa)	HeLa and CaSki (cervix) cancer cell lines	Induction of cell death by apoptosis	(23)
Epinephelus lanceolatus egg (giant grouper)	Protease N	<5 kDa	Ca9-22 and CAL 27 (oral cancer cell lines)	Decrease of cell viability of both cell lines and induction of apoptosis of Ca9-22 cells	(24)
Lates calcalifer (barramundi fish)	Alcalase	-	CaCo2 (human colon) and HepG-2 (liver) cancer cell lines	Antiproliferative activity	(25)
Brassica campestris (Rapeseed)	Neutral proteases, B. subtilis and A. elegans	WTP (408.2 Da)	HepG-2 (liver cancer cell line)	Antiproliferative activity and induction of apoptosis	(26)
Raja porosa (skate)	Alcalase and trypsin	FIMGPY (726.9 Da)	HeLa cell line	Antiproliferative activity by induction of apoptosis	(27)
Spirulina platensis	Pepsin, trypsin and chymotrypsin	HVLSRAPR	MCF-7 (breast), HepG-2 (liver) and SGC-7901 (gastric) cancer cell lines	Antiproliferative activity	28)
Pleurotus eryngii (oyster mushroom)	-	-	BT-549 cells (breast), Hela-229 cells (cervical), and HGC-27 (stomach) cancer cell lines	Cell growth inhibition	(29)

A large number of anticancer peptides (ACP) have been reported from plant sources. Luna-Vital et al. (14) identified five peptides extracted from common bean by the sequential enzymatic system Pepsin-Pancreatin which exerted antiproliferative effects on human colon cancer cell lines (HCT-116, RKO and KM12L4) by modification of proteins involved in either cell cycle arrest or induction of apoptosis. Xue et al. (20) reported the extraction of an ACP of 1.155 kDa from chickpea, by enzymatic hydrolysis with Flavorzyme, that reported antiproliferative activity; the concentration of the protein p53 was increased in response to the interaction of the peptide with the breast cancer cell lines MCF-7 and MDA-MB-231. The increase of p53 might regulate signaling pathways leading to target cell apoptosis and mitochondrial oxidative phosphorylation. Zheng et al. (21) reported the extraction of nine peptide fractions from *D. catenatum* by enzymatic hydrolysis with alcalase and trypsin. From the nine fractions extracted, A_3 demonstrated the highest antiproliferative activity on liver (HepG-2), gastric (SGC-7901) and breast (MCF-7) cancer cell lines by inhibiting growth up to 70%. The three most abundant peptides in the A_3 fraction were RHPFDGPLLPPGD (1.416 kDa), RCGVNAFLPKSYLVHFGWKLLFHFD (2.994 kDa) and KPEEVGGAGDRWTC (1.504 kDa), to which the antiproliferative activity could be attributed. Wang et al. (26), identified an ACP of 408.2 Da with the amino acid sequence WTP. This peptide was extracted from rapeseed by bacteria and enzyme synergy on solid-state fermentation and then, it was tested on a liver cancer cell line (HepG-2). The results showed that the identified peptide significantly changed the morphology of the cancerous cells *in vitro* and caused apoptosis. Interesting results have been reported by ACPs extracted from animal sources. In a study, Umayaparvathi et al. (16) identified three bioactive peptides from an oyster, all of which reported anticancer activities, such as cell growth inhibition, apoptotic morphological changes and oxidative DNA damage. From rohu eggs, Chalamaiah et al. (17) reported the antiproliferative activity of protein hydrolysates (PH), obtained by hydrolysis with pepsin, on a colon cancer cell line (Caco-2); the results reported that the PH showed dose dependent inhibitory effect on Caco-2 cells. Chi et al. (18) isolated two peptides from the blood clam muscle and the peptide identified with the sequence WPP (398.44 Da) showed strong cytotoxicity in a dose dependent matter on PC-3 (human prostate), DU-145 (human prostate), H-1299 (lung) and HeLa (cervical) cancer cell lines and significantly changed the morphology of PC-3 cells; in addition, WPP has the capacity to eliminate excessive ROS, therefore, preventing the development of cancers caused by a large number of free radicals. From goat spleens, Su et al. (19) reported the isolation of and ACP (≈8 kDa) that significantly inhibited the growth of HCT116 (human colon) cancer cell line after a 4-6 day treatment; furthermore, treatment of HCT116 with the ACP for 6-12 h induced apoptosis by increasing the expression of poly(ADP-ribose) polymerase (PARP) and p53, and decreased the expression of Mcl-1. In HeLa and CaSki cervix cancer cell lines, Theansungnoen et al. (23) tested two peptides, KT2

(NGVQPKYKWWKWWKKWW) and RT2 (NGVQPKYRWWRWWRRWW), derived from freshwater crocodile; both ACPs were observed to induce cell death in HeLa cells. Yang et al. (24) tested bioactive peptides with a molecular weight <5 kDa, obtained from roe protein hydrolisates derived from giant grouper (*Epinephelus lanceolatus*); the results showed the peptides can inhibit cell proliferation of two cervical cancer cell lines (Ca9-22 and CAL27) in a dose dependent manner and induce apoptotic morphology by depolarization of the mitochondria and ROS generation. Pan et al. (27) separated an hexapeptide, FIMGPY (726.9 Da), from skate (*Raja porosa)* cartilage protein hydrolysate and its anticancer activity was evaluated in HeLa cells; cell viability tests indicated that FIMGPY strongly exhibited high dose-dependent antiproliferative activity by induction of apoptosis on the cancer cell line, since the Bac/Bacl-2 ratio and intensity of caspase-3 were significantly higher.

There is an evident relationship between the amino acid sequence and the activity of bioactive peptides. Song et al. (15) identified a bioactive peptide with anticancer activity, determined its sequence (YALPAH), synthetized it along with three analogous peptides (YALRAH, YALPAR and YALPAG) and evaluated their activity in PC-3 cells. Furthermore, the peptide with highest positive net charge (YALRAH) exhibited the strongest antiproliferation activity and the one with lower molecular weight (YALPAH) was proposed to be responsible for higher cytotoxicity. In another study, Tada et al. (30) reported similar results when they replaced a single amino acid on a peptide, histidine, for one with a higher positive charge, arginine; the results showed that the cytotoxic activity was increased 1.2- to 1.9-fold than the original peptide. Cationic peptides could disrupt the cancer cell membrane due to their interaction with the characteristic anionic components. The proposition that lower molecular weight peptides could display better cytotoxicity is because they demonstrate greater motility and diffusivity, which contribute to increased molecular interactions with cancer cell components (31).

There is enough scientific evidence that validates the anticancer activity of bioactive peptides to continue with studies directed towards the effect of peptides in clinic trials. However, there are still limitations that have to be analyzed, *i. e*., the mechanism of action of ACPs in the peptide-membrane level. Also, most of the tests to evaluate the anticancer activity of peptides are with *in vitro* models and very few are *in vivo*, which is why there is a lack of toxicologic information. For the above mentioned, it is very important to continue the identification of ACPs including the analysis of its amino acidic sequence and its specific mechanism of action.

2. Immunomodulatory activity of bioactive peptides

In diseases, like cancer, were the immunomodulatory system is suppressed it is important to review bioactive peptides with immunomodulatory activities.

2.1 Brief overview of the immune system

The immune system is the collection of cells, organs, tissues and molecules that function to defend the human body against bacterial, fungal, parasitic, viral infections and from the growth of tumor cells; the coordinated response of the immune system against infectious molecules is known as the immune response. The immune system is classified into two functional categories: innate and adaptive immunity. Innate immunity is non-specific and the first line of defense through anatomic barriers (skin, mucosity), physiologic (changes in pH, temperature), cellular (macrophages, leukocytes, dendritic cells, natural killer cells) or inflammatory components (cytokines, complement, interferons, prostaglandins) (12).

Adaptive immunity is highly specific against potentially harmful antigens. This type of immunity is classified into two categories: cell-mediated and antibody-mediated (also called humoral) immunity. In cell-mediated immunity, T lymphocytes are effector cells that mediate the cellular immune response by secreting cytokines and interacting with antigen presenting cells (APCs). T lymphocytes are divided in helper T (Th) cells, cytotoxic (Tc) cells and regulatory (Treg) cells. Tc cells, also called CD8+, recognize endogenous antigens associated to the mayor histocompatibility complex (MHC) class I in order to kill cancer cells and cells infected with viruses. Th cells, also called CD4+, recognize exogenous antigens associated to the MHC class II. CD4+ cells secrete cytokines (IFN-y), interleukins (IL-2, IL-4, IL-5, IL-6, IL-10, IL-13 and IL-25) and participate in the activation of B cells, T cells and other immune cells such as macrophages. Treg cells are responsible for the suppression of the immune response and the prevention of autoimmune disorders (32).

2.2 Peptides with immunomodulatory activities

The National Cancer Institute defines an immunomodulatory agent as a substance that stimulates or suppresses the immune system and may help the body fight cancer or other diseases. Most immunomodulatory pharmaceuticals are not adequate for chronic or preventive use; moreover, the side effects associated with the use of allopathic drugs and their high cost have enforced the need for alternative treatments (33). The discovery of immune modulating peptides from food proteins could provide an efficient and effective strategy (34).

Recently, immunomodulatory peptides have been extracted from a wide range of natural sources. These sources include fish (25, 34-39), whey (40, 41), amarant (41), egg (17, 43) and rice (44). Among the methods by which a peptide with immunomodulatory activities can be obtained, the *in vitro* hydrolysis of protein using proteolytic enzymes, is the most accepted; the enzymes used are mainly trypsin, Alcalase, pepsin, papain, pancreatin, chymotrypsin, Flavourzyme and Neutrase.

Numerous *in vitro* and *in vivo* assays have been successfully employed to evaluate de immunomodulatory activities of bioactive peptides. Most of the testing to evaluate the *in vivo* effects of immunomodulatory peptides (IP) have been conducted in mouse models, since their immune responses resemble the most to that of humans (Table 2).

Table 2. Bioactive peptides from natural sources with immunomodulatory activity.

Source	Enzyme used to produce hydrolysate	Peptide sequence and/or molecular weight	Animals or cancer cell lines used in tests	Immunomodulatory activity	Reference
In vivo					
Epinephelus lanceolatus egg (giant grouper)	Pepsin, trypsin and alcalase	<10 kDa	BALB/c mice	Increased cytotoxicity of NK cells, phagocytosis of macrophages, level of serum IgA and splenic CD4+ and CD8+	(34)
Shark proteins	Trypsin and chymotrypsin	<10 kDa	BALB/c mice	Enhanced gut barrier function and down regulated uncontrolled-inflammatory reactions	(35)
Cyprinus carpio egg (common carp)	Pepsin, trypsin and Alcalase	Peptides from 5-90 kDa	BALB/c mice	Enhanced proliferation of spleen lymphocytes, NK cell cytotoxicity, mucosal immunity and splenic CD4+ and CD8+	(36)
Ovalbumine, lysozyme, ovomucoid and whole egg white proteins	Pepsin, Neutrase and Alcalase	<10 kDa	BALB/c mice	Reduced lymphocyte proliferation and production of IL-13 and IL-10, and decreased secretion of TNF-α.	(43)
Alaska pollock	Trypsin	PTGADY (622 Da)	Kunming (KM) mice	Enhanced innate and adaptive immune responses	(39)
Coix glutein	Pepsin	≤ 3 kDa	ICR and BALB/c mice	Spleen index increase and influence in average body weight of mice	(45)
Salvia hispanica	Pepsin and pancreatin	<10 kDa	BALB/c mice	Decreased production of pro-inflammatory cytokines: TNF-α and IL-6	
In vitro* and *ex vivo					
Lates calcarifer (seabass) gelatin	Alcalase	-	U937 and RAW 264.7	Significantly reduced IL-6 and IL-1β production and showed DNA protective effects.	(25)
Salmon pectoral fin	Pepsin	PAY (349.15 Da)	RAW 264.7	Inhibited production of NO and PEG_2 and attenuation of pro-inflammatory cytokines	(37)
Aluterus monoceros (unicorn leatherjacket) gelatin	Glycyl endopeptidase from papaya latex	-	U937 and RAW 264.7 a	Protected DNA damage and reducted production of pro-inflammatory cytokines and NO	(38)
Whey protein	Alcalase	-	Spleen cell suspension	Enhanced splenocyte proliferation activity	(40)
Whey β-lactoglobulin	Trypsin	<1, 1-2, 2-5 and 5> kDa	Human Peripheral blood mononuclear cells (PBMC)	Increased secretion of IFN-γ and TGFβ. Stimulation of macrophages.	(41)
Amaranth protein	Pepsin and pancreatin	RSHK (527 Da), NRPWWWHPGGGG GGGGLGAGT (2034 Da), HGSEPFGPR (984 Da), RPRYPWRYT (1295 Da) and RDGPFPWPWYSH (1545 Da)	THP-1 and RAW 264.7 cells	Reduced TFN-α and COX-2 secretion and expression of p65 NF-κB	(42)
Coix glutein	Pepsin	≤ 3 kDa	ICR and BALB/c peritoneal macrophages and RAW 264.7 cell line	Increased secretion of NO from macrophages and attenuated immune response	(45)
Lupine protein	Izyme AL and Alcalase	-	THP-1 monocytes	Decreased the expression of proinflammatory cytokines and increased the expression of anti-inflammatory marker genes. Favored of M2 polarization and macrophage survival.	(46)
Rana chensinensis	Papain, trypsin, Neutral protease, pepsin and alkaline protease	-	RAW 264.7 cells	Enhanced macrophage phagocytic activities, NO secretion and production of pro-inflammatory cytokines	(47)
Paphia undulata (clam) meat	Alkaline protease	PHTC, VGYT, EF, LF, EGAK, WI, WL	Isolated splenocytes from mice	Enhanced lymphocyte proliferation	(48)
Tilapia mince, casein and pea protein	Virgibacillus halodenitrificans SK1-3-7 proteinase	-	THP-1 macrophages	Suppressed expression of pro-inflammatory cytokines and enhanced innate immunity by induction of IL-1β and COX-2	(49)
Egg yolk livetin	Pepsin and Alcalase	<10 kDa	RAW 264.7	Reduced inflammatory responses and pro-inflammatory cytokines. Also, enhanced the phagocytic activity of macrophages	(50)
Salvia hispanica seeds	Pepsin and pancreatin	Molecular weight from <1 to 10> kDa	BALB/c peritoneal macrophages	Decreased pro-inflammatory and increase anti-inflammatory cytokines	(51)
Rice protein	Trypsin	YGIYPR (767 Da)	RAW 264.7	Enhanced macrophage proliferation	(44)

Chalamaiah et al. (34) obtained low molecular mass (<10 kDa) peptides from giant grouper eggs and evaluated its immunomodulatory effects on BALB/c mice; both innate and adaptive immune responses were tested. The IP obtained by enzymatic hydrolysis with pepsin significantly increased NK cell cytotoxicity and the level of serum immunoglobulin A (IgA); the mucosal immunity (S-IgA) in the small intestine lumen was significantly enhanced by the IP obtained with pepsin and Alcalase, thus, enhancing immunosurveillance and preventing invasion of pathogens. The IP from the trypsin hydrolisates significantly increased the percentage of splenic CD4+ and CD8+, thus, improving the immune response against pathogens and cancer cells. From shark proteins, Mallet et al. (35) obtained small peptides (<10 kDa), by hydrolysis with trypsin and chymotrypsin. which were fed to BALB/c mice; the results showed that the IP enhanced the gut barrier function by up-regulation of IgA and cytokines (IL-6 and TNF-α) production; in addition, the increase of TGF-β and IL-10 contribute to the down-regulation of uncontrolled-inflammatory response. Chalamaiah et al. (36) reported the isolation of three hydrolysates from common carp eggs which contained peptides ranging from 5 to 90 kDa. After a 45-day treatment of the Balb/c mice with the pepsin hydrolysate (0.5 g/kg b.w.), the levels of NK cell cytotoxicity and mucosal immunity were significantly increased; whereas, the treatment with the Alcalase hydrolysate induced significant increases in the percentage of splenic CD4+ and CD8+. Lozano-Ojalvo et al. (42) assessed the effects of IP (<10 kDa) obtained from hydrolysates of ovalbumin, lysozyme, ovomucoid and whole egg white which resulted in reduction of ConA-stimulated (node cells stimulated with concanavalin A) lymphocyte proliferation, production of Th2 cytokines, such as IL-10 and IL-13, and decreased the secretion Th1 cytokine TNF-α; additionally, the IP considerably inhibited IgG1-class switching induced by LPS and counteracted the release of ROS. A study carried out by Hou et al. (39) isolated a hexapeptide with an amino acid sequence of PTGADY (622 Da) which significantly increased the production of IL-2, IL-4 and IL-6, and also increased the proliferation rate (≈42%) of splenic lymphocytes in immunosuppressed mice. Recently, Ling-Ling et al. (45) obtained a low molecular weight (≤ 3 kDa) IP from Coix glutein protein by enzymatic hydrolysis with pepsin and its effects were tested in ICR and BALB/c mice. The results showed that on the group of mice treated with 800 mg/kg of the IP, the spleen index was significantly increased and on the group of mice treated with 400 mg/kg of IP, the serum malondialdehyde (MDA) concentration was significantly lower than the blank control; MDA represents lipid peroxidation, which contributes to processes of host defense, inflammation and tissue damage. Additionally, the concentration of IP influenced the average body weight of the mice, *i.e.*, the 200 mg/kg and 400 mg/kg groups body weight were higher than the blank. Recently, Laviada-Castillo et al. (52) obtained five peptide fractions, with molecular weights ranging from <1 to >10 kDa; these bioactive peptides were extracted by enzymatic hydrolysis with the sequential system Pepsin-Pancreatin and

tested in T1D mice model. The 1-3 kDa peptide fraction exhibited the highest inhibition of pro-inflammatory cytokines (TNF-α and IL-6).

The immunomodulatory activity of bioactive peptides has also been evaluated in established cell lines, such as RAW 264.7 (macrophage cell line), U937 (human monocytic model), THP-1 (human monocyte cell line) and jurkat T cells (human T lymphocyte model). Ahn et al. (37) studied the anti-inflammatory potential of a tripeptide (PAY) in RAW 264.7 cells and reported that it reduced the production of NO and PEG_2 in 63.8% and 45.33%, respectively; additionally, PAY significantly reduced the production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6. Recently, Karnjanapratum et al. (38) and Sae-leaw et al. (25) reported IPs from different sources that exhibited protection to DNA damage by H_2O_2 in U937 cells and demonstrated immunomodulatory potential by reducing production of IL-1β, IL-6 and NO in RAW 264.7 cells. Rodríguez-Carrio et al. (41) reported that peptides with low molecular weight increased the proliferation of resting cells in a dose dependent manner; also, one of the fractions obtained, DR (5> kDa), increased the production of IFN-γ, a cytokine involved in the control of intracellular pathogens. Furthermore, relative low doses of short peptides could generate anti-inflammatory M2 macrophages and increase TGF-β secretion, key mechanisms for the control of inflammatory conditions. From amaranth protein, Montoya-Rodriguez et al. (42) identified five peptide sequences with a molecular weight below 2.5 kDa. These IPs significantly prevented inflammation by reducing TNF-α secretion in THP-1 and RAW 264.7 cells by over 30%, with collateral reduction of PGE_2 and COX-2. Also, phosphorylation of IKK-α was significantly reduced with consequential decrease in phosphorylation of IκB-α, resulting in a reduction in expression of NF-κB subunits in the nucleus by over 60%. Ling-Ling et al. (45) evaluated immunomodulatory peptides with molecular weight lower than 3 kDa and the results showed that the stimulation index and the acid phosphatase of mice splenocytes were increased in a dose-dependent manner; also, the secretion of NO was stimulated in RAW 264.7 macrophages and cell excessive activation was attenuated. Acid phosphatase (AP) is one of the central enzymes in the degradation of phagocytized pathogens, thus, it may serve as a marker for macrophage activity and its digestion ability In another study, Millán-Linares et al. (46) evaluated two peptides obtained by enzymatic hydrolysis with two different proteases: Izyme AL and Alcalase, which reported the attenuation of the expression of proinflammatory cytokines (TNF-α, IL-6, IL-1β) and an increased expression of anti-inflammatory marker gene CCL18 (chemokine (C-C motif) ligand 18)); furthermore, both IPs favored M2 polarization of macrophages (associated with repair and resolution of the inflammatory response), decreased a 50% the production of nitric oxide and promoted survival of THP-1-derived macrophages. Huang et al. (47) reported the extraction of IPs from the Chinese brown frog (*Rana chensinensis)* and the evaluation of

its immunomodulatory activity. The results showed that the IPs enhanced the phagocytic activity of macrophages, increased the production of TNF-α, IL-1β and IL-6 and NO in RAW264.7 cells, and the levels of NF-κB were also elevated; NF-κB plays a critical role in the activation of immune cells by upregulating the expression of cytokines. He et al. (48) reported the extraction of seven peptides (PHTC, VGYT, EF, LF, EGAK, WI, WL) which reported enhancement of lymphocyte proliferation activity in RAW 264.7 cells; also, the amino acids contained in the peptides are related to DPPH scavenging activity. Wu et al. (53) studied the effect of low molecular weight peptides in isolated lymphocytes and the results showed that there was an increase in the proliferation rate of treated cells; also, there was an improvement in the phagocytic activity of macrophages and its capacity to produce NO and secrete pro-inflammatory cytokines. From tilapia mince (TM), casein (CH) and pea (PH) protein hydrolysates, Toopcham et al. (49) reported that TM enhanced innate immunity through induction of pro-inflammatory cytokines (IL-1β and COX-2) expression; CH suppressed TNF-α, IL-1β, IL-8, IL-6 and COX-2, and PH reduced IL-6 and TNF-α responses in LPS-induced THP-1 macrophages. In another study, Meram & Wu (50) showed that peptides with low molecular weight (<10 kDa) significantly reduced the inflammatory response by inhibiting production of NO, IL-6, IL-1β, and TNF-α, and the phagocytic activity of the macrophages was enhanced. Recently, Chan-Zapata et al. (51) evaluated bioactive peptides from *Salvia hispanica* seeds on BALB/c peritoneal macrophages; the >10 and 1-3 kDa fractions exhibited significant decrease of NO, TNF-α, IL-1β and IL-6 production in a concentration-dependent manner, where the 1-3 kDa fraction showed the lowest levels of cytokines. As for anti-inflammatory cytokines, the 1-3 kDa fraction reported the highest production of IL-10. In reference to the structure of the IPs, it has been reported that the positively charged region may act as a chemokine (54); also, this region interacts directly with DNA and may potentially affect gene activity (55).

Unlike ACPs, there is a wide range of studies where immunomodulatory peptides are evaluated in *in vivo* models; however, the lack of information regarding the particular mechanism of action and toxicologic information remains.

3. Current status of bioactive peptides

There are thousands of peptides that present anticancer and immunomodulatory activities but, unfortunately, only a small number of these are currently in clinical trials. Lau & Dunn (56) maintain a comprehensive database of peptides that have entered clinical studies, which contains information on 484 peptides. From the total amount of peptides on the database, 68 have been approved in the United States, Europe and/or Japan, eight have been withdrawn and 155 peptides are currently in active clinical development. It is relevant to highlight that between 1980 and 2010, the trend for peptides in clinical trials gradually went upward; from the start of 2010 to 2017, 13 peptides were approved.

Currently the areas of highest concentration of peptide development are those of high interest to the pharmaceutical industry, one of them being oncology. Remarkably, for the oncology area, the number of approved peptides is not proportional to those in development, since a lot of peptides have entered trials but only few have been approved (56). On Table 3 are listed some of the peptides with anticancer activity that are in clinical trials and those that have been approved until 2017. It is possible to observe that from the 22 anticancer peptides in development, 45% are still in pre-clinical trials and only 9% have reached phase III. From the approved peptides, it is not surprising that most of them are for prostate cancer therapy since, by 2018, that was the most prevalent type of cancer on males worldwide (7). It is also noticeable that not only pharmaceuticals are involved on the development of peptides, but also universities such as the University of Bradford from and the University of Salford, both from England.

Table 3. Anticancer peptides with indication of the phase and the therapeutic condition for which they are developed. Modified from Lau & Dunn (56) and Felício et al. (57).

Product name	Company	Phase	Therapeutic condition
ANG-4043	AngioChem Co.	Preclinical	Brain metastases
CLS-001	Cadence Pharmaceuticals Inc. Carrus Capital Corp. Cutanea Life Sciences Inc. Migenix Inc.	II	Vulvar intraepithelial neoplasia
GRN-1201	Green Peptide Co.	I	Solid tumors
ICT01-2588	Incanthera Ltd.	I	Vascular disrupting agents
	University of Bradford	Preclinical	Breast cancer
		Preclinical	Colorectal cancer
		Preclinical	Lung cancer
		Preclinical	Prostate cancer
ICT03-Es5	Incanthera Ltd.	I	Solid tumors
	University of Saldford	Preclinical	Breast cancer
		Preclinical	Liver cancer
		Preclinical	Non-small cell lung cancer
ICT04-CYP	Incanthera Ltd.	Preclinical	Bladder cancer
	University of Bradford	Preclinical	Colorectal cancer
ITK-1	FUJIFILM Co.	III	Glioblastoma
	Green Peptide Co. Kurume University	III	Prostate cancer
Oncopore™	Lytix Biopharma AS	I	Solid tumors
Paclitaxeltrevatide	AngioChem Co.	II	Brain metastases
		II	Glioblastoma
		II	Glioma
WT-2725	Sumitomo Dainippon Pharma Co.	I	Hematological malignancies
	Sunovion Pharmaceuticals Inc.	I	Solid tumors
Leuprorelin	-	Approved in 1984	Prostate cancer
Triptorelin	-	Approved in 1986	Prostate cancer Breast cancer
Goserelin	-	Approved in 1987	Breast cancer Prostate cancer
Histrelin	-	Approved in 1991	Prostate cancer
Abarelix	-	Approved in 2003	Prostate cancer
Degarelix	-	Approved in 2008	Prostate cancer

In spite of the peptides' potential use in the treatment of cancer, they present certain inconveniences such as low bioavailability when administered orally. Although oral administration is the current hallmark for drug delivery (58), peptides administered through this route present poor stability since physiological conditions, gastric acids, complex gastric enzymes and the complex gastrointestinal environment make peptides vulnerable to degradation or inactivation (59). To overcome these challenges, modifications of peptides should be an acceptable option. Currently, there is a wide range of strategies designed to improve peptides' anticancer and immunomodulatory effects (60) that have proved useful *in vitro* and *in vivo*, such as aminoacid substitution (30) and structural fusion of peptides (61), without compromising the peptide efficiency. Additionally, improvements on computational biology will enhance peptide development.

4. Future perspectives from peptides as therapeutic adjuvants for cancer

Over the last decade, there has been a significant breakthrough in the development and modification of targeted antitumor drugs (62). The discovery of bioactive peptides that could affect a tumor and inhibit its development is a mayor contribution to the creation of antitumor diet therapies with high selectivity and efficacy (63). Such therapeutics have several additional benefits when compared to conventional treatments, such as better organ or tumor permeability; its production is more cost effective than drugs and, due to the fact that the principal components of bioactive peptides are amino acids, they have lower toxicity. Also, when compared to full-length proteins and antibodies, bioactive peptides are less immunogenic and more stable at room temperature, which makes prolonged storage a viable option (64, 65).

Despite their potential use in anticancer diet therapies, the commercial application of bioactive peptides from natural sources has been deferred due to several reasons: insufficiency of appropriate and scalable production methods, adequate analysis of mechanisms of action, variable pharmacokinetics profiles and absence of well-designed clinical trials that can be used as evidence of their therapeutic potential. For these reasons, further studies are required to define the future use of bioactive peptides in the treatment of cancer. Such knowledge will allow the categorization of these products, which would ease their commercialization as diet therapeutics (60, 66).

5. Conclusion

Currently, finding anticancer drugs that are highly effective and have low toxicity is of main interest. The evaluation of bioactive peptides with anticancer and immunomodulatory activities have been reported by a wide range of researchers and the results show promising results for their use in complementary medicine due to their unique potential. However, there are still several limitations for their commercial distribution. First, the cost of anticancer peptides is too high due to their complicated extraction and purification techniques, which also limits their commercial mass production. Also,

although it is reported that peptides are not toxic to normal cell lines, further pharmacokinetic and toxicological profiles are crucial, which can be obtained by *in vivo* essays and clinical trials. Due to the known advantages of the anticancer peptides, this line of research offers promising results as a therapeutic alternative.

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CONCLUSIONES

El grado de hidrólisis del hidrolizado enzimático de *S. hispanica* conseguido con el sistema secuencial Pepsina-Pancreatina® fue 33.79 ± 2.14%. Asimismo, el contenido proteico de los derivados proteínicos <1, 1-3 y 3-5 kDa, obtenidos a partir de la ultrafiltración del hidrolizado enzimático, fue de 0.074 ± 0.02, 0.069 ± 0.01 y 0.059 ± 0.02 mg/mL, respectivamente.

Los derivados proteínicos, <1, 1-3 y 3-5 kDa, fueron secuenciados y se obtuvieron 1201, 1625 y 1511 secuencias peptídicas, respectivamente.

El derivado proteínico con mayor efecto supresor de la viabilidad celular de las líneas de cáncer MCF-7, Caco2, PC-3 y HepG2, fue el derivado <1 kDa a una concentración de 1 mg/mL. En la línea celular hFB no se reportó efecto citotóxico por lo que los derivados proteínicos son selectivos a células de cáncer.

Al ser el derivado proteínico <1 kDa el de mayor actividad anticancerígena, se analizaron sus secuencias peptídicas a través de un análisis multicriterio y se determinó que el péptido con secuencia KLKKNL, listado como número uno del rank obtenido, tiene mayor posibilidad de presentar actividad anticancerígena al cumplir con los criterios teóricos de un péptido con dicha actividad.

ANEXOS

1. Introducción a artículos elaborados

1.1 Actividad anticancerígena de los derivados proteínicos de Salvia hispanica

Actualmente los tratamientos convencionales para el cáncer ocasionan serios efectos secundarios en los pacientes y podrían estar involucrados en el aumento de la mortalidad por dicha enfermedad. Por tal motivo se propone el uso de los tratamientos complementarios, de los cuales han ganado gran interés los derivados proteínicos, ya que han reportado beneficios a la salud y actividad anticancerígena. Los derivados proteínicos son generalmente obtenidos de fuentes con alto contenido proteico, como es el caso de la *S. hispanica,* una planta endémica de México cuyas semillas contienen hasta un 26% de proteína. Por lo tanto, el presente artículo plantea la extracción de derivados proteinicos de *S. hispanica* y la evaluación de su actividad anticancerígena en líneas celulares de cáncer de mama, colon, próstata e hígado.

1.2 Peptidos bioactivos como terapeúticos adyuvantes para cáncer

Las terapias convencionales para el cáncer ocasionan serios efectos en los pacientes que disminuyen significativamente su calidad de vida. Actualmente, se propone el uso de los derivados proteínicos como tratamientos complementarias a dichas terapias ya que presentan ciertos beneficios como selectividad a las células, baja toxicidad para el paciente, entre otras. A lo largo de este artículo de revisión, se describen los derivados proteinicos más recientes que han reportado actividad anticancer. Uno de los efectos secundarios del cáncer es la supresión del sistema inmune, por lo que de igual manera se consideró importante la recopilación de derivados proteínicos con actividad inmunomoduladora. Además, en el presente artículo se discuten los obstáculos actuales que se enfretan para el uso de los derivados proteínicos como tratamiento a enfermedades como el cáncer, y cuáles son las perspectivas a futuro de los mismos.

2. Evidencia de envío del articulo “Actividad anticancerígena de los derivados proteínicos de *Salvia hispanica”* a Journal of Food Science

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