Centro de Investigación en Alimentación y Desarrollo, A.C.

ACTIVIDAD ANTICANCERÍGENA DE PÉPTIDOS DE HOJA DE *Moringa oleifera* Lam. EN CÉLULAS DE CÁNCER DE COLON

Por:

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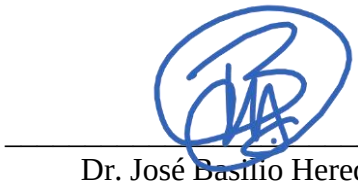

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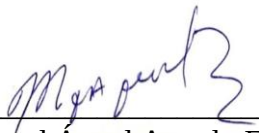

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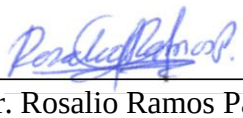

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RESUMEN

Los péptidos son fracciones proteicas. Entre sus actividades biológicas, destacan su potencial antioxidante, antiinflamatorio y antiproliferativo asociados a un efecto anticancerígeno. El cáncer colorrectal se ha sugerido como un modelo para evaluar la bioactividad de estas moléculas, debido al destino fisiológico de las mismas. La principal causa de aparición de esta enfermedad se asocia a una alta concentración de radicales libres, los cuales provienen de la alta tasa metabólica de las células del colon y también de estímulos inflamatorios. Con base en lo anterior, los péptidos con potencial anticancerígeno pueden funcionar como agentes preventivos y/o de tratamiento. Ya se han estudiado péptidos en modelos de cáncer de colon, principalmente de leguminosas. Sin embargo, estas no son consideradas como idóneas, debido a que sus proteínas son causantes de alergias alimentarias. Por lo que nuevas fuentes vegetales han sido propuestas, entre estas, la hoja de *Moringa oleifera*. El objetivo de esta investigación fue determinar la actividad anticancerígena de péptidos generados a partir de proteína de hoja de *Moringa oleifera*. Se extrajo proteína a partir de la hoja y fue hidrolizada mediante digestión gastrointestinal *in vitro*. Los péptidos fueron identificados por nano-HPLC-MS/MS. La capacidad antioxidante fue determinada por DPPH, ABTS, ORAC, FRAP y actividad antioxidante celular en células de cáncer de colon (Caco-2). Para evaluar el efecto antiinflamatorio, se evaluó el efecto de los péptidos sobre la producción de óxido nítrico en macrófagos RAW 264.7. Finalmente, se determinó su actividad antiproliferativa en Caco-2. Obteniéndose la secuencia de 14 péptidos, los cuales inhibieron los radicales DPPH y ABTS en un 45.70 y un 93.09%, respectivamente; y mostraron una actividad en ORAC y FRAP de 3.27 y 1.435 mM TE/g, respectivamente. A una concentración de 100 μg/ml, inhibieron la producción de óxido nítrico de macrófagos en un 30.51%. Concentraciones de 500 μg/ml no mostraron efecto citotóxico sobre células sanas del colon (CCD-18Co) y redujeron la actividad oxidante celular y la proliferación celular en Caco-2 en 71.51 y 90.20%, respectivamente. Los hallazgos sugieren que los péptidos de la hoja de *Moringa oleifera* podrían tener el potencial de funcionar como agentes preventivos para esta enfermedad y como agente de tratamiento debido a su capacidad para inhibir la proliferación celular. Por lo tanto, estas moléculas podrían ser un componente anticancerígeno eficaz en alimentos y medicamentos.

Palabras clave: péptidos, *Moringa oleifera*, cáncer de colon.

ABSTRACT

Peptides are protein fractions. Among its biological activities, its antioxidant, anti-inflammatory and antiproliferative potential associated with an anticancer effect stand out. In this sense, colorectal cancer has been suggested as a suitable model to evaluate the bioactivity of these molecules, due to their physiological fate. The main cause of the appearance of this disease is associated with a high concentration of free radicals, which come from the high metabolic rate of the cells of the colon and also from inflammatory stimuli. Based on the above, peptides with anticancer potential can function as preventive and/or treatment agents. Peptides have already been studied in colon cancer models, mainly from legumes. However, these are not considered suitable because their proteins are the cause of food allergies. Therefore, new plant sources have been proposed, including the *Moringa oleifera* leaf. The objective of this research was to determine the anticancer activity of peptides generated from *Moringa oleifera* leaf protein. Protein was extracted from the leaf and hydrolyzed by *in vitro* gastrointestinal digestion. Peptides were identified by nano-HPLC and MS/MS. Antioxidant capacity was determined by DPPH, ABTS, ORAC, FRAP, and cellular antioxidant activity in Caco-2 colon cancer cells. To assess the anti-inflammatory effect, the effect of peptides on nitric oxide production in RAW 264.7 macrophages was measured. Finally, its antiproliferative activity on Caco-2 was determined. Sequence analyses revealed 14 peptides, which inhibited DPPH and ABTS radicals by 45.70 and 93.09%, respectively and showed an ORAC and FRAP activity of 3.27 and 1.435 mM TE/g, respectively. At a concentration of 100 µg/ml, they inhibited the production of nitric oxide by macrophages by 30.51%. Concentrations of 500 µg/ml showed no cytotoxic effect on healthy colon cells (CCD-18Co) and reduced Caco-2 cellular oxidant activity and cell proliferation by 71.51 and 90.20%, respectively. These findings suggest that *Moringa oleifera* leaf peptides could have the potential to function as preventive agents for this disease and as a treatment agent due to their ability to inhibit cell proliferation. Therefore, these molecules could be an effective component of functional foods targeting colorectal carcinoma.

Keywords: peptides, *Moringa oleifera*, colorectal cancer.

1. SINOPSIS

1.1. Justificación

En la actualidad, las enfermedades crónico-degenerativas son la principal causa de muerte a nivel mundial. En el caso del cáncer, este es responsable por un estimado de 10 millones de decesos al año. Los tipos de cáncer más frecuentes son de mama, pulmón y colon. Este último ocupa el segundo lugar en cuanto a muertes causadas por cáncer con un total de 916 000 defunciones registradas en 2020. Se espera que estas cifras aumenten rápidamente al ritmo del crecimiento de la población, incremento de la esperanza de vida y la adopción de hábitos considerados como perjudiciales. El principal tratamiento para esta enfermedad es cirugía en conjunto con terapia coadyuvante en la cual se administran agentes químicos. Estos medicamentos atacan indiscriminadamente a todo tipo de células en rápida división, afectando no solo células cancerosas lo que promueve la aparición de efectos adversos que afectan la calidad de vida del paciente, por lo que se ha sugerido evaluar terapias coadyuvantes alternas, como el uso de compuestos nutracéuticos. En este sentido, diferentes estudios científicos respaldan la correlación que existe entre el consumo de proteína de origen vegetal y la disminución en la incidencia de diferentes tipos de cáncer, principalmente el cáncer de colon. Lo anterior, se asocia a la formación de péptidos en el tracto gastrointestinal debido a la acción de enzimas digestivas. Los péptidos actúan como antioxidantes, antiinflamatorios, inhibidores de enzimas, inductores de apoptosis y/o promotores de arresto del ciclo celular, siendo consideradas como moléculas con potencial anticancerígeno al tener la capacidad de prevenir o tratar esta enfermedad. Las investigaciones asociadas a péptidos con potencial anticancerígeno se han enfocado desde hace aproximadamente cinco años a aquellos obtenidos de fuentes vegetales, principalmente de leguminosas y cereales. Con respecto a las leguminosas, estas contienen proteínas alergénicas, reacción que puede asociarse a las secuencias peptídicas limitando su aceptación y por lo tanto su uso. En cuanto a las proteínas de los cereales y sus derivados, la tendencia a consumir productos sin gluten ha hecho disminuir grandemente su aceptación y demanda, incluso de aquellos provenientes de cereales sin gluten, ya que se sabe que pueden estar contaminados con gluten durante procesamiento, transporte y manejo.

Por lo anterior, se ha propuesto el estudio de péptidos provenientes de otras fuentes vegetales, como es el caso de la hoja de la planta de *Moringa oleifera*, la cual contiene hasta un 34% de proteína y presentan un perfil proteico bajo en compuestos anti nutrimentales. A partir de su proteína, se han generado péptidos altamente antioxidantes y reactivos con la habilidad de penetrar diferentes tipos celulares y la capacidad de llegar directamente al colon. Por lo anterior, el cáncer de colon se vuelve un modelo ideal para el estudio de su capacidad anticancerígena. Por otro lado, la *Moringa oleifera* es un árbol ampliamente distribuido en toda la costa del pacífico de México, asegurándose su acceso al mismo. Además de lo mencionado anteriormente, es necesario resaltar la importancia de apoyar a la investigación de nuevos compuestos que faciliten la prevención y el tratamiento de enfermedades crónico-degenerativas, como el cáncer de colon, cuya incidencia aumenta año con año. Finalmente, cuando se trata del estudio de compuestos a considerar como nutracéuticos, es esencial llevar a cabo una validación mediante el método científico y de esta manera promover el desarrollo de suplementos o medicamentos que generen aceptación y confianza.

1.2. Antecedentes

1.2.1. Péptidos Bioactivos

Por muchos años las proteínas presentes en los alimentos fueron consideradas únicamente como un macronutriente responsable de proveer energía. Sin embargo, su estudio ha permitido conocer su papel en la salud humana. Uno de los principales mecanismos de acción es mediante la liberación de péptidos. Los péptidos son fragmentos proteicos conformados por entre 2 y 50 aminoácidos que permanecen unidos mediante enlaces peptídicos (Karami y Akbari-Adergani, 2019). Una vez liberadas de su proteína original, estas moléculas pueden actuar como agentes antitrombóticos, antihipertensivos, inmuno-moduladores, anticancerígenos y antioxidante, lo cual se debe principalmente a características como su tamaño, especificidad y secuencia (Daliri *et al*., 2027). Lo anterior depende tanto de las fuentes a partir de las cuales se obtienen los péptidos, como de los métodos de producción o generación (Fields *et al*., 2009).

1.2.2. Métodos de Generación de Péptidos Bioactivos

1.2.2.1. Ocurrencia natural. Los péptidos de ocurrencia natural son aquellos que no requieren ser producidos, si no que ya se encuentran como tal en la matriz alimenticia por lo tanto su método de obtención consiste en su recuperación mediante técnicas que serán discutidas más adelante. Entre los péptidos de ocurrencia natural más estudiados se encuentra: 1) la lunasina, aislada de cereales y leguminosas y considerado como un agente quimiopreventivo, 2) la vglicina, proveniente de semillas de guisantes y catalogado como promotor de muerte de células cancerígenas y 3) los inhibidores de tripsina que se encuentran en casi todas las fuentes vegetales y que modulan diversas rutas metabólicas (Avilés-Gaxiola *et al*., 2020)

1.2.2.2. Fermentación. La fermentación es una técnica muy antigua que en sus inicios se utilizó por su capacidad para conservar los alimentos. Actualmente se sabe que mejora el perfil nutracéutico de los mismos, mediante la generación de diferentes compuestos, incluidos los péptidos. Los péptidos se pueden producir mediante el uso de un microorganismo o mediante enzimas proteolíticas de microorganismos aislados (Rajapakse *et al*., 2005). Si bien la fermentación era un proceso no controlado, en la actualidad estos procesos han sido optimizados, siendo posible controlar sus parámetros, incluyendo temperatura, pH, tiempo y tamaño del inóculo. La fermentación se utiliza principalmente para producir péptidos a partir de productos lácteos utilizando cultivos iniciadores o no iniciadores. Las bacterias del ácido láctico también se utilizan para la producción de péptidos antioxidantes de origen vegetal, especialmente *Lactobacillus plantarum*, ya que puede hidrolizar varias proteínas produciendo numerosos oligopéptidos diferentes (Mechmeche *et al*., 2017).

1.2.2.3. Síntesis química. La síntesis química es un proceso llevado a cabo en reactores en fase sólida. Aquí, el primer aminoácido se une a un soporte o resina a través de una molécula enlazadora. Posteriormente al complejo anterior se le elimina el grupo N-terminal y se acopla un aminoácido nuevo. Este proceso de desproteger un aminoácido y acoplar el siguiente, se repite hasta completar

la secuencia deseada (Pérez-Espitia *et al*., 2012). A pesar de las ventajas que ofrece este método, es más conveniente una vez que se ha identificado una estructura peptídica con determinado potencial bioactivo proveniente de una fuente natural. Además, los péptidos obtenidos por este método no siempre se asemejan a la conformación química adoptada por los obtenidos de fuentes naturales y en muchas ocasiones no son estables en sistemas biológicos y son más susceptibles a degradación (Bray, 2003).

1.2.2.4. Hidrólisis enzimática. La hidrólisis enzimática de proteínas es la forma más común y eficaz de producir péptidos bioactivos. Se utiliza una enzima sola o una combinación. Cada enzima tiene un sitio de corte específico dentro de la proteína y también cada una trabaja en condiciones particulares de temperatura y pH. El grado de hidrólisis de cada enzima depende de factores controlables como el tiempo de hidrólisis, la concentración y la combinación de enzimas (Kullman, 2018). La hidrólisis enzimática es el método más efectivo para liberar péptidos antioxidantes y anticancerígenos. Varias proteínas alimentarias como el suero de leche, el huevo, las lentejas y las proteínas de los cereales han sido hidrolizadas. Se han utilizado enzimas de fuentes fúngicas y bacterianas, por ejemplo, alcalasa y enzimas vegetales como la papaína (Bhat *et al*., 2015; Korhonen y Pihlanto, 2006). Sin embargo, las proteasas más utilizadas son las enzimas digestivas del tracto gastrointestinal: pepsina, tripsina y quimotripsina. Lo anterior, debido a que diversas investigaciones señalan que mediante su uso se genera un perfil de péptidos con mayor actividad biológica entre las cuales destacan las actividades antioxidante y anticancerígena. Por otro lado, el uso de estas enzimas asemeja lo que ocurriría en un sistema vivo tras la digestión de proteínas alimenticias (Marciniak *et al*., 2018). Mediante el uso de enzimas gastrointestinales se han obtenido péptidos con potencial anticancerígeno de fuentes como soya, frijol, garbanzo, arroz, maíz, trigo, avena, cebada, centeno, *Gloriosa superba* y nuez. Entre los principales modelos en los que se ha estudiado la bioactividad de estos péptidos, se encuentran cultivos celulares de cáncer de colon como HT-29, HCT-116 y Caco-2 (Avilés-Gaxiola *et al*., 2020).

1.2.3. Métodos para la Purificación e Identificación de Péptidos Bioactivos

Una vez que se producen los péptidos, es necesario separarlos de otros compuestos que puedan encontrarse en la muestra, como las proteínas no hidrolizadas o pobremente hidrolizadas. La principal forma de separarlos es mediante ultrafiltración. Sí se necesita el fraccionamiento de péptidos muy pequeños, se usan técnicas cromatografías y cuando el objetivo es la identificación se emplea además la espectrometría de masas (Ortiz-Martinez *et al*., 2017).

1.2.3.1. Ultrafiltración. La ultrafiltración es un proceso de filtración por membrana que se utiliza una vez obtenido el hidrolizado con la finalidad de fraccionar y concentrar los péptidos por peso molecular. Por lo general, se utilizan membranas de corte de peso molecular de 5, 10 y 50 kDa para obtener fracciones peptídicas <5, 5-10 y 10-50 kDa. Lo anterior, con el propósito de analizar la capacidad biológica de acuerdo con el tamaño. Para la separación de péptidos <1 kDa se utiliza la nanofiltracción. En esta técnica la separación está basada en repulsión electrostática (Bazinet y Firdaous, 2009).

1.2.3.2. Métodos cromatográficos. La cromatografía en sus distintas modalidades se utiliza para una mayor purificación y separación de los péptidos. los métodos cromatográficos aprovechan la diversidad fisicoquímica de estas moléculas. Es decir: carga, punto isoeléctrico, hidrofobicidad y tamaño. Para los péptidos, la separación se ha logrado mediante el uso de gradientes de fase móvil, ya sea cambiando la concentración del solvente utilizado una técnica llamada cromatografía de fase reversa, o cambiando la concentración de sal en una técnica conocida como cromatografía de intercambio iónico (Sila y Bougatef, 2016).

Cromatografía en fase reversa (RP-HPLC, por sus siglas en inglés). La RP-HPLC ha sido considerada como una técnica bien estudiada para la separación de moléculas pequeñas y se ha convertido en el método de elección para la purificación de péptidos provenientes de fuentes naturales, principalmente de digestión enzimática y también para la purificación de péptidos naturales y sintéticos. Durante la RP-HPLC, los péptidos se separan según su hidrofobicidad,

característica que dependerá de la secuencia y conformación de los aminoácidos. Una vez que la mezcla de péptidos está dentro de la columna, se adsorben en la fase hidrófoba, correspondiente a la fase estacionaria. Posteriormente, los péptidos se desorben cuando la fase orgánica alcanza la concentración óptima para cada péptido (Hoofnagle *et al*., 2016).

Cromatografía de intercambio iónico. Fue diseñada para la separación de moléculas ionizables, como proteínas y por lo tanto péptidos. Esta técnica incluye dos fases, una móvil compuesta por una matriz orgánica inerte de carga opuesta a los solutos que se pretenden separar, y otra móvil compuesta por un sistema tampón acuoso en el que se introduce la muestra a resolver. Debido a su baja selectividad, la cromatografía de intercambio iónico se utiliza principalmente para obtener muestras de péptidos más puras y suele ir seguida de una HPLC preparativa para un proceso de separación adicional (Skoczylas *et al*., 2017).

1.2.3.3. Caracterización. La cromatografía líquida es uno de los métodos más utilizados para separar y caracterizar péptidos al combinarla con un detector UV. Los enlaces peptídicos absorben la luz ultravioleta entre 210 y 220 nm, mientras que los aminoácidos aromáticos lo hacen entre 250 y 290 nm. Por otro lado, le espectrometría de masas en tándem (MS/MS, por sus siglas en inglés) es considera como la técnica más adecuada para la identificación de secuencias de aminoácidos al producir iones y separarlos por relación masa-carga (Taylor y Johnson, 2001). Las técnicas de ionización más utilizadas para la caracterización de péptidos en MS/MS son la ionización por electro pulverización (ESI) y la desorción/ionización láser asistida por matriz (MALDI). ESI produce pequeñas gotas cargadas de un medio líquido bajo la influencia de un campo eléctrico y MALDI crea los iones mediante el uso de una matriz de absorción de energía láser (Nadler *et al*., 2017). El analizador de masas más empleado para la caracterización de péptidos es el de tiempo de vuelo (TOF), tradicionalmente acoplado a MALDI (Chui *et al*., 2015).

La Figura 1 muestra un esquema general con los pasos a seguir para obtener péptidos bioactivos

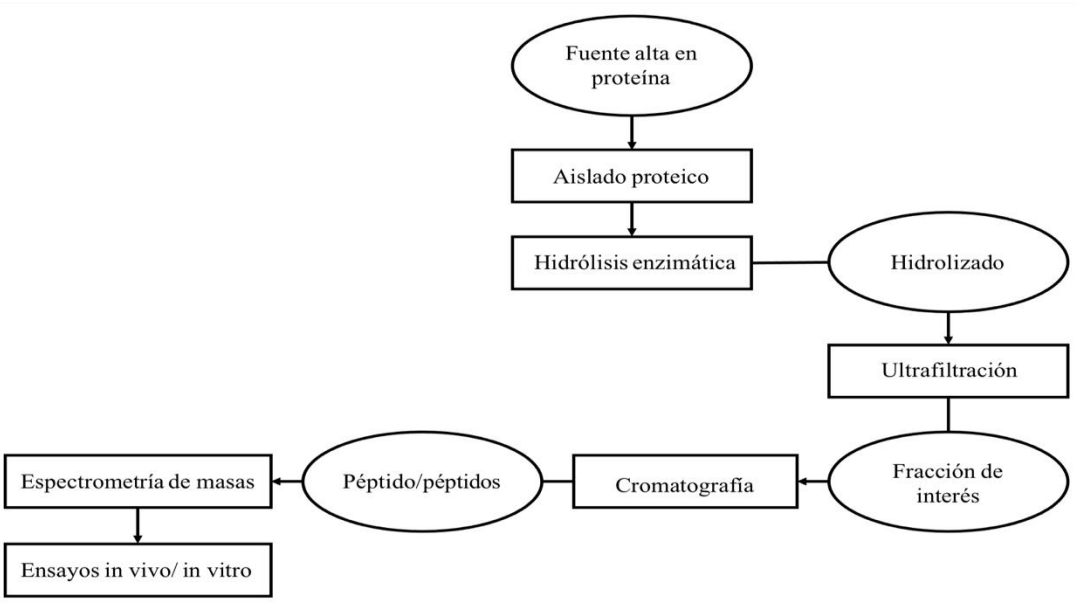

Figura 1. Esquema general con los pasos a seguir para obtener péptidos con potencial bioactivo.

1.2.4. Fuentes de Obtención de los Péptidos

1.2.4.1. Fuentes animales. La determinación de que existen fracciones proteicas con la capacidad de influenciar la salud humana surgió del estudio de hidrolizados proteicos de origen animal y por su alto contenido en proteína y su composición de aminoácidos, fueron por muchos años las fuentes más populares para la obtención y estudio de estas moléculas. La primera investigación fue publicada en 1950. En esta, se reportan los péptidos caseinofosfopeptidos como capaces de aumentar la calcificación ósea independiente de vitamina D en lactantes con raquitismo (Korhonen y Pihlanto, 2006). Además de la leche, que ha sido la fuente animal más utilizada para la obtención de péptidos, estos se han producido a partir de alimentos como huevo, queso, carne, subproductos de la industria pesquera e incluso sangre bovina (Sánchez y Vázquez, 2017). En posteriores estudios, las funciones más importantes atribuidas a los péptidos de origen animal fueron: transporte de minerales, estimulantes del sistema inmune, antihipertensivos, controladores de los niveles de colesterol, acción opioide, inhibición de agregación plaquetaria y actividad fungicida y bactericida. La mayoría de los péptidos de origen animal han sido generados por la acción de enzimas digestivas. Especialmente de enzimas como pepsina, tripsina y quimotripsina (Albenzio *et al*., 2017).

En años recientes se ha hecho conciencia del impacto ambiental relacionado a la producción de proteína animal y a los problemas de salud que se asocian a su consumo excesivo, por lo que su estudio como fuente de biomoléculas ha disminuido considerablemente (Aiking y Boer, 2020).

1.2.4.2. Fuentes vegetales. En los últimos años el estudio de péptidos bioactivos se ha centrado en los provenientes de plantas. Esto, debido a que la producción de proteína vegetal es mucho más económica y sostenible en comparación con la de fuente animal. Asimismo, la demanda de proteínas de origen vegetal está aumentando, ya que su consumo está asociado a una dieta más saludable. Por otro lado, existen fuentes de proteína vegetal mucho más diversas en comparación con la proteína animal (Marsh *et al*., 2013).

La mayor cantidad de péptidos bioactivos obtenidos a partir de plantas, provienen de leguminosas. Estas, representan el 27% de la producción agrícola primaria del mundo y suministran alrededor del 15% de la proteína a la población mundial. Otro gran porcentaje de péptidos bioactivos de origen vegetal, provienen de cereales. Los cereales son la fuente de energía más importante a nivel mundial aportando cantidades significativas de carbohidratos, proteínas, vitaminas B y minerales (Ortiz-Martinez *et al*., 2014).

Durante mucho tiempo, las legumbres y los cereales fueron las principales fuentes para obtener péptidos bioactivos. Sin embargo, la obtención de péptidos a partir de leguminosas es algo limitada ya que se ha observado que estos pueden conservar la fracción alergénica de varias de sus proteínas. En el caso de los cereales, el mercado demanda sus proteínas cada vez en menor medida debido a la tendencia en el consumo de alimentos libres de gluten. Por lo anterior, las investigaciones recientes se han centrado en la obtención de péptidos de otras fuentes vegetales con alto contenido proteico (Bustamante *et al*., 2017). Entre las fuentes evaluadas han sido nuez, raíces de árboles como el de *Gloriosa superba*, camote dulce y hojas de alto valor nutrimental como de rosal silvestre y *Moringa oleifera* (Avilés-Gaxiola *et al*., 2020).

1.2.5. *Moringa Oleifera* como Fuente de Péptidos Bioactivos

M. oleifera es una planta angiosperma que pertenece a la familia Moringaceae. Esta familia está

compuesta por solo 13 especies, entre las que destaca *M. oleifera.* El árbol de *M. oleifera* es nativo de la región del Himalaya y de la India y hoy en día se encuentra principalmente en climas tropicales y subtropicales (Asia, América Latina, El Caribe, Islas del Pacífico, etc.) debido a que se caracteriza por ser resistente a condiciones adversas, llegando a crecer hasta 10 m de altura. A pesar de que no existen registros oficiales de la siembra de este árbol, se cree que la India es el principal productor ya que comunidades como Andhra Pradesh, Karnataka y Tamil Nadu dedican a su producción 156.6, 102.8 y 74.09 km^2, respectivamente (Ravani *et al*., 2017).

Al árbol de *M. oleifera* se le ha atribuido la capacidad para prevenir hasta 300 enfermedades y por lo tanto se le han dado nombres como "árbol de la vida" y "árbol milagroso". La primera civilización en documentar estos beneficios fue la egipcia, la cual elaboraba aceites, medicamentos, perfumes y artículos de cuidado facial a partir de sus diferentes secciones anatómicas, incluso se le dio uso como fertilizante (Elgamily *et al*., 2016).

Todas las partes del árbol de *M. oleifera* son adecuadas tanto para consumo animal como humano por lo que, en países como aquellos en el sur de Asia y África, ha sido ampliamente utilizado. Sin embargo, en América era un árbol principalmente ornamental, hasta hace pocos años que se conocen sus beneficios y comenzó a formar parte de diversos estudios científicos (Olson y Alvarado-Cárdenas, 2016).

Hoy en día, las secciones más consumidas son las hojas y las semillas debido a su alto contenido proteico (Alain Mune Mune *et al*., 2016). La proteína de la hoja se ha propuesto como suplemento dada su composición de aminoácidos. Contiene los 10 aminoácidos esenciales tirosina, metionina, treonina, fenilalanina, valina, leucina, isoleucina, histidina, triptófano y lisina. Debido a esto, las hojas de *M. oleifera* se ofrecen a niños en diferentes platillos y formulaciones, a infantes durante la alimentación complementaria y a madres en proceso de lactancia (Olson y Alvarado-Cárdenas, 2016).

El perfil proteico de las hojas de *Moringa oleifera* está compuesto principalmente por proteínas de más de 29 kDa y otras en el rango de 14-20 kDa. Se ha extraído proteína a partir de la hoja de *M. oleifera* mediante precipitación con sulfato de amonio, precipitación con acetona y mediante la técnica de extracción básica y precipitación ácida, siendo la primera técnica la que genera péptidos con más alta pureza (Paula *et al*., 2017)

A partir de hojas de *M. oleifera*, se han aislado 2 péptidos de ocurrencia natural. Estos fueron designados con el nombre de morintides 1 y 2, ambos de 44 aminoácidos de longitud y con la

capacidad de inhibir fuertemente el crecimiento de hongos patogénicos como *Alternaria alternata* y *Alternaria brassiciola* (Kini *et al*., 2017).

También se han generado péptidos de la hoja de *M. oleifera* mediante hidrólisis enzimática. En este sentido, las enzimas utilizadas han sido tripsina, quimotripsina, pepsina, alcalasa, bromelaina, flavourzima, dispasa, papaina y Protamex. Estos péptidos exhiben actividades biológicas *in vitro* como: actividad antioxidante, antidiabética, antihipertensiva, antifúngica y antibacterial. En cuanto a ensayos *in vivo*, hidrolizados de hoja de *M. oleifera* han mostrado reducir los niveles de glucosa en ratones diabéticos (Paula *et al*., 2017).

Con relación a la actividad antioxidante, se ha encontrado que los péptidos provenientes de la hoja de *M. oleifera* generados con alcalasa o con enzimas digestivas contrarrestan el efecto del estrés oxidativo en ensayos como DPPH, TAC y FRAP. En cuanto a los péptidos generados por enzimas digestivas, se determinó que tienen la capacidad de proteger contra los daños provocados por el estrés oxidativo a enterocitos mediante la inhibición de la formación de malondialdehido (Lin *et al*., 2019; Yun *et al*., 2020). Respecto a la actividad *in vivo*, péptidos de hoja de *M. oleifera* redujeron el estrés oxidativo en ratas también mediante la inhibición de la formación de malondialdehido (Paula *et al*., 2017). En estos estudios se ha establecido como parte de la conclusión que, dada esta propiedad, estos hidrolizados deberían ser estudiados más a fondo como posibles agentes preventivos, como ingredientes de alimentos funcionales, belleza y cuidado de la piel.

En cuanto al potencial de estas, se ha sugerido su estudio como anticancerígenos debido a la actividad antibacterial que presentan ya que se ha establecido que aquellos péptidos capaces de promover la muerte o inhibir el crecimiento de bacterias patógenas, tiene potencial de ejercer el mismo efecto en células cancerígenas. Lo anterior dada la similitud de varias características bioquímicas entre estos dos tipos celulares, principalmente la carga negativa de las membranas. En este sentido, péptidos de hoja de *M. oleifera* presentaron actividad antimicrobiana contra las bacterias *Staphylococcus aureus*, Escherichia *coli* y *Bacillus subtilis* las cuales han mostrado su resistencia a múltiples medicamentos (Shakir *et al*., 2019). Por otro lado, la actividad antioxidante reportada por estos péptidos se relaciona a la prevención de cáncer por la asociación entre el estrés oxidativo y esta enfermedad.

El árbol de *M. oleifera* está ampliamente distribuido en el mundo, lo que lo convierte en una materia prima accesible. Las cualidades nutricionales de sus diferentes partes han sido ampliamente

estudiadas y reportadas, lo que ha llevado a la identificación de diversos compuestos nutracéuticos, así como su función en términos de salud. Los péptidos se encuentran entre las moléculas nutracéuticas de *M. oleifera* de más reciente estudio. En este sentido, poco se ha evaluado respecto a ellas, en comparación con otras moléculas. Sin embargo, lo encontrado hasta el momento da pie a seguir estudiando su efecto en diversas enfermedades, sugiriendo su futuro uso tanto en la industria alimenticia como farmacéutica.

1.2.6. Uso de los Péptidos Bioactivos

En los últimos años las personas han buscado sustituir productos de la dieta, la belleza y el área de salud por opciones de origen natural. Entre las alternativas más destacadas se encuentran compuestos químicos provenientes de fuentes vegetales como antocianinas, carotenoides, polifenoles y péptidos (David *et al*., 2015). En este sentido, los péptidos son las moléculas de más reciente estudio y como se comentó anteriormente, destaca la investigación referente a péptidos e hidrolizados de origen vegetal, donde cada vez aparecen más fuentes nuevas para su obtención y/o generación.

La primera aplicación que se le dio a los hidrolizados proteicos fue en la industria de los alimentos. Aquí, se han utilizado hidrolizados y péptidos individuales como modificadores de consistencia, emulsificadores, estabilizadores de color, endulzantes y para incrementar el sabor agradable de ciertos alimentos, mejorando el producto final que se le ofrece al consumidor. En este sentido, los péptidos de origen vegetal han sido señalados como preferidos ya que son altamente solubles en formulaciones alimenticias. Por otro lado, algunos hidrolizados con alta capacidad antioxidante, han sido utilizados como ingrediente para inhibir la peroxidación lipídica de alimentos previniendo sabores desagradables y la distorsión de la coloración (Esfandi *et al*., 2019).

La industria cosmética también ha hecho uso de los péptidos debido a que en los últimos años la formulación de sus productos se dirige a enfoques más particulares por lo que distintas moléculas de origen natural han sido incorporadas. Específicamente los péptidos han sido preferidos por características como su biodisponibilidad y estabilidad. Entre los distintos usos que se les han dado destacan: promotores de síntesis de colágeno, antioxidantes y antiarrugas (Ledwoń *et al*., 2021).

En cuanto a la industria farmacéutica, los péptidos han sido ampliamente estudiados y diversas investigaciones muestran que benefician de forma general la salud humana y de forma particular ayudan al tratamiento y prevención de diversas enfermedades. Por lo anterior se ha considerado su uso como parte de la formulación de alimentos funcionales, nutracéuticos e incluso como medicamentos (Gianfranceschi *et al*., 2018). La mayoría de los estudios publicados se enfocan en su efecto como antimicrobianos, antiinflamatorios, antihipertensivos, antioxidantes y anticancerígenos. En particular, el enfoque de los péptidos como moléculas anticancerígenas ha sido de gran interés debido a que esta enfermedad se ha posicionado como una de las más mortales. Además, todo tratamiento existente (radioterapia, quimioterapia e inmunoterapia) ha sido considerado como "inadecuado" por no lograr el objetivo de reducir los casos y por presentar altos índices de reincidencia. Por lo anterior, la industria farmacéutica se ha enfocado en estudiar un sinfín de moléculas, tanto sintéticas como naturales que ayuden a disminuir la mortalidad provocada por los distintos tipos de cáncer (Ng y Lee, 2020). Al inicio, los péptidos más estudiados en diversos modelos de cáncer fueron aquellos de ocurrencia natural provenientes de soya, como la lunasina y los inhibidores de tripsina, pero posteriormente se fue reportando el efecto prometedor de hidrolizados de diversas fuentes vegetales (Zaky *et al*., 2021). Se ha encontrado que algunos péptidos tienen la capacidad de matar células cancerígenas y los tipos de cáncer que más han servido como modelo de estudio han sido de mama, de próstata, de hígado y de colon. En este sentido, el cáncer colorrectal ha sido considerado como un modelo idóneo para el aprovechamiento de la bioactividad de péptidos debido al destino fisiológico que pueden tener estas moléculas una vez consumidas y por el impacto de esta enfermedad (Kiela y Ghishan, 2016). Lo anterior debido a que los enterocitos, las células responsables de la absorción de los nutrientes en el intestino delgado, absorben aminoácidos libres, así como di y tripéptidos mediante el cotransportador PepT1 Hfl/péptido. Sin embargo, estas células solo pueden absorber el 0.1 % de los péptidos más largos, y la mayoría van al intestino grueso (Vermeirssen *et al*., 2004). En cuanto a los colonocitos, las células presentes en el colon, existe evidencia de transporte transcelular de péptidos más grandes (de 3 o más aminoácidos) (Renukuntla *et al*., 2013).

1.2.7. Cáncer Colorrectal

1.2.7.1. Epidemiología del cáncer colorrectal. El cáncer colorrectal es el tercer cáncer más común después de mama y pulmón con alrededor de 1.93 millones de casos reportados anualmente. Por otro lado, en el 2020 se registraron 935000 decesos asociados a esta enfermedad. Durante mucho tiempo, el cáncer colorrectal tuvo una mayor incidencia en países en desarrollo. Sin embargo, hoy en día casi el 55% de los casos se han presentado en países desarrollados como Australia, Nueva Zelanda y en el caso particular de EUA, el cáncer colorrectal es la principal forma de cáncer. Se ha establecido que lo anterior es el resultado del estilo de vida que promueve la adopción de una dieta poco saludable (Arnold *et al*., 2017).

Su incidencia es aproximadamente un 25% mayor en hombres que en mujeres, debido principalmente a una mayor exposición de este grupo a factores de riesgo como alcoholismo y tabaquismo. Por otro lado, cuanto menor es el nivel socioeconómico de la persona, mayor es el riesgo de incidencia. Esto se relaciona con el hecho de que la población de bajos recursos tiene dietas inadecuadas y desconocimiento sobre la prevención de enfermedades crónico-degenerativas. La edad es otro factor que influye en gran medida en la incidencia y el desarrollo del cáncer colorrectal. Si bien es muy poco probable que las personas menores de 40 años lo desarrollen, después de esta edad, el riesgo aumenta aproximadamente un 2% cada año (Favoriti *et al*., 2016).

Otros factores de riesgo son la inactividad física, así como una dieta pobre en cuanto al consumo de frutas y verduras. Se ha registrado que las personas involucradas en la práctica deportiva o en trabajos que requieren actividad física equivalente a caminar 4 h por semana, presentan un menor riesgo a padecer cáncer colorrectal (Ghafari *et al*., 2016).

Asimismo, otro factor de riesgo es la herencia mendeliana, la predisposición familiar y el historial médico personal. Por ejemplo, las personas que padecieron cáncer de ovario, colitis ulcerosa y enfermedad de Crohn, tienen más probabilidades de desarrollarlo. Se espera que la incidencia mundial de esta enfermedad aumente debido a la occidentalización y al aumento de la esperanza de vida (Arnold *et al*., 2017).

1.2.7.2. Bases moleculares del cáncer de colon. La tumorgénesis del cáncer colorrectal se inicia en

la mucosa del colon cuando existe un trastorno en la replicación y la renovación celular que da como resultado la aparición de grupos con anomalías proliferativas, bioquímicas y biomoleculares. La mucosa colorrectal está constituida por 3 elementos: epitelio, lámina propia y muscularis mucosae. El epitelio gastrointestinal normal se organiza a lo largo de un eje de criptas/vellosidades. En el fondo de las criptas, se localiza un *pool* de células madre que son capaces de auto-renovarse y son pluripotentes (De Leon y Di Gregorio, 2001). Estas células migran a lo largo del eje cripta/vellosidad, diferenciándose simultáneamente en todos los linajes epiteliales del colon. En unos 14 días llegan a la parte superior de la vellosidad y sufren muerte celular programada. Sin embargo, en las primeras etapas del cáncer colorrectal, estas células se vuelven incapaces de reprimir la síntesis de ADN durante la migración, desarrollando una mayor capacidad para proliferar y una pérdida de estabilidad genómica que incluye inestabilidad cromosómica (cambios en el número y estructura de copias cromosómicas), inestabilidad de microsatélites, metilación aberrante del ADN e incapacidad de llevar a cabo correctamente procesos de reparación. Todo lo anterior se asocia principalmente a la presencia excesiva de radicales libres y procesos inflamatorios en la zona (Ewing *et al*., 2014).

Estos fenómenos se traducen en la pérdida de la integridad genómica al tiempo que se acumulan múltiples mutaciones dando lugar a una enfermedad muy heterogénea a nivel molecular. El cáncer colorrectal comenzará como un pólipo adenomatoso benigno, que se convertirá en un adenoma avanzado con displasia de alto grado y luego progresará a un cáncer invasivo. La mutación más común en cáncer colorrectal es la inactivación del gen que codifica la proteína APC, proteína que controla la concentración de β-catenina y la interacción con la E-cadherina. La segunda mutación genética clave es la inactivación de la vía p53, mediante la cual se pierde el control del ciclo celular. La tercera, es la inactivación de la señalización de TGF-β, responsable de mediar la detención del crecimiento y la apoptosis (Jass, 2007).

1.2.8. Péptidos y Cáncer Colorrectal

Debido a que las terapias que existen actualmente para prevenir o tratar el cáncer de colon han mostrado ser poco efectivas en reducir la mortalidad y la incidencia de esta enfermedad, nuevas

alternativas han sido estudiadas. En este respecto, la biología molecular ha permitido el estudio de los péptidos, ya sea de manera individual o en forma de hidrolizados, como una alternativa. Estas investigaciones han permitido conocer como funcionan (Xie *et al*., 2020).

1.2.8.1. Blancos moleculares de los péptidos en cáncer de colon. La actividad anticancerígena de los péptidos depende de su composición de aminoácidos. Los péptidos cargados positivamente interactúan con las membranas de las células cancerosas cargadas negativamente, pudiendo destruirlas selectivamente mediante la formación de micelas. Lo anterior, ya que las células sanas tienen membranas neutras, mientras que las células cancerosas contienen fosfatidilserina, un componente cargado negativamente, en su superficie (Zhang *et al*., 2019). Los péptidos que son solubles a través de las membranas de las células cancerosas e interactúan a niveles intracelulares contienen aminoácidos hidrofóbicos. Además, los aminoácidos aromáticos y cargados negativamente promueven la penetración de los péptidos en estas células, aunque el mecanismo aún no se comprende. Se han informado varias ventajas de los péptidos como posibles agentes anticancerígenos en comparación con los medicamentos de quimioterapia utilizados actualmente, como: un cruce más fácil de las barreras biológicas, una gama más amplia de objetivos, menos efectos secundarios, menor acumulación en los tejidos y menor toxicidad (Pan *et al*., 2020). Esta última característica indica que los péptidos poseen una alta seguridad clínica ya que sus productos de degradación son aminoácidos, los cuales son utilizados como nutrientes por las células sanas (Pan *et al*., 2020). Los péptidos de origen vegetal, como la soya, el arroz y la nuez, han mostrado un mayor efecto citotóxico en líneas celulares de cáncer de colon en comparación con otras, como cáncer de pulmón, sangre, mama e hígado, por lo que se cree que existe un efecto selectivo (Avilés-Gaxiola *et al*., 2020). Los mecanismos más reportados por los cuales los péptidos vegetales ejercen actividad anticancerígena, tanto como agentes preventivos como de tratamiento, se describen brevemente a continuación.

1.2.8.2. Péptidos anticancerígenos como agentes de tratamiento del cáncer de colon. Muerte celular: la apoptosis puede desencadenarse por vías extrínsecas o intrínsecas mediadas por caspasas. Hasta ahora, los péptidos vegetales que se ha informado que promueven la apoptosis por

la vía extrínseca son inhibidores de tripsina. Estos inducen la apoptosis en las líneas celulares de cáncer colorectal humano HT-29, SW620 y DLD1 a través de la vía apoptótica mediada por el receptor extrínseco (Fas/FasL) (Li *et al*., 2014). Se ha reportado que otros péptidos de origen vegetal pueden promover la muerte de células de cáncer colorectal mediante la vía intrínseca. Aquí, varias proteínas están involucradas: la proteína p53 promueve la activación de Bax, que se une a la membrana de la mitocondria, lo que permite la liberación del citocromo C. Esta molécula promueve la activación de la caspasa-9, que a su vez activará la caspasa-3, responsable de las características apoptóticas como la asimetría de la membrana plasmática, la condensación de la cromatina y la alteración del ADN. Otro mecanismo observado es la disminución de la expresión de TNFR1, un receptor transmembrana que induce la activación de proteínas antiapoptóticas a través de la señalización de la subunidad NF-kB p65. El bloqueo de NF-kB para el tratamiento del cáncer colorectal se ha convertido en una estrategia atractiva (Luna-Vital *et al*., 2016). Por otro lado, hay hidrolizados vegetales que promueven la apoptosis a través del aumento de los niveles de expresión de la proteína pro-apoptótica Bax y la disminución de los niveles de expresión de las proteínas anti-apoptóticas Bcl-2 y Mcl-1. Finalmente, se han encontrado péptidos vegetales que activan los mecanismos de reparación del ADN y que suelen verse afectados en el cáncer colorrectal (Allaoui *et al*., 2019).

Arresto del ciclo celular: Las proteínas involucradas en la progresión del ciclo celular se han estudiado ampliamente para el desarrollo de fármacos contra el cáncer, especialmente las ciclinas dependientes de cinasas (CDK), que regulan directamente la progresión del ciclo celular (Otto y Sicinski, 2017). CDK2, por ejemplo, impulsa la progresión de las células a las fases S y M, y su sobreexpresión se asocia con el crecimiento tumoral en múltiples tipos de cáncer, como el colorrectal. Existen péptidos de origen vegetal que reducen la expresión de CDK2. Por otro lado, Dentro de la célula, CDK2 es inhibido por la proteína p21. Algunos hidrolizados vegetales son capaces de regular positivamente a p21(Zhang *et al*., 2014).

Finalmente, el crecimiento neoplásico también se promueve a través de las ciclinas D, que median las señales extracelulares con la proliferación celular. La sobreexpresión de ciclina D1 da como resultado un crecimiento celular rápido al promover la transición del ciclo celular de G1 a S. Este evento está asociado con diferentes tipos de cáncer, como el colorectal. Péptidos de origen natural de leguminosas disminuyen la expresión de ciclina D1, controlando el crecimiento celular (Li *et al*., 2014)

1.2.8.3. Péptidos anticancerígenos como agentes preventivos de cáncer de colon. Inflamación: La inflamación crónica está involucrada en el desarrollo de tumores malignos en el colon. Se ha descubierto, por ejemplo, que altas concentraciones de óxido nítrico, son responsables del desarrollo de cáncer colorrectal. Hidrolizados derivados de la digestión gastrointestinal *in vitro* de proteína vegetal han logrado inhibir la producción de este compuesto en hasta 45% en macrófagos inducidos a un proceso inflamatorio mediante el uso de lipopolisacáridos. El aminoácido glutamina se ha implicado en esta actividad antiinflamatoria (González-Montoya *et al*., 2018).

Antioxidante: las especies reactivas de oxígeno (ERO) se generan como resultado del metabolismo celular. Sin embargo, el estilo de vida puede influir en una sobreproducción de estas, teniendo un efecto adverso en las macromoléculas, principalmente en el ADN. Las ERO reaccionan con el material genético de las células provocando la aparición de mutaciones somáticas que al acumularse promueven la aparición de células malignas. Además, estas moléculas pueden influir en el patrón de expresión génica, siendo promotores de la expresión de oncogenes. Por lo anterior, se les ha considerado como una de las causas de la aparición de diversos tipos de cáncer, como el cáncer de colon. Esto debido a que mucha evidencia sugiere que el consumo de antioxidantes previene la iniciación de esta enfermedad (Carini *et al*., 2017). En cuanto a la actividad antioxidante de los péptidos, estos pueden actuar como agentes reductores, quelantes de metales, extintores de oxígeno, donadores de hidrógeno, etc. y, además, inhiben las enzimas involucradas en la producción de radicales libres. La actividad antioxidante de los péptidos depende de su secuencia. Entre los aminoácidos que están más estrechamente relacionados con la actividad antioxidante de un péptido se encuentran ácido glutámico, glicina, alanina, leucina y fenilalanina. El ácido glutámico es un aminoácido negativo debido a un exceso de electrones y tiene actividad inhibidora de los radicales libres. Debido a esta propiedad también inhibe la oxidación mediada por metales (Torres-Fuentes *et al*., 2015). En cuanto a los aminoácidos hidrofóbicos como leucina, alanina y glicina, como se mencionó anteriormente, estos promueven el paso de péptidos al interior de las células. Por otro lado, contienen anillos de imidazol, que son donantes de protones. En particular, la leucina reduce el Fe^{3+} y tiene una cadena lateral alifática larga que interactúa con las cadenas acilo de los ácidos grasos (Chunkao *et al*., 2020). En cuanto a la fenilalanina, su anillo aromático funciona como donante de protones. El grupo hidroxilo fenólico de la tirosina también actúa como donante de hidrógeno, pudiendo apagar los radicales. Este aminoácido también elimina los radicales peroxilo generados durante el ensayo AAPH y también se ha asociado con valores altos de actividad antioxidante celular, principalmente porque tiene una mayor capacidad para eliminar

los radicales peroxilo, en comparación con otros aminoácidos. Se ha reportado que los péptidos que contienen tirosina tienen el doble de actividad antioxidante celular en comparación con aquellos que no la tienen en su estructura (Jin *et al*., 2016).

1.3. Hipótesis

1. Mediante la digestión gastrointestinal *in vitro* de proteína extraída a partir de hoja de *M. oleifera*, se generan péptidos bioactivos mayores a cinco aminoácidos, conformados por aminoácidos hidrofóbicos en una proporción igual o mayor al 20%.
2. El hidrolizado obtenido a partir de proteína de hoja de *M. oleifera* tiene propiedades antiinflamatorias por lo que reducen en más del 20% la producción de óxido nítrico de macrófagos.
3. El hidrolizado obtenido a partir de proteína de hoja de *M. oleifera* ejerce efecto antioxidante por métodos espectrofotométricos y en células de cáncer de colon
4. El hidrolizado obtenido a partir de proteína de hoja de *M. oleifera* tiene efecto antiproliferativo en células de cáncer de colon.

1.4. Objetivo General

Determinar la actividad anticancerígena de péptidos generados mediante digestión gastrointestinal in vitro de proteína de hoja de *M. oleifera*.

1.5. Objetivos Específicos

1. Obtener proteína de alta pureza a partir de hojas de *M. oleifera*.
2. Generar péptidos a partir de la proteína de hojas de *M. oleifera*.

3. Determinar el perfil de los péptidos obtenidos a partir de hoja de *M. oleifera*.
4. Evaluar el efecto de los péptidos obtenidos a partir de hoja de *M. oleifera* sobre la viabilidad celular y la producción de óxido nítrico en macrófagos.
5. Evaluar la actividad antioxidante de los péptidos obtenidos a partir de hoja de *M. oleifera* mediante técnicas espectrofotométricas y actividad antioxidante en células de cáncer de colon.
6. Evaluar la actividad anti proliferativa de los péptidos obtenidos a partir de hoja de *M. oleifera* en células de cáncer de colon.

1.6. Sección Integradora del Trabajo

En esta tesis se incluyen cuatro artículos: dos de revisión y dos originales. El primer artículo es de revisión y se encuentra publicado en la revista *Plant Foods for Human Nutrition*. Este artículo de revisión, permitió definir el estado del arte de la investigación. En este se establecen las principales causas de aparición de la enfermedad y se presenta evidencia de varios mecanismos a través de los cuales los péptidos bioactivos ejercen actividad antitumoral en modelos de cáncer colorrectal *in vitro* e *in vivo*. También se informa el estado actual de las principales técnicas de producción de péptidos, así como las limitaciones y perspectivas futuras. Así como que, la actividad anticancerígena de los péptidos depende de la composición de sus aminoácidos y longitud y los principales mecanismos de acción se asocian con su capacidad de promover apoptosis (manipulando la expresión de genes como p53 y Bcl-2), arresto del ciclo celular (cambiando la concentración de ciclinas y ciclinas dependientes de cinasas), control de procesos inflamatorios (disminuyendo la concentración de citocinas pro inflamatorias) y modificación de patrones de invasión y metástasis (inhibiendo serin proteasas). Como conclusión se plantea que, los péptidos podrían tener un impacto positivo en la prevención y el tratamiento del cáncer colorrectal. Sin embargo, aun son necesarios más estudios para evaluar sus efectos en ensayos clínicos tanto con sujetos sanos como con pacientes con cáncer y asegurar una transferencia exitosa del laboratorio al mercado de la industria farmacéutica. El segundo artículo corresponde a un artículo original y se encuentra publicado en *South African Journal of Botany*. En este artículo se incluyeron los primeros tres objetivos específicos del proyecto de investigación, presentando como resultados la

actividad de los péptidos de las hojas de *M. oleífera* (1.33 mg/ml) para inhibir los radicales DPPH y ABTS en un 45.70 y un 93.09%, respectivamente, y la actividad ORAC de 3.27 mM Trolox equivalente/g. Así como, el efecto no citotóxico para los macrófagos RAW 264.7 inducidos por lipopolisacáridos y, a una concentración de 100 µg/ml, la inhibición de la producción de óxido nítrico en un 30.51%. Así también, se reporta la secuencia de 14 nuevos péptidos, en orden de mayor a menor abundancia: LAYKPPG, YHSEVPV, WPPTFEQPK, LLGFDNR, QVWPTPGLK, FTKDDEWSCFPF, VEQNLVPGLK, TMMLMT, VQLPGWRVFP, SYLPPLSAEVTAK, TMKGPPDTLQ, MPWHEQ, LTAPGQATLPT y LLTPEGPKE. Como conclusión, se sugiere que los péptidos de las hojas de *M. oleifera* liberados por la digestión gastrointestinal *in vitro* podrían ser un recurso potencial para los componentes antioxidantes y antiinflamatorios naturales. Sin embargo, se necesitan más experimentos *in vivo*. En el tercer artículo se incorporaron los dos últimos objetivos de investigación del proyecto. Este artículo fue enviado a la revista *South African Journal of Botany* de la cual aun no se tiene respuesta. En este manuscrito se reporta la actividad antioxidante y anti proliferativa de los péptidos de hoja de *M. oleifera* en un modelo de cáncer de colon (Caco-2). Como resultados, se presenta que los péptidos de hojas de *M. oleifera* exhibieron una actividad FRAP de 1435 µmol TE/g. A una concentración de 500 µg/ml los péptidos no mostraron efecto citotóxico sobre CCD-18Co (células sanas de colon) y disminuyeron la actividad oxidante celular y la proliferación celular hasta en un 71.51 y 90.20% en Caco-2, respectivamente. Como conclusiones, se propone que los péptidos de la hoja de *M. oleifera* liberados por la digestión gastrointestinal *in vitro* podrían tener el potencial de funcionar como agentes preventivos para esta enfermedad, especialmente debido a su efecto antioxidante celular y como agente de tratamiento debido a su capacidad para inhibir la proliferación celular. Destacando que estas moléculas podrían ser un componente eficaz de los alimentos funcionales dirigidos al carcinoma colorrectal y posiblemente, con más estudios, como una terapia coadyuvante. El cuarto artículo, corresponde a un artículo de revisión, el cual se encuentra en preparación para la revista *Food Research International*. En este artículo de revisión se incluye la información referente al papel que juega la secuencia de aminoácidos, la estructura secundaria, la carga neta y la hidrofobicidad de los péptidos de origen vegetal, respecto a su bioactividad; entre ellas, la actividad antioxidante y anticancerígena. Como resultado de un análisis informático, se encontró que los aminoácidos que más se repiten entre los péptidos vegetales antioxidantes son el ácido glutámico, la glicina, la alanina, la leucina y la fenilalanina, así como el mecanismo particular de cada uno de estos

aminoácidos. Por otro lado, se determinó la secuencia secundaria ideal y la longitud más común entre péptidos antioxidantes. En cuanto a péptidos anticancerígenos, se determinó que los aminoácidos que más se repiten entre los péptidos vegetales reportados con actividad anticancerígena son el ácido glutámico, la leucina, la serina, la fenilalanina y la alanina. Además de una mayor actividad asociada a péptidos con carga neta positiva. Como conclusión, se plantea que los péptidos de origen vegetal son moléculas con gran potencial contra enfermedades transmisibles y no transmisibles. Asimismo, estos se han convertido en la mejor opción debido a la gran diversidad de fuentes. Este artículo de revisión surge de la necesidad de asociar las 14 estructuras obtenidas con las actividades antioxidantes, antiinflamatoria y anticancerígena que se estudiaron en este proyecto de investigación, así como conocer más a fondo el funcionamiento real de estas moléculas.

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2. PEPTIDES IN COLORECTAL CANCER: CURRENT STATE OF KNOWLEDGE

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REVIEW ARTICLE

Peptides in Colorectal Cancer: Current State of Knowledge

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Abstract
Colorectal cancer (CRC) is the second most deadly and the third most commonly diagnosed cancer in the world. CRC treatment is mainly based on surgery, chemotherapy, and even though the probability of complications after surgery is very low, chemo drugs affect the patient's quality of life. Multiple studies have shown a strong correlation between diet and the onset and progression of CRC. Thus, the consumption of dietary nutraceuticals for its treatment and prevention has been suggested as a promising option. Peptides have increasingly become of interest in human health due to their antioxidant, antihypertensive, and anticancer potential. In recent years, there have been extensive reports on peptides with anti-tumor activity, and some studies suggest that peptides modulate cell proliferation, evasion of cell death, and metastasis in malignant cells. Plant-derived peptides such as soybean, bean, and rice have received main attention. In this review, we show evidence of several mechanisms through which bioactive peptides exert anti-tumor activity over *in vitro* and *in vivo* CRC models. We also report the current status of major production techniques, as well as limitations and future perspectives.

Keywords Colorectal cancer · Bioactive peptides · Legumes · Cereals

Abbreviations

BBTIs	Bowman-Birk trypsin inhibitors
CDKs	Cyclin-dependent kinases
CDK2	Cyclin-dependent kinase 2
CRC	Colorectal cancer
GI	Gastrointestinal

Introduction

Cancer is the second leading cause of death globally and currently responsible for an estimated 9.6 million deaths every year. According to the Global Cancer Observatory, the most common cancers are lung, breast, and colorectal. Colorectal cancer (CRC) caused an estimated of 862,000 deaths worldwide in 2018 [1]. CRC treatment consists of surgery where the colorectal damaged fraction is removed; after this surgery, it is common that patients submit to adjuvant therapy like chemotherapy [2]. Chemotherapy indiscriminately attacks all rapidly dividing cells, causing adverse effects like infections, anemia, memory loss, heart disease, among others [2]. These side effects compromise the patient's quality of life, so the development of new therapies and strategies for CRC treatment is essential. Various meta-analyses have reported an inverse association between the consumption of foods of plant origin and the risk of developing CRC [3–5].

Similarly, human studies have shown that the consumption of a minimum of 12.3 g/day of cereals, 603 g/day of fruits and vegetables, as well as 3–5 cups of legumes *per* week, decreases the risk of CRC onset and progression [6]. This effect has been associated with plant-origin molecules, especially to their non-digestible fraction, which is mainly composed of fiber, starch, phenolic compounds, and bioactive peptides [7]. Peptides are protein fractions from 2 to 100 amino acids linked by peptide bonds. They are naturally found in food sources and can also be produced by protein enzymatic hydrolysis or fermentation [8].

For a long time, animal products were considered as the main source of protein. Nevertheless, since plants represent a more economical and sustainable source of protein, the main

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target of current peptide research is focused on plant sources with high protein content, such as cereals and legumes [9]. Bioactive peptides can affect numerous physiological functions. For example, they may act as antihypertensive agents or as opioid agonists or antagonists. Furthermore, antithrombotic, immunomodulating, antioxidative, and anticancer activity has been reported [10]. Regarding the anticancer effects, peptides promote apoptosis, necrosis, cell cycle arrest, and metastasis suppression [11]. Numerous protein hydrolysates and natural-occurring peptides with anticancer activity have been obtained. In this review, we will focus on plant-based peptides with antiproliferative and anticancer activity over CRC *in vitro* and *in vivo* models.

Plant Peptides Production

Some peptides are naturally found in plant sources, such as Vglycin, lunasin, and Bowman-Birk trypsin inhibitors (BBTIs). In contrast, other peptides are obtained by fermentation or enzymatic hydrolysis from plant protein [8]. As for naturally occurring peptides, some limitations of their use are their low concentration and complex isolation and purification process. Therefore, it is suggested to obtain them through other techniques such as chemical synthesis using solid-phase techniques or recombinant DNA technology [12, 13]. As for chemical synthesis, it is easily automated, facilitates peptide purification, and is less expensive compared to recombinant technologies. Nevertheless, this process faces challenges such as low yields due to peptides insolubility issues and the use of hazardous reagents during the process [12]. On the other hand, recombinant peptides are produced by designing expression vectors that include localization and purification signals and by introducing them into bacteria such as *Escherichia coli* in highly controlled and large-scale processes [13]. Enzymatic hydrolysis is preferred over fermentation because the latter is a non controlled process, while in the enzymatic hydrolysis, parameters such as pH, temperature, time, and enzyme concentration are controlled [14]. During enzymatic hydrolysis, enzymes from both microorganisms and plants have been used, like alcalase and papain, respectively [15, 16]. Nevertheless, the most commonly used proteases are the digestive ones (trypsin, chymotrypsin, pancreatin, and pepsin), which aim to simulate gastrointestinal (GI) conditions [17, 18].

Pathogenesis of CRC

Both environmental and genetic factors influence the development of CRC. Only around 25% of CRC cases are due to inherited mutations in well-known cancer-related genes (*APC*, *MUTYH*, *MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM*), and the rest of the cases are mostly associated to lifestyle risk factors like diet, smoking, and alcohol use [19]. CRC mutations include chromosomal and microsatellite instability, as well as altered methylation patterns, which promote the alteration of several molecular signaling pathways, such as the Wnt/APC/β-catenin. This pathway plays a dominant role in the initiation of CRC and its characterized by the inactivation of the *APC* gene, which results in the β-catenin release. The Wnt signaling pathway, acting through β-catenin, modulates cellular processes, including survival, apoptosis, proliferation, differentiation, and cell adhesion [20]. The passage from an early adenoma to an intermediate one is characterized by phosphatidylinositol-3-kinase pathway alteration in which the *KRAS* gene is activated [21]. As for advanced adenomas, they are characterized by the inactivation of the transforming growth factor-β pathway [21]. Another very important gene is p53, which is mutated in 50% of all CRCs and mediates the progression from adenoma to cancer [22]. Furthermore, the presence of immune system cells in the large intestine area leads to an inflammatory state which fosters multiple cancer hallmarks [23].

Mechanisms of Action of Plant-Derived Peptides on CRC Models

The anticancer activity of peptides depends on their amino acid composition. Positively charged peptides interact with negatively charged cancer cell membranes, being able to destroy them selectively by micelle formation. The above, since healthy cells have neutral membranes, whereas cancer cells contain a negatively charged component (phosphatidylserine) on their surface [24]. Peptides that are soluble across cancer cell membranes and interact at intracellular levels contain hydrophobic amino acids. Also, aromatic and negatively charged amino acids promote penetration of the peptides into cancer cells, although the mechanism is not yet understood [25, 26]. Peptides larger than three amino acids have been considered for study in CRC models because although enterocytes absorb free amino acids as well as di and tripeptides by the PepT1 Hfl/peptide cotransporter, these cells can only absorb 0.1% of longer peptides, with the majority going to the large intestine [27]. As for colonocytes, there is evidence of transcellular transport of larger peptides, especially hydrophobic ones [28]. Several advantages of peptides have been reported as potential anticancer agents compared to currently used chemotherapy drugs, like an easier crossing of biological barriers, a wider range of targets, fewer side effects, lower accumulation in tissues, and lower toxicity [29]. This last feature indicates that peptides possess a high clinical safety since their degradation products are amino acids, which are used as nutrients by the cells [29]. *In silico* analyses have shown that peptides with a possible toxic effect are only those containing Cys, Pro, Asp, and His in high

proportions [30]. Peptides from plant sources, such as soybean, rice, and walnut, have shown a greater cytotoxic effect in colon cancer cell lines compared to others, such as lung, blood, breast, and liver cancer, for what is believed there is a selective effect against CRC [17, 31–35]. The mechanisms by which plant peptides exert anticancer activity are briefly described below. Although this information was determined over *in vivo* or *in vitro* CRC models, the molecular mechanisms studied in the majority of the included research articles are general for all types of cancer.

Cell Death

Apoptosis

Apoptosis can be triggered either by the caspase-mediated extrinsic or intrinsic pathways [36]. So far, the only plant peptide that has been reported to promote apoptosis by the extrinsic pathway is a fragment of mung bean (*Vigna radiata*) BBTI. It induced apoptosis in the human CRC cell lines HT-29, SW620, and DLD1 through the extrinsic receptor (Fas/FasL)-mediated apoptotic pathway [37]. As for the intrinsic pathway, several proteins are involved: here, the protein p53 promotes the activation of Bax, which becomes mitochondria membrane-bound, allowing cytochrome C release. This molecule promotes the activation of caspase-9, which in turn will activate the executioner caspase-3, responsible for apoptotic hallmarks such as plasma membrane asymmetry, chromatin condensation, and DNA disruption [38]. A bean (*Phaseolus vulgaris*) peptide (GEGSGA), as well as *Gloriosa superba* hydrolysate, were reported to promote apoptosis by p53 upregulation; this protein is the most studied target against CRC [39, 40]. GEGSGA apoptotic effect was also due to the decreased expression of tumor necrosis factor receptor type 1, a transmembrane receptor that induces the activation of anti-apoptotic proteins through subunit nuclear factor-kB p65 signaling. Blocking nuclear factor-kB for CRC therapy has become an attractive strategy [40]. On the other hand, Vglycin and sweet potato (*Ipomoea batatas*) hydrolysates promoted apoptosis through increased expression levels of the apoptotic protein Bax. They decreased expression levels of anti-apoptotic proteins Bcl-2 and Mcl-1. It is reported that Bax expression is positively correlated with better survival in advanced CRC [15, 41]. Soybean lunasin, which is found in a proportion of 4.4 mg *per* 70.5 g of soy protein, and amaranth (*Amaranthus mantegazzianus*) hydrolysates induced caspase-3 activation and cleavage of Poly (ADP-ribose) polymerase by acting as adapter molecules [42–44]. Previously it was reported an amaranth peptide that matched more than 60% with lunasin sequence, which may be the reason for the similar observed activities [43]. Cytochrome C release can also be promoted by a change in mitochondria membrane potential, which can be caused by mitochondrial increased oxygen species concentration. A bean peptide (GLTSK) and fenugreek (*Trigonella foenum-graecum*) hydrolysate promoted cell death by this mechanism [26, 45]. Peptides mainly promote mitochondrial increased reactive oxygen species production by the impairment of the mitochondrial respiratory chain and the intracellular chelation of iron, which may trigger a Fenton-type reaction that can lead to the generation of the highly reactive hydroxyl radical [26]. In the particular case of fenugreek hydrolysate, it activated DNA repair mechanisms, which are usually affected in CRC and are the ones promoting microsatellite instability [45, 46].

Necrosis

It has been reported that necrotic cell death can be driven by a defined molecular pathway mediated by receptor-interacting protein 1 and 3 and mixed lineage kinase domain-like pseudokinase [47]. Since cancer treatment is mainly based on the use of chemotherapy drugs, promoting apoptosis, cancer cells have developed evasion mechanisms in order to survive. Accordingly, other programmed cell death modes have gained attention as potential therapeutic approaches. Even though necrosis has not been considered to treat cancer due to inflammatory responses, its clinical significance is increasing as an emerging tool to overcome cancer cells acquired apoptosis-resistance [48]. So far, peptides from amaranth are the only ones reported to promote necrosis over CRC cells [44]. However, because the exact mechanism has not been thoroughly studied, further research is recommended. The research articles included in this review have focused on determining whether peptides promote apoptosis and cell cycle arrest. Further oomic studies would be convenient to assess if or not peptides promote programmed necrotic cell death. The above can be determined by detecting the presence of phosphorylated receptor-interacting protein 1 and 3 using the Western Blot technique [49].

Cell Cycle Arrest

Proteins involved in cell cycle progression have been extensively studied for the development of anticancer drugs, especially cyclin-dependent kinases (CDKs), which directly regulate cell cycle progression [50]. Cyclin-dependent kinase 2 (CDK2) drives the progression of cells to the S- and M-phases and its overexpression is associated with tumor growth in multiple cancer types, such as CRC. It was reported that CDK2 expression was downregulated by Vglycin, promoting cell cycle arrest [41]. Vglycin also decreased proliferating cell nuclear antigen expression. Proliferating cell nuclear antigen, a DNA polymerase δ auxiliary protein, plays an important role in colorectal neoplastic progression, and it correlates with high-grade dysplasia and size [51]. Inside the cell, CDK2 is inhibited by the CDK inhibitor p21 [50]. P21 expression,

which is regulated by p53, was increased by the bean peptide GEGSGA, leading to cell cycle arrest at the G1-S transition. GEGSGA phosphorylated Ser15 of p53, leading to its activation [26]. P21 was also upregulated by sweet potato hydrolysate leading to cell cycle arrest at G2-M transition. Also, sweet potato hydrolysate increased the expression of the CDK inhibitor p27 [15]. Downregulation of p27 is caused by increased ubiquitin-mediated proteasomal degradation in CRC and has been associated with poor prognosis [52]. Neoplastic growth is also promoted through D cyclins, which mediates extracellular cues with cell proliferation. Overexpression of cyclin D1 results in rapid cell growth by promoting cell cycle transition from G1 to S. This event is associated with different types of cancer, such as CRC [53]. In colon carcinoma cells, β-catenin mediates cyclin D1 expression. A fragment of mung bean BBTI and Vglycin decreased cyclin D1 expression by blocking the β-catenin signaling pathway [37, 41].

Invasion

Serine proteases are a group of proteases involved in extracellular matrix degradation and, therefore, in cancer progression. Serine protease inhibitors are considered as a potential chemopreventive agent, especially during the early stages of CRC, so they have been extensively investigated [54]. Pea and lentil (*Lens culinaris*) BBTIs significantly inhibited serine proteases in a dose-dependent manner, influencing the growth of human CRC negatively. In contrast, non-malignant colonic fibroblasts were unaffected [55, 56]. Lentil BBTI activity is associated with Gln18 and His45 interacting with serine proteases active site [56, 57]. Wnt/APC/β-catenin signaling also plays an essential role in CRC progression. There is a positive correlation between the expression of β-catenin and the most invasiveness of the cell line [20]. Mung bean BBTI fragment decreased β-catenin protein levels, inhibiting tumor growth [37].

Anti-Inflammatory Properties of Plant-Derived Peptides

Chronic inflammation is involved in the development of malignant CRC tumors. Nitric oxide and increased expression of cyclooxygenase-2 with concomitant overproduction of eicosanoids, such as prostaglandin D2, have been found responsible for the development of CRC [23]. Peptides derived from *in vitro* gastrointestinal digestion of germinated soybean inhibited nitric oxide and prostaglandin D2 production up to 45 and 95%, respectively, in lipopolysaccharide-induced macrophages RAW267.4. The amino acid glutamine had been implicated in the anti-inflammatory activity of these peptides since it was found on the identified peptides (QQQQQGGSQSQ, QEPQESQQ, QQQQQGGSQSQSQKG, and PETMQQQQQQ) [7]. Also, the soybean peptides lunasin and VPY have been reported with anti-inflammatory activity, but their mechanisms have not been fully elucidated [58]. Anti-inflammatory peptides have also been obtained from *in vitro* GI digestion of chia (*Salvia hispanica* L.) seeds. The 1–3 kDa fraction reduced nitric oxide, H_2O_2, interleukin-1β, −6, and 10, and tumor necrosis factor-α production [59]. Figure 1 summarizes the key cellular process affected by plant-derived peptides in colon cancer cell lines.

In Vitro and *in Vivo* Studies

Peptides from Legumes

Soybean Peptides

Soybean seed defatted flour is the source from which most of the reported bioactive peptides have been obtained. One of the most studied soybean bioactive compounds is the BBTI. Of all legumes, soybean is the one with the highest trypsin inhibitor activity (94.1 units/mg) [60]. Soybean BBTI inhibited up to 80% of Caco-2 and HT-29 cell lines growth [61]. BBTIs have been of great interest in the study of CRC treatment since proteolytic enzymes do not digest it in the GI tract, and their structure and function are maintained [62]. A 75% growth inhibition in Caco-2, HCT-116, and HT-29 cells was reported by a soybean 18 kDa peptide obtained after enzymatic hydrolysis [31]. When comparing hydrolysates from soybean protein obtained either by GI-simulated digestion alone or by an alcalase hydrolysis followed by GI-simulated digestion, it was determined that when there was no alcalase previous treatment, a 7-fold higher concentration was needed to achieve the same cell growth inhibitory effect [7, 31]. The highest bioactivity given by the previous alcalase hydrolysis may be given by the preference of this enzyme for sites containing hydrophobic residues, increasing its exposure, which enhances the ability of soybean peptides to cross the cellular lipid membrane of the CRC cells [25].

Pea Peptides

Pea seed extracts are potentially pharmacologically active against CRC, and this activity has been attributed to compounds such as Vglycin and BBTI. Vglycin is a 37 amino acid polypeptide with six half-cysteine residues interconnected by three pairs of disulfide bonds; this peptide inhibited CT-26, SW480, and NCI-H716 cells growth by up to 82%. Vglycin was also reported to inhibit CRC growth by 38% when administered in a dose of 20 mg/kg/day for 21 days to male mice [41]. Vglycin has a very rigid structure due to its high number of disulfide bonds. This property allows it to survive both to the acidic conditions and the action of proteolytic enzymes within the stomach and small intestine, permitting significant amounts to reach the colorectal

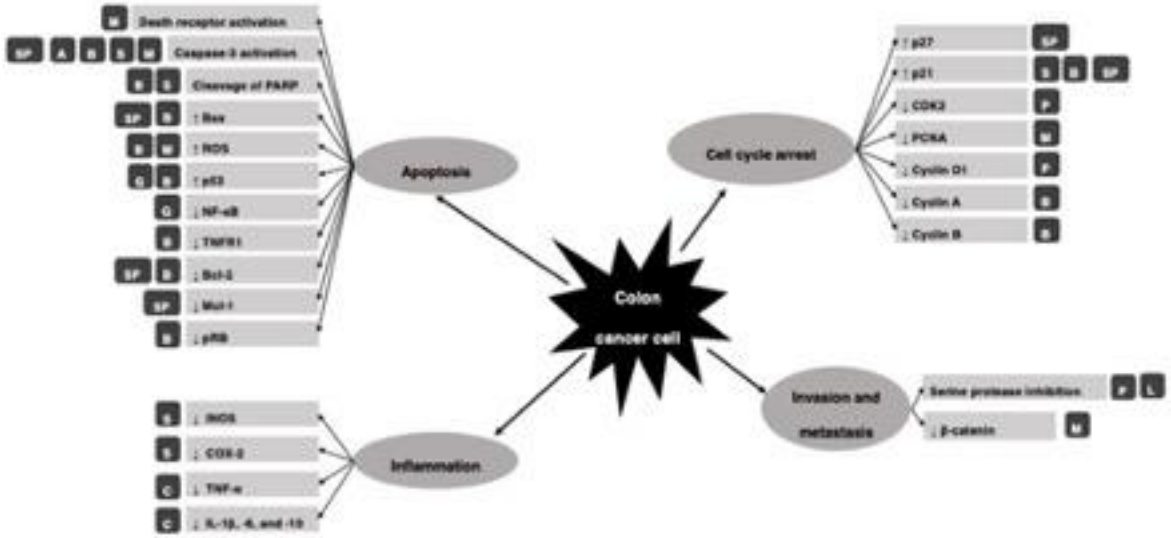

Fig. 1 Molecular targets of plant peptides involved in their efficacy against colorectal cancer (M: mung bean, SP: sweet potato, A: amaranth, B: bean, S: soybean, G: *Gloriosa superba*, C: chia, P: pea, and L: lentil). PARP: Poly (ADP-ribose) polymerase, ROS: Reactive oxygen species, NF-kB: Nuclear factor-kB, TNFR1: Tumor necrosis factor receptor type 1, pRB: Phosphorylated retinoblastoma protein, iNOS: Inducible nitric oxide synthase, COX-2: Cyclooxygenase-2, TNF- α: Tumor necrosis factor-α, IL-1β, -6, and -10: Interleukin-1β, -6 and 10, CDK2: Cyclin-dependent kinase 2, PCNA: Proliferating cell nuclear antigen

fraction in their active form [63]. In regard to BBTI, pea is one of the legumes with the lowest trypsin inhibitor activity (2.20 units/mg). Pea BBTI significantly affected HT20 cell line proliferation in a dose-dependent manner while not affecting colonic fibroblasts growth [55].

Bean Peptides

A fragment of mung bean BBTI was targeted to colon cancer cells by binding it to the peptide WIFPWIQ via a recombinant plasmid expressed in *Escherichia coli* strain BL21. WIFPWIQ binds specifically to GRP78, a glucose-regulated protein expressed in CRC cell membranes but not in healthy ones. The fused peptides were able to inhibit cell growth in HT-29, SW620, and DLD1 cells with no adverse effects over normal human colon epithelial cells and to reduce the tumor volume and mass in female mice [37]. The main advantage of this molecule as a therapeutic agent is its high selectivity. Mung bean trypsin inhibitor activity is low compared to that of soybean (1.83 units/mg) [64]. Luna-Vital et al. [40] have investigated the anticancer activity of hydrolysates obtained by GI *in vitro* digestion from the non-digestible fraction of bean. The most effective fraction was the <500 kDa one, inhibiting HCT-116 and RKO cell line proliferation in more than 80%. This effect was associated with the most abundant peptides in the hydrolysate: GLTSK, LSGNK, GEGSGA, MPACGSS, and MTEEY. GLTSK and GEGSGA had the highest cytotoxic activity, inhibiting HCT116 cell proliferation by 17 and 8%, respectively, in a dose-dependent manner [40]. Nevertheless, since these five peptides exert a synergistic effect, it is convenient to use the entire hydrolysate [26].

Other Legume Sources

Use of 30 mg/kg of chickpea (*Cicer arietinum*) protein hydrolysate for 28 days promoted an 87% reduction of the aberrant crypts of CRC induced male mice in a dose-dependent manner [65]. This finding suggests that chickpea protein hydrolysates might confer a protective effect against CRC. Also, it would be interesting to observe the effect of chickpea protein consumption before hydrolysis. These proteins were subjected to two GI hydrolysis, the first *in vitro* and the second one *in vivo*. Anticancer properties of BBTI from lentil have been investigated; for instance, lentil BBTI is a double-headed trypsin/chymotrypsin inhibitor of 7 kDa formed by 47 amino acids. It inhibited up to 80% of HT29 cell's growth in a dose-dependent manner while unaffecting the growth of colonic fibroblasts [56, 57]. Lentin trypsin inhibitor activity is considered as high, being up to 7.40 units/mg [66].

Peptides from Cereals

Rice (*Oryza sativa*) Peptides

A bioactive pentapeptide (EQRPR) isolated from rice bran protein after GI *in vitro* digestion was reported to inhibit in more than 80% the growth of HCT-116 cells in a dose and time-dependent manner [33–35]. The fact that a single peptide

Table 1 Anticancer potential of plant peptides over *in vivo* and *in vitro* colorectal cancer models

Sources	Obtaining method	Final Product	Bioactivity assays			Reference
			Model	Antiproliferative effect	Mode of action	
Amaranth	Pepsin and pancreatin	Hydrolysate	• *In vitro* (HT29)	• IC_{50}: 0.30 mg/mL	• Necrosis	[44]
Bean	Expressed in *Escherichia coli*	A single peptide (WIFPWIQL)	• *In vivo* (nude mice)	• ↓ of tumor mass by 60%	• Cell cycle arrest (G1) • Apoptosis (death-receptor and mitochondrial pathways)	[37]
Bean	Pepsin and pancreatin	A single peptide (GEGSGiA)	• *In vitro* (HCT116)	• IC_{50}: 134.6 μM	• Cell cycle arrest (G1) • Apoptosis (loss of mitochondrial membrane potential)	[26, 40]
Chickpea	Gastrointestinal (GI) *in vitro* and *in vivo* digestion	Hydrolysate	• *In vivo* (mice)	• ↓ of aberrant crypts by 85%	–	[65]
Fenugreek	Purafect and Esperase	–	• *In vitro* (Caco2/TC7)	• ↓ cell growth by 55% (1 mg/mL)	• Cell cycle arrest (G1) • Apoptosis (mitochondrial permeabilization)	[45]
Gloriosa superba rhizome	Pepsin	<3 kDa peptides	• *In vitro* (SW620)	• ↓ cell growth by 50% (30 ng/mL)	• Apoptosis (upregulation of p53 and downregulation of nuclear factor-κB)	[39]
Lentil	Expressed in *Pichia pastoris*	7.5 kDa Bowman-Birk trypsin inhibitor (BBTI)	• *In vitro* (HT29)	• IC_{50}: 32 μM	–	[56]
Pea	–	Vglycin	• *In vitro* (NCL-H716, CT-26, and SW480) • *In vivo* (mouse)	• IC_{50}: 3.62, 4.21, and 3.68 μM • ↓ of tumor growth by 38%	• Cell cycle arrest (G1/S) • Apoptosis	[41]
Pea	Expressed in *Pichia pastoris*	BBTI	• *In vitro* (HT29)	• IC_{50}: 31 μM	• Inhibition of serine proteases	[55]
Rice	*Alcalase digestion*	A single peptide (EQRPR)	• *In vitro* (HCT-116)	• ↓ cell growth by 84% (600–700 μg/ml)	–	[33–35]
Soybean	Alcalase followed by GI digestion	Three peptides of 10–50 kDa	• *In vitro* (HCT-116)	• ↓ cell growth by 73% (800 μg/ml)	–	[31]
Soybean	Alcalase followed by GI digestion	A 18 kDa peptide	• *In vitro* (HCT-116)	• ↓ cell growth by 80% (700 mg/mL)	–	[32]
Soybean	Pepsin and pancreatin	15 peptides: NNDDRDS, VVNPDNNEN, LSSTEAQQS, NAENNQRN, IKSQSES, EEPQQPQQ, GQSSRPQD, LAGNQEQE, NLKSQQA, QEPQESQQ, SQRPQDRHQ, QQQQQGGSQSQ, QQQQQGGSQSQKG, PETMQQQQQQ, and SDESTESETEQA	• *In vitro* (Caco-2, HT-29, and HCT-116)	• IC_{50}: 10.3, 14.9, and 15.2 mg/mL	–	[7]
Soybean	Chemical synthesis	Lunasin	• *In vitro* (HCT-116)	• IC_{50}: 107.5 μM	• Cell cycle arrest in G1 (p21 upregulation) •Apoptosis (caspase-3 activation and	[43]

Table 1 (continued)

Sources	Obtaining method	Final Product	Bioactivity assays		Reference
			Model	Antiproliferative effect	Mode of action
					cleavage of Poly (ADP-ribose) polymerase)
Sweet potato	Alcalase digestion	<3 kDa fraction	• *In vitro* (HT-29)	• ↓ cell growth by 43.87% (100 g/mL)	• Cell cycle arrest in G2/M (p21 up-regulation) • Apoptosis (Bcl-2 downregulation, Bax upregulation, and caspase-3 activation)	[15]
Walnut	Chymotrypsin digestion	Hydrolysate	• *In vitro* (HT-29)	• IC_{50}: 650 μg/mL	–	[17]
Walnut	Papain digestion	A single peptide (CTLEW)	• *In vitro* (Caco-2)	• IC_{50}: 0.65 mg/mL	• Apoptosis	

exerted such a high anticancer capacity is a great advantage since it can be easily synthesized or produced, which may contribute to the development of more efficient and specific drugs.

Other Cereal Sources

The first investigation of peptides obtained from plant sources and tested over CRC cell lines was carried out with wheat (*Triticum* spp) prolamins hydrolysates [67]. Wheat hydrolysates inhibited 50% of the proliferation of apical Caco-2 cells in an *in vivo* simulation. The cells were divided into two compartments, the apical and the basolateral, using a cyclopore polyester membrane. Cyclopore polyester membranes are not recommended since when cells reach confluency, they produce a tight impermeable monolayer, preventing the adequate diffusion of compounds from the apical to the basolateral compartment. One of the most recent studies in this field was carried out with an amaranth hydrolysate obtained by GI simulation. An IC_{50} of 0.30 ± 0.07 mg/mL was reported after a 24 h treatment over HT-29 cells [44]. Due to the high popularity of amaranth in recent years, its protein has been incorporated into the formulation of different functional foods that promote health benefits.

Peptides from Other Plant Sources

Gloriosa superba hydrolysate fraction <3 kDa obtained by simulated GI digestion inhibited up to 40% SW620 cell growth [39]. Although these peptides exert significant inhibitory activity, their bioactivity is lower than that of cereals and legumes. Walnut hydrolysate, obtained by simulated GI digestion followed by proteinase K hydrolysis, inhibited HT-29 cell growth by 76% [17]. On the other hand, a single walnut peptide obtained by papain hydrolysis (CTLEW) inhibited by itself up to 80% Caco-2 cell line growth without inhibiting non-cancerous IEC-6 cells growth [16]. Even though walnut peptides have high anticancer activity, the main disadvantage of their use is the high cost of the raw material. Despite having a low protein content, sweet potato protein has also been considered as a source of bioactive peptides. The antiproliferative activity of sweet potato peptides has been tested after hydrolysis by different enzymes. Among these enzymes are proleather, ASI, neutrase, papain, pepsin, and alcalase [15]. From these, alcalase and pepsin produced the peptides with the highest (80%) and the lowest (50%) antiproliferative activity in HT-29 cells, respectively. The fraction with the highest antiproliferative effect was the <3 kDa one [15]. However, since sweet potato is a root with such a low protein content, obtaining the hydrolysate needed amount could result in a costly process. Table 1 summarizes the most relevant information regarding the anti-cancer potential of plant peptides tested over *in vivo* and *in vitro* CRC models.

Limitations and Future Perspectives

Peptides must be bioavailable to exert the bioactive effect, which is a limitation to their bioactivity due to possible modifications caused by the pH of the gastrointestinal tract; and also, the biochemical changes by brush border peptidases and further microbiota transformation [28]. The use of nanocarriers has been suggested as they act as controlled delivery systems [68]. For proteins and peptides colonic delivery, the polymer chitosan has been proven as an adequate material. Chitosan is a polysaccharide with reported capacity for protein/peptide entrapment, and the glycosidases secreted by colonic bacteria execute random hydrolysis of the 1,4 glycosidic bond [68]. In order to increase the dose of peptides reaching the colon, it is recommended for loaded chitosan nanoparticles to be coated with materials resistant to gastrointestinal enzymatic hydrolysis, such as Eudragit S100 [69]. Also, peptides have poor solubility and are easily hydrolyzed. Therefore, artificial modifications of peptides have been suggested. Peptides coupling with polyethylene glycol molecules improve their solubility and hydrolysis. AG10 is another molecule that can be linked to peptides and also protect them from proteases [11]. Due to the structural diversity of peptides, studies are still needed to identify the amino acids responsible for these effects. As a result, some authors have suggested using tools like molecular docking and *in silico* research [30]. Moreover, there are still limitations in obtaining and purifying bioactive peptides to a large scale, so once the amino acid sequences are identified, they can be more easily obtained by using recombinant DNA technology [11].

Conclusions

Finding an effective cancer treatment is one of the greatest challenges for medicine. In recent years there has been an extensive search aimed at identifying and obtaining anticancer compounds of natural origin. The diverse structures of proteins present in plants provide a large number of bioactive peptides. These peptides have the potential to be used as pharmaceuticals for the treatment of cancer. For example, they could be combined with conventional drugs, minimizing the problem of chemo drug resistance and avoiding recurrence. Although we believe that in the upcoming years, peptides will have a major impact on the treatment of cancer, there are still several obstacles to overcome. Further studies are necessary to evaluate their effects in clinical trials with both healthy subjects and patients with cancer. More research is still needed to verify the beneficial effects of peptides in order to successfully transfer research from the laboratory to patients to control the growing burden of this disease effectively.

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Compliance with Ethical Standards

Conflict of Interest The authors declare they have no conflict of interest.

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3. ANTIOXIDANT AND ANTI-INFLAMMATORY PROPERTIES OF NOVEL PEPTIDES FROM *MORINGA OLEIFERA* LAM. LEAVES

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Antioxidant and anti-inflammatory properties of novel peptides from *Moringa oleifera* Lam. leaves

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ABSTRACT

Moringa oleifera is a tree used as a medicinal herb by several populations. Due to their curative and preventive properties, all parts have been studied, especially the leaves. They have been found to act as antithrombotic, antihypertensive, anticancer, immunomodulating, and antioxidant agents. This study was aimed to characterize and evaluate the antioxidant and anti-inflammation activities of *Moringa oleifera* leaves protein hydrolysate obtained by *in vitro* gastrointestinal digestion. The antioxidant activity of these peptides was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and Oxygen Radical Absorbance Capacity (ORAC) assays. RAW 264.7 macrophages were stimulated with lipopolysaccharides, and the effect of the peptides over nitric oxide production was measured to assess the anti-inflammatory effect. Furthermore, peptides were identified by nanoscale liquid chromatography coupled to tandem mass spectrometry. *Moringa oleifera* leaves peptides (1.33 mg/ml) inhibited DPPH and ABTS radicals by 45.70 and 93.09%, respectively, and had an ORAC activity of 3.27 mM Trolox equivalent/g. They were not cytotoxic to lipopolysaccharides-induced RAW 264.7 macrophages and, at a concentration of 100 μg/mL inhibited the nitric oxide production by 30.51%. The sequences of 14 novel peptides were identified. Our findings suggest that *Moringa oleifera* leaves peptides released by *in vitro* gastrointestinal digestion might be a potential resource for natural antioxidant and anti-inflammatory components. However, further *in vivo* experiments are needed.

1. Introduction

Moringa oleifera Lam. (MO), also known as "the miracle tree," is a tree species belonging to the Moringaceae family, within the order Brassicales. The Moringaceae family has 13 species, with MO as the most cultivated and studied one (Tshabalala et al., 2019). MO is native to sub-Himalayan tracts of Northern India, where it was first described as a medicinal herb. Later, it was distributed to Africa and Ethiopia. Nowadays, since the tree can grow in various conditions, it is also found in distant places such as Latin America and the Pacific Islands, among other countries (Bartíková et al., 2020). In Africa particularly, folk medicine uses MO (a.k.a. panacea) to treat and prevent more than 300 diseases such as cancer, diabetes, malaria, dementia, hypercholesterolemia, Parkinson's, and asthma, among others. Also, MO has been used to combat child malnutrition (Granella et al., 2021; Matic et al., 2018).

Due to their preventive and curative properties, all MO parts have been studied, particularly leaves, which are an important dietary source for humans (Nouman et al., 2020). Consumption of MO leaves may enrich the human diet in bioactive components, for example, biologically active peptides. Bioactive peptides are released during gastrointestinal digestion and are small compounds with biological activity, for example, antithrombotic, antihypertensive, immunomodulating, anticancer, and antioxidant agents (Görgüç et al., 2020; Lin et al., 2019; Paula et al., 2017; Yun et al., 2020).

There is increased interest in the use of plant peptides for the treatment of oxidative stress. Overproduction of free radicals and oxidants can cause oxidative stress and oxidative damage to biological molecules promoting cancer, cardiovascular disease, and neurological

Abbreviations: MO, Moringa oleifera; MOP, Moringa oleifera leaves protein; MOPH, Moringa oleifera leaves protein hydrolysate; NO, nitric oxide; LPS, lipopolysaccharides; DH, degree of hydrolysis; SDS-PAGE, sodium dodecyl sulfate gel electrophoresis; DPPH, 2,2-diphenyl-1-picrylhydrazyl assay; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid assay; ORAC, Oxygen radical absorbance capacity assay

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diseases (Pizzino et al., 2017). Natural antioxidants are the subject of increasing interest for their role in preventing diseases. Several studies have shown that antioxidant and anti-inflammatory peptides have a protective effect against ROS, contributing to reducing oxidative stress (Saenjum et al., 2012).

MO leaves have high amounts of crude protein (23.0 to 30.3%) composed of the essential amino acids methionine, phenylalanine, threonine, leucine, valine, histidine, isoleucine, lysine, and tryptophan (Aderinola et al., 2018; Granella et al., 2021; Su and Chen, 2020). Therefore, they may be a good source of bioactive peptides. MO leaves protein hydrolysate (MOPH) is hypoglycemic; nevertheless, its full bioactive potential is unknown (Paula et al., 2017).

For this reason, this study aimed to evaluate the antioxidant and anti-inflammation activities of MOPH obtained by *in vitro* gastrointestinal digestion. The antioxidant activity of MOPH was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and Oxygen Radical Absorbance Capacity (ORAC) assays. RAW 264.7 macrophages were stimulated with lipopolysaccharides (LPS), and the effect of MOPH over nitric oxide (NO) production was measured to assess the anti-inflammatory effect. Furthermore, novel peptides were identified within MOPH by nanoscale liquid chromatography-coupled to tandem mass spectrometry (nano LC-MS/MS).

2. Material and methods

2.1. Chemical reagents

Protein extraction, hydrolysis, hydrolysis degree, antioxidant assays, peptide identification, and Griess reagents: pepsin, tris (hydroxymethyl)aminomethane hydrochloride (TRIS-HCl), NaCl, poly (vinylpolypyrrolidone) (PVPP), phenylmethanesulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), ammonium sulfate, pepsin, HCl, $NaHCO_3$, NaOH, pancreatin, phenolphthalein, formaldehyde, ABTS, Trolox, $K_2S_2O_8$, DPPH, fluorescein, 2,2'-azobis-(2-amidino-propane) dihydrochloride (AAPH), K_2HPO_4, KH_2PO_4, H_3PO_4, formic acid, acetonitrile, sulfanilamide, N(1-naphthyl) ethylenediamine dihydrochloride, indomethacin, and LPS were purchased from Sigma-Aldrich (MO, USA). Electrophoresis reagents were purchased from Bio-Rad (CA, USA). Murine macrophage cell line RAW 246.7 was purchased from American Type Culture Collection (VA, USA). Cell culture reagents were purchased from GIBCO (MA, USA). [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) was purchased from Promega Co (WI, USA).

2.2. Plant material

The present investigation was conducted on 20 plants of *Moringa oleifera* Lam. marked from a larger number of plants cultivated in a family-owned Farm of Culiacán, Sinaloa, México (24° 51'37.0" N / 107°12'59,6" W and 86 m above sea level). The farm has sandy-loam soil, which was irrigated at regular intervals as required. The selected plants were three years old, and after harvest, samples were taken into the laboratory. They were first soaked in gentle commercial detergent for 15 min and washed in running tap water, followed by washing them in double-distilled water. Samples were further disinfected with chloeine (150 ppm), and right after, they were double washed in double-distilled water. The fresh young leaves were excised from the plants. After cleaning, leaves were dried at a temperature of 40 °C for 72 h in an Excalibur 3526T food dehydrator (Ca, USA). The dried leaves were milled in a KRUPS Gx41011 coffee grinder (CDMX, Mexico). MO leaves flour was stored at 4 °C in sealed containers until further use.

2.3. Characterization of Moringa oleifera leaves flour

Moisture, ash, crude protein content (N x 6.25), crude fiber, and crude fat were performed according to AOAC official methods 925.10, 923.03, 978.02, 962.09 and 963.15, respectively (AOAC, 1992). Nitrogen free extract was calculated by difference: 100-(% moisture + % ash + % protein + % fiber + % fat).

2.4. Moringa oleifera leaves protein extraction

MO leaves flour protein was extracted in 0.05 M Tris-HCl buffer pH 8.0 containing 2% (W/V) PVPP, 0.15 M NaCl, 0.01 M EDTA, and 0.001 M PMSF, 1:5 (w/v). The solution was agitated at 4 °C for 30 min and subsequently filtered through a cheesecloth layer. The solid retained fraction was discarded, and the filtrate was centrifugated at 10000 rpm and 4 °C for 30 min. The supernatant was precipitated at 90% saturation of $(NH_4)_2SO_4$ at 4 °C for 18 h. The precipitate was centrifugated at 10000 rpm and 4 °C for 30 min. The pellet was recovered and dissolved in double distilled water and dialyzed with a membrane of molecular weight cut-off (MWCO) of 2 kDa against double distilled water at 4 °C. The dialysate was further lyophilized, and MO leaves protein (MOP) was stored at -20 °C until further analyses.

2.5. Digestion of Moringa oleifera leaves protein

The simulated gastrointestinal digestion of MOP was done based on the modified method from Minekus et al. (2014). The protein isolate was suspended in pepsin solution (2000 units per ml) 1:20 (w/v) at 37 °C and pH 3 during 2 h of continuous stirring to simulate passage through the stomach. pH was revised and adjusted with HCl 1 M every 30 min. Afterward, pH was adjusted to 7.0 with NaOH 1 M, and subsequently, $NaHCO_3$ and pancreatin were added at a concentration of 0.1 M and 100 units per ml, respectively, to simulate intestinal fluid. The solution was stirred for 2 h at 37 °C, while pH was revised and adjusted with NaOH 1 M every 30 min until it was placed on ice to stop the reaction. The enzymes were inactivated by heating the hydrolysate for 20 min at 80 °C. Later, the hydrolysate was centrifuged at 4000 rpm, and 37 °C for 20 min and the supernatant was recovered and ultrafiltered with a 5 kDa MWCO to remove enzymes and undigested protein. The permeated was recovered, lyophilized and stored at -20 °C until further analyses.

2.6. Degree of hydrolysis of Moringa oleifera leaves protein after gastrointestinal digestion

The degree of hydrolysis (DH) of the MOP after *in vitro* gastrointestinal digestion was calculated using the relationship between α-amino nitrogen and total nitrogen according to the following equation:

$$DH\ (\%) = \frac{\alpha - amino\ nitrogen}{Total\ nitrogen} \times 100$$

The α-amino nitrogen represents the proportion of nitrogen that was once part of a peptide bond and then is found in the free state; it was quantified by the Sorensen formol titration technique (Silva and Silveira, 2013). Total nitrogen represents bound nitrogen within peptide bonds, as determined by the AOAC official method 978.02 (AOAC, 1995).

2.7. Electrophoretic profile of Moringa oleifera leaves protein and protein hydrolysate

Electrophoretic profile was determined for MOP and MOPH. MOP was dissolved in double distilled water to a concentration of 0.9 mg/mL. This aliquot (aliquot #1A) was subsequently combined with denaturing buffer (1:1) (aliquot #1B). MOPH was dissolved in

double distilled water to a concentration of 10 mg/ml (aliquot #2A). Subsequently, aliquot #2A was combined with denaturing buffer (3:1) (aliquot #2B). The denaturing buffer was prepared with 5% β-mercaptoethanol (v/v), 2% sodium dodecyl sulfate w/v, 10% glycerol v/v, and 0.025% bromophenol blue w/v in 62.5 mM Tris-HCl pH 6.8. 20 μl of both aliquots, #1B and #2B, were boiled at 100 °C for 5 min and loaded on a discontinuous sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (stacking section at 6% and the resolving section at 15%) according to the protocol described by Laemmli (1970) using a Mini electrophoresis cell Protean II from Bio-Rad (CA, USA). Protein separation was carried out at 120 V until the dye reached the lower edge of the gel before staining with Coomassie Brilliant Blue R-250, according to Neuhoff et al. (1985). The gel picture was obtained by using an Axygen gel documentation system BL-Imagen (MA, USA).

2.8. Antioxidant activity of Moringa oleifera leaves protein and protein hydrolysate

ORAC assay: the oxygen radical absorbance capacity was measured for MOP and MOPH. They were dissolved in phosphate buffer according to the protocol developed by Huang et al. (2002). AAPH was used as the peroxyl radical generator and fluorescein as the fluorescent probe. Trolox was used as a standard, and its curve ranged from 6.5 to 125 μM Trolox equivalent (TE)/g. The reaction mixture contained 25 μl of the sample/blank (phosphate buffer)/ Trolox standard curve, 75 μl of 0.8 M AAPH, and 200 μl of 0.106 μM fluorescein. The sample/ blank (phosphate buffer)/ Trolox standard curve and fluorescein were pre-incubated at 37 °C for 15 min. APPH was added to start the reaction. The fluorescence was measured every 70 s for 70 min (485 nm excitation and 580 nm emission) in a Synergy HT multi-detection microplate reader from BioTek (VT, USA). The values were calculated using the regression equation that relates Trolox concentration and the net area under the fluorescein decay curve. The results are expressed as mM TE/g.

DPPH scavenging capacity: DPPH radical scavenging assay was carried out according to Karadag et al. (2009) for MOP and MOPH. They were dissolved in double-distilled water. Briefly, 20 μl of the samples were placed in a 96-well microplate. Then, 280 μl of DPPH was added and left to incubate for 30 min in the dark. Absorbance was measured at 540 nm in an Epoch microplate spectrophotometer from Biotek (VT, USA). Water was used as a blank, and the results were expressed as the percentage of DPPH inhibition at a concentration of 1.33 mg of the sample per ml.

ABTS assay: the assay was carried out according to Thaipong et al. (2006) for MOP and MOPH. They were dissolved in double-distilled water. The ABTS was first dissolved in double distilled water to 7.4 mM. Subsequently, the ABTS stock solution reacted in the dark with a 2.6 mM potassium persulfate solution [1:1, v/v] for 16 h at room temperature. 100 μl of the resulting solution were diluted in 2900 μl of methanol (reaction solution). 15 μl of the samples were placed in a 96-well microplate. Then, 285 μl of the reaction solution was added and left to incubate for 2 h in the dark. Absorbance was measured at 734 nm in an Epoch microplate spectrophotometer from Biotek (VT, USA). Water was used as a blank, and the results were expressed as the percentage of ABTS inhibition at a concentration of 1.33 mg of the sample per ml.

2.9. In vitro anti-inflammatory activity of Moringa oleifera leaves protein hydrolysate

Cell culture: RAW 264.7 macrophages were grown in Dulbecco's Modified Eagles medium (DMEM)/F12 supplemented with 7.5% heat-inactivated fetal bovine serum (FBS) and GlutaMax. No antibiotics were used. RAW 264.7 macrophages were plated and incubated in a humidified atmosphere (5% CO_2 at 37 °C). Cells were subcultured by scraping and seeding them in 75 cm^2 flasks.

Treatment of RAW 264.7 macrophages with LPS: macrophages were plated into 96-well plates at a density of 2.5×10^4 cells per well and incubated for 24 h at 37 °C and 5% CO_2 in 200 μl of DMEM/F12 medium supplemented with 7.5% heat-inactivated FBS. Subsequently, macrophages were incubated for 2 h either with the extracts at various concentrations (1-100 μg/ml), vehicle (dimethyl sulfoxide, 0.5%, V/V) or indomethacin (30 ug/ml.). After that, macrophages were incubated with LPS at a concentration of 10 μg/ml (20 h at 37 °C). Finally, the cell-free supernatants were collected and stored at -20 °C until NO quantification.

Determination of NO production: nitrite was used as an indicator of NO production in the culture medium. It was measured according to the Griess reaction. 50 μl of each cell culture supernatants were mixed with 100 μl of the Griess reagent (50 μl of 0.1% N(1-naphthyl) ethylenediamine dihydrochloride in 2.5% of phosphoric acid and by 50 μl of 1% sulfanilamide) during 10 min at room temperature. Subsequently, the optical density was measured (540 nm) using a microplate reader (Yang et al., 2012). Nitrite concentrations were calculated by comparison with a standard curve of $NaNO_2$ prepared in a fresh culture medium.

Cell viability: macrophages were plated in 96-well plates at a density of 1.2 × 104 cells/well for 24 h. Subsequently, cells were treated with MOPH (0-100 μg/ml) and later incubated for 24 h. MTS assay was used in order to determine cell viability. 20 μl of MTS was added to each well, and macrophages were then incubated (4 h, 5% CO_2 at 37 °C). Optical density was measured on a microplate reader at 490 nm.

2.10. Characterization of Moringa oleifera leaves protein hydrolysate

MOPH peptides identification by nano LC-MS/MS: ultrapure water was prepared from a Millipore purification system (MA, USA). MOPH was dissolved and desalted using Ziptip C18 resin and further lyophilized to near dryness. MOPH was resuspended in 20 μl of 0.1% formic acid before LC-MS/MS analysis. For peptides resolution, the Ultimate 3000 nano UHPLC system (ThermoFisher Scientific, USA) was used; the trapping column was a PepMap C18, 100A, 100 μm × 2 cm, 5 μm and the analytical column a PepMap C18, 100A, 75 μm × 50 cm, 2 μm. The loaded sample amount was 2 μg. Mobile phase A was composed of 0.1% formic acid in water; mobile phase B was composed of 0.1% formic acid in acetonitrile. The LC linear gradient was as follow: from 6% to 9% B for 8 min, from 9% to 14% B for 16 min, from 14% to 30% B for 36 min, from 30% to 40% B for 15 min, and from 40% to 95% B for 3 min, eluting with 95% B for 7 min. The total flow rate was 250 nl/min. For mass spectrometry, the full scan was performed between 300–1,650 m/z at the resolution of 60,000 at 200 m/z, the automatic gain control target for the full scan was set to 3e6. The MS/MS scan was operated in Top 20 mode using the following settings: resolution 15,000 at 200 m/z; automatic gain control target 1e5; maximum injection time 19 ms; normalized collision energy at 28%; isolation window of 1.4 Th; charge state exclusion: unassigned, 1, > 6; dynamic exclusion 30 s. For data analysis, MS raw file was analyzed with PEAKS STUDIO X. The parameters were set as follows: the protein variable modifications were dioxidation (M), oxidation (HW), and deamination (variable); the enzyme specificity was set to as none. The precursor ion mass tolerance was set to 10 ppm, and MS/MS tolerance was 0.05 Da. Only high confident identified peptides were chosen for downstream peptide sequencing analysis.

Peptide structures and physicochemical properties were predicted using the PepDraw tool. The potential biological activity of the peptides was predicted by using the BIOPEP database. Swisstarget algorithm was used to predict targets of the peptides.

2.11. Statistical analysis

Each experiment was conducted in triplicate, and the standard deviations were calculated. Data were reported as mean ± standard

Table 1
Proximate composition of *Moringa oleifera* leaves flour.

Component	Content (%)
Protein	27.77 ± 0.31
Fat	8.09 ± 0.02
Ash	11.49 ± 0.07
Moisture	2.46 ± 0.03
Crude fiber	23.34 ± 0.05
Nitrogen free extract	1.32 ± 0.31

deviations. Data were subjected to analysis of variance (ANOVA) using the statistical software Minitab from MiniTab Inc. (PA, USA). Means were compared using Tukey's multiple comparison test at $p \leq 0.05$.

3. Results

3.1. Characterization of Moringa oleifera leaves flour

The proximate composition of MO leaves flour is presented in Table 1. The main component was protein, while moisture showed the lowest content.

3.2. Degree of hydrolysis of Moringa oleifera leaves protein

The DH for MOP after enzymatic digestion has not been reported before. The DH of MOP by Sorensen formol titration technique and the AOAC total nitrogen official method was 3.53 ± 0.29%. This result confirms that MOP underwent hydrolysis by pancreatin and pepsin.

3.3. Electrophoretic profile of Moringa oleifera leaves protein and of Moringa oleifera leaves protein hydrolysate

Electrophoresis was performed to identify the characteristic protein pattern of MOP and determine whether peptides were produced after the hydrolysis process. SDS-PAGE analysis revealed that MOP is composed of several protein bands (Fig. 1a). The majority of them showed a mass of over 20 kDa, and two strong bands between 10 and 15 kDa and 5 and 10 kDa were identified. From what is shown, MOP was susceptible to pepsin and pancreatin digestion (Fig. 1b).

Table 2
Antioxidant activity of *Moringa oleifera* leaves protein, and *Moringa oleifera* leaves protein hydrolysate.

Assay	MOP	MOPH
DPPH (%)*	36.68 ± 4.86[a]	45.70 ± 3.57[a]
ABTS (%)*	67.18 ± 0.67[b]	93.09 ± 0.07[a]
ORAC (mM TE/g)	1.81 ± 0.21[b]	3.27 ± 0.24[a]

* At a concentration of 1.33 mg/ml

3.4. Antioxidant activity of Moringa oleifera leaves protein and of Moringa oleifera leaves protein hydrolysate

The percentage of DDPH and ABTS inhibition and mM of TE/g of MOP and MOPH values are given in Table 2. The results showed that both MOP and MOPH could scavenge ABTS radical cation and exhibited strong ORAC assay activity. However, in both assays, MOPH presented significantly greater antioxidant activity. As for DPPH radical scavenging, no significant differences were found between MOP and MOPH.

3.5. In vitro anti-inflammatory activity of Moringa oleifera leaves protein hydrolysate

3.5.1. Cell viability as affected by Moringa oleifera leaves protein hydrolysate

The potential toxic effect of MOPH was evaluated with the MTT assay. The results are shown in Fig. 2, where the cell viability of RAW264.7 macrophages was not affected by MOPH in any

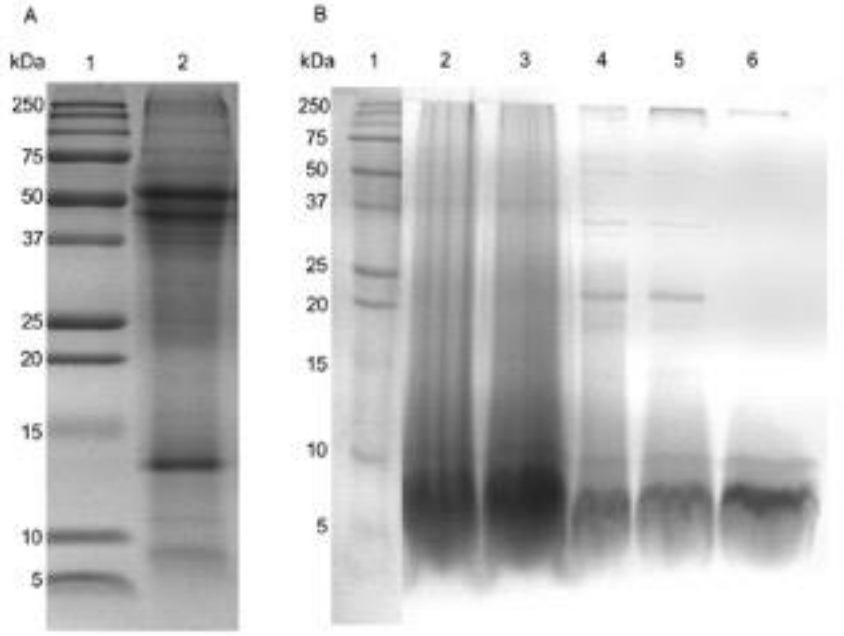

Fig. 1. Assessment of the in vitro digestibility of *Moringa oleifera* leaves protein (MOP) by electrophoresis SDS-PAGE 15%. A. 1) Marker. 2) MOP. B. 1) Marker, 2) MOP after 1 h of pepsin digestion. 3) MOP after 2 h of pepsin digestion. 4) MOP after 2 h of pepsin digestion and 1 h of pancreatin digestion. 5) MOP after 2 h of pepsin digestion and 2 h of pancreatin digestion. 6) ultrafiltered and centrifuged MOP hydrolysate.

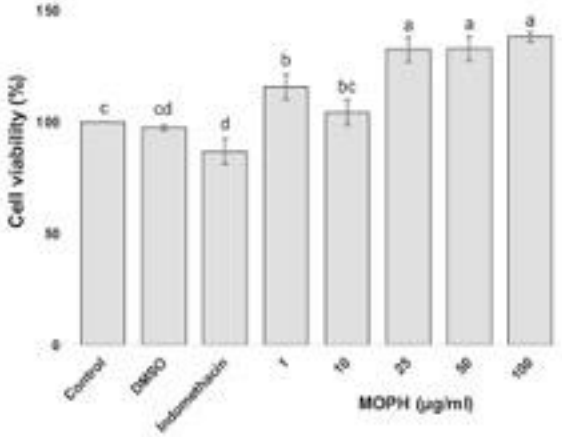

Fig. 2. Effect of *Moringa oleifera* leaves protein hydrolysate on the viability of LPS-stimulated RAW 264.7 cells. The values are expressed as the mean ± SD.

concentration (from 1 to 100 µg/ml). Therefore, these concentrations were used in the subsequent experiment.

3.5.2. Determination of Nitric oxide production by LPS stimulated RAW264.7 macrophages after Moringa oleifera leaves protein hydrolysate exposure

The effect of different concentrations of MOPH (0 to 100 µg/ml) on NO production by LPS stimulated RAW264.7 macrophages is shown in Fig. 3. A minimum amount of NO was produced by macrophages when cultured in medium alone (control). LPS-stimulated macrophages released NO at a level of 31.93 ± 0.27 µM. Only the concentrations of 50 and 100 µg/ml of MOPH significantly reduced by 15.68 and 30.51%, respectively, the NO that resulted from LPS exposure.

3.6. Moringa oleifera leaves protein hydrolysate characterization

The sequences, physicochemical properties, and biological potential of the main peptides present in MOPH are listed in Table 3. The identified sequences of 14 peptides showed a mass of 726.31 to 1520.63 Da, a pI between 3.01 to 9.84, hydrophobicity that ranks between 5.14 to17.14 Kcal * mol $^{-1}$, and a net charge from -2 to +1. The main bioactivities predicted for the identified peptides are angiotensin I-converting enzyme and dipeptidyl peptidase IV inhibitor, as well as an antioxidant. The chemical structures of the 14 peptides are presented in Fig. 4.

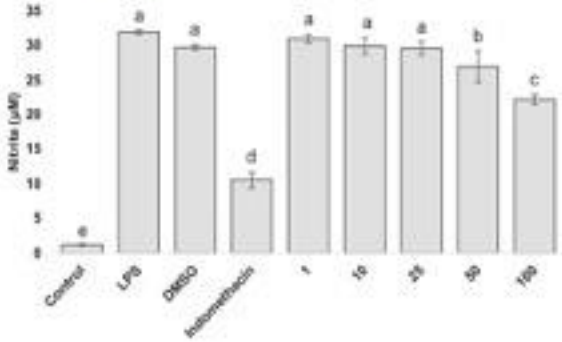

Fig. 3. Effect of *Moringa oleifera* leaves protein hydrolysate on NO production in LPS-stimulated RAW 264.7 cells. The values are expressed as the mean ± SD.

4. Discussion

4.1. Moringa oleifera *leaves proximate composition*

Wide ranges of values have been reported in the literature for the different constituents of MO leaves. These values depend on the solar radiation, humidity, type of soil, harvest time, and geographic area (Firenzuoli and Gori, 2007). Protein, fat, ash, crude fiber, and nitrogen-free extract contents in MO leaves are near or within the range of values previously reported (Alves et al., 2017; Evum and Sanusthan, 2014; Leone et al., 2018; Leone et al., 2016; Su and Chen, 2020; Sultana, 2020). Protein, carbohydrate, high fiber, and low-fat content of MO leaves makes MO leaves flour a great source of nutrients. The ash content in MO leaves flour is considered a measure of the mineral content (Sultana, 2020). As for fiber, it is mainly cellulose with small amounts of lignin (Su and Chen, 2020).

4.2. *Degree of hydrolysis of* Moringa oleifera *leaves protein after pepsin and pancreatin digestion*

The DH is defined as the proportion of cleaved peptide bonds within a protein hydrolysate (El et al., 2015). The degree of hydrolysis by proteolytic enzymes of the gastrointestinal tract usually varies from 3.26 to 36.41% (Silvestre et al., 2013). The value here reported for MOP after pepsin and pancreatin digestion is near the DH reported for other plant sources of protein when using these enzymes, such as lupin, with a value of 3.37% (Schlegel et al., 2019). Other sources, such as whey protein concentrates, showed 2-25% DH by pepsin and trypsin (proteolytic enzymes), depending on the process duration (Kim et al., 2007). Regarding the time used in this research, the result is similar to what has already been reported for digestive enzymes (Schlegel et al., 2019; Kim et al., 2007; Shu et al., 2018). Moreover, when the food is a protein concentrate or isolate, the DH is around 5%, close to the MOPH value (Vidal et al., 2018).

The DH for MOPH is considered low; it may depend upon *Moringa oleifera* leaves protein being poor in certain bonds such as those of trypsin and pepsin cleavage (C-terminal of lysine and arginine and after phenylalanine and leucine, respectively) or that these bonds are unavailable for the digestive enzymes (Silvestre et al., 2013). A low value of the DH indicates protein that is not highly nutritious since it suggests the formation of peptides greater than three amino acids. However, this does not compromise the peptides' bioactivity (Silvestre et al., 2013). For example, high DH does not always translate into increases in the antioxidant capacity of the hydrolysates since hydrolysates with low DH values have shown high antioxidant capacity (Vidal et al., 2018).

4.3. *Electrophoretic profile of* Moringa oleifera *leaves protein and of* Moringa oleifera *leaves protein hydrolysate*

SDS-PAGE analysis showed that protein bands around 50-55 and 10-15 kDa in size were predominant in MOP. These bands may be the constituent of Rubisco, the most abundant soluble protein in chloroplasts, making up to 50% or more of all proteins in plant leaves. Rubisco is composed of eight small subunits of around 14 kDa and eight large subunits of around 56 kDa (Ma et al., 2009). The MOPH electrophoretic profile shows that almost all MOP underwent hydrolysis since its characteristic protein bands disappeared. Also, the centrifugation and ultrafiltration processes removed undigested protein.

4.4. *Antioxidant activity of* Moringa oleifera *leaves protein, and* Moringa oleifera *leaves protein hydrolysate*

MOPH has a higher antioxidant activity than MOP in terms of ABTS, DPPH, and ORAC assays. These suggest that the enzymatic hydrolysis may have caused an increase in exposed amino acid

Table 3
Main peptide sequences in *Moringa oleifera* leaves protein hydrolysate identified by nanoLC-MS/MS.

Sequence	Mass (Da)	pI	HydrophobicityKcal * mol^{-1}	Net Charge	BIOPEP	Swiss Target
1 LAYKPPG	744.42	9.78	+10.67	+1	Dipeptidyl peptidase IV inhibitor and angiotensin-converting enzyme inhibitor	Neurotensin receptor 1: Family A G protein-coupled receptor
2 YHSEVPV	829.40	5.06	+12.83	-1	-	Cystinyl aminopeptidase; protease
3 WPPTPEQPK	1000.46	3.01	+9.17	-1	Antioxidant	HLA class I histocompatibility antigen A-3; Surface antigen
4 LLGFDNR	833.44	6.74	+11.14	0	-	Integrin alpha-IIb / beta-3: Receptor membrane
5 QVWPTPGLK	1020.57	9.84	+9.35	+1	Angiotensin-converting enzyme inhibitor	Angiotensin-converting enzyme; protease
6 FTKDDEWSCTPF	1520.63	3.68	+15.22	-2	-	Neurokinin 1 receptor; Family A G protein-coupled receptor
7 VEQNLVPGLK	1095.63	6.81	+13.82	0	Angiotensin-converting enzyme inhibitor	Cathepsin D: protease
8 TMMLMT	726.31	5.28	+5.14	0	Antioxidant	HLA class I histocompatibility antigen A-3; Surface antigen
9 VQLPGWRVFP	1197.66	11.11	+5.94	+1	Angiotensin-converting enzyme inhibitor	Neuropilin-1 (by homology); Secreted protein
10 SYLPPLSAEVTAK	1374.74	6.57	+13.11	0	Dipeptidyl peptidase IV inhibitor and angiotensin-converting enzyme inhibitor	HLA class I histocompatibility antigen A-3; Surface antigen
11 TMKGPPDTLQ	1086.54	6.46	+15.12	0	Angiotensin-converting enzyme inhibitor	HLA class I histocompatibility antigen A-3; Surface antigen
12 MPWHEQ	826.34	5.06	+12.01	-1	Antioxidant	Gastrin releasing peptide receptor; Family A G protein-coupled receptor
13 LTAPGQATLPT	1068.58	5.36	+9.35	0	Dipeptidyl peptidase IV inhibitor. angiotensin-converting enzyme inhibitor and anticancerogenic	HLA class I histocompatibility antigen A-3; Surface antigen
14 LLTPEGPKE	982.53	4.08	+17.14	-1	-	HLA class I histocompatibility antigen A-3; surface antigen

residues, promoting an increase in scavenging activity (Bamdad et al., 2017). Most antioxidant peptides have been reported to contain a high proportion of hydrophobic amino acids, which is the case with most of the identified peptides (Zou et al., 2016). Hydrophobic amino acids increase peptides solubility in non-polar environments, and therefore a better interaction with free radicals is promoted (Saisavoey et al., 2019). There are different amino acids with known antioxidant activity, such as tyrosine and glycine, which act through hydrogen atom transfer. ORAC is a hydrogen atom transfer-based method that measures peptides' ability to quench peroxyl radical by H-donation (Esfandi et al., 2019; Li et al., 2017). Peptides containing one of these amino acids are 1, 2, 4, 5, 7, 9, 10, 11, 13, 14 (Table 3). As for peptide 1, it contains both. The antioxidant activity of any MOPH has not yet been reported before. As for ORAC, MOPH had a value of 3.27 ± 0.24 mM TE/g. Other plant sources such as defatted peanut meal hydrolysate and hemp seed hydrolysate have shown values of 1.35 and 0.7 mM TE/g, respectively (Zheng et al., 2012; Logarušić et al., 2019).

On the other hand, the amino acids cysteine, tryptophan, histidine, phenylalanine, and tyrosine acts mainly by single electron transfer. DPPH and ABTS are single electron transfer-based methods (Esfandi et al., 2019; Li et al., 2017). Peptides containing one or more of these amino acids are 1, 2, 3, 4, 5, 6, 9, 10 and 12 (Table 3). As for DPPH, MOPH at a concentration of 1.33 mg/ml inhibited in 45.70% the DPPH radical. Similar values have been reported for other plant sources such as oat, barley, and tomato hydrolysates, which DPPH radical quenching at the same concentration was 46.55, 53.2 and 33.25%, respectively (Bamdad and Chen, 2013; Bamdad et al., 2011; Esfandi et al., 2019; Meshginfar et al., 2018; Tsopmo et al., 2010).

As for ABTS radical scavenging activity, MOPH had a value of 93.09%. Hydrolysates for other plant sources such as sorghum and gluten meal for the same amount of hydrolysate had ABTS radical scavenging activity values of 66 and 93%, respectively (Xu et al., 2019) (Hu et al., 2020).

4.5. Anti-inflammatory activity of Moringa oleifera leaves protein hydrolysate

4.5.1. Cell viability as affected by Moringa oleifera leaves protein hydrolysate

Activated macrophages are important for humoral immunity as they remove necrotic debris and cells via phagocytosis to alleviate an injury or kill a pathogen (Hirayama et al., 2018). MOPH hydrolysate showed no cytotoxicity in LPS-induced RAW 264.7 macrophages. Instead, they promoted their growth. As for this, peptides have been considered low or nontoxic molecules since their degradation products are amino acids, which may be used as nutrients by the cells, promoting their growth (Avilés-Gaxiola et al., 2020; Gupta et al., 2013).

4.5.2. Nitric oxide production as affected by Moringa oleifera leaves protein hydrolysate

Inhibition of NO production is important in the therapeutic management of inflammatory diseases because it is an inflammatory response to oxidative stress by cells such as macrophages, endothelial cells, and neurons. When it is overproduced, it may play a critical role in diseases such as neurodegenerative disorders and diabetes mellitus due to DNA damage (Sharma et al., 2007). NO production was increased when macrophages were stimulated with LPS. MOPH in the higher concentration (100 μg/ml) significantly diminished NO production. A molecule that reduces NO production can suppress oxidative signaling and alleviate inflammatory response (Bamdad et al., 2017). Other peptides have been shown to inhibit NO by directly scavenging and modulating cellular inflammatory pathways (Saisavoey et al., 2019). One of the amino acids associated with a NO inhibitory effect is arginine, which inhibits nitric oxide synthase (Vitecek et al., 2012). Peptides 4 and 9 contain arginine (Table 3). Other amino acids that have been found in common anti-inflammatory peptide structures are leucine and glycine (Saisavoey et al., 2019). At least one of these amino acids was found in peptides 1,4, 5,

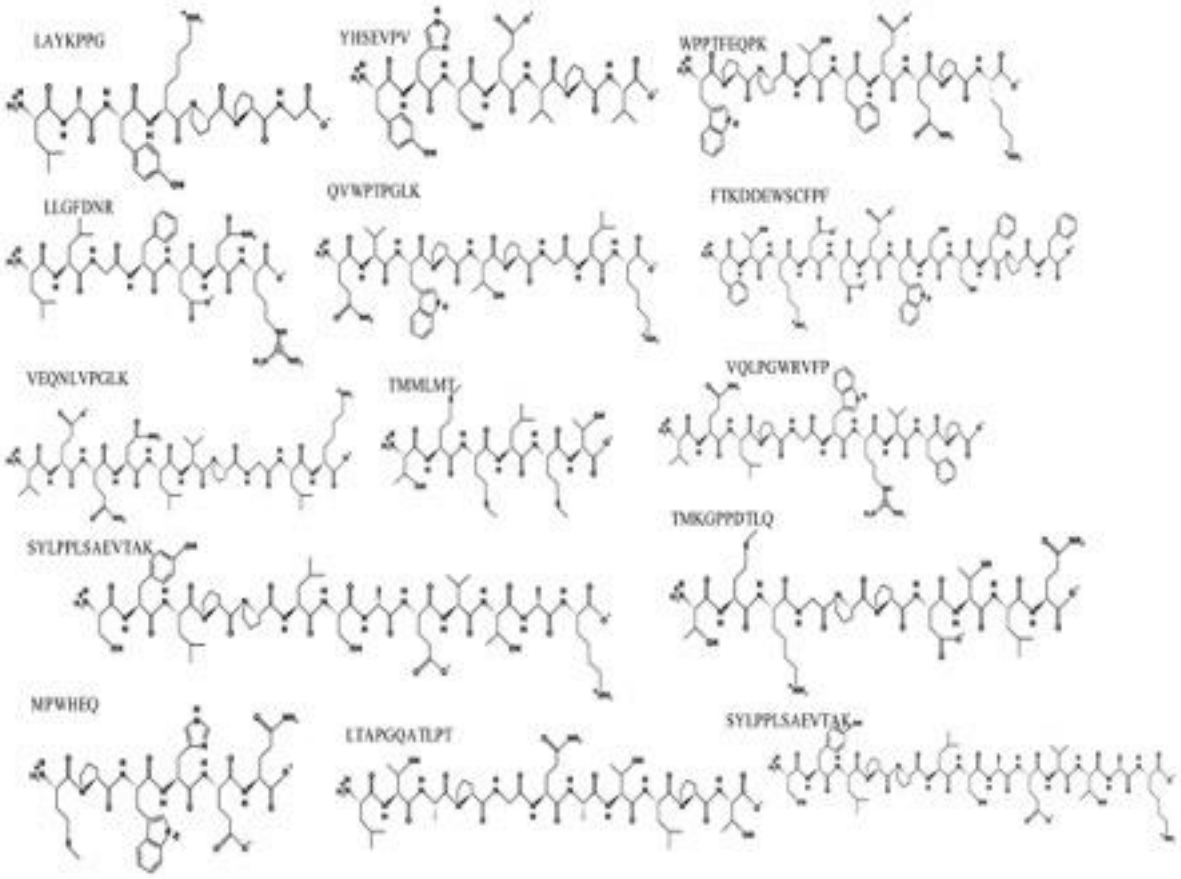

Fig. 4. *Moringa oleifera* peptides chemical structures.

7, 8, 9, 10, 11, 13 and 14 (Table 3); while both of them were detected in peptides 1, 4, 5, 7, 9, 11, 13 and 14 (Table 3).

4.6. *Moringa oleifera* leaves protein hydrolysate characterization

The sequence of peptides from MO leaves had not been previously reported. The analysis of the main peptides of the MOPH using the BIOPEP database showed the presence of angiotensin I-converting enzyme and dipeptidyl peptidase IV inhibitory peptide sequences. These peptides could be further evaluated for their preventive potential against chronic diseases. The presence of amino acids associated with antioxidant and anti-inflammatory activity was expected; this is widely described in sections 4.4 and 4.5.2.

5. Conclusions

MO leaves are a source of bioactive compounds such as peptides produced by enzymatic hydrolysis. The present study demonstrated, for the first time, the antioxidant and anti-inflammatory activities of MOPH. With the information reported in this article, it can be concluded that the MOPH obtained by the use of digestive enzymes may be potentially used as foods/nutraceuticals for improving human health and for treating oxidative and inflammatory-related disorders. However, further *in vitro* and *in vivo* experiments are needed to confirm their benefits and safety before being used by humans.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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4. MORINGA OLEIFERA LEAF PEPTIDES: ANTIOXIDANT AND ANTIPROLIFERATIVE ACTIVITY TO HUMAN COLON CANCER CACO-2 CELL LINE

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4.1. Abstract

Reactive oxygen species are produced as part of cellular metabolism. However, lifestyle can promote an excess in their concentration, generating oxidative stress and damaging biological molecules. In addition, free radicals react with DNA promoting the accumulation of mutations and resulting in the appearance of malignant cells. Therefore, natural antioxidants have been suggested as an alternative to prevent and treat this disease since they modulate cell proliferation processes. Natural antioxidants are classified into different categories, including peptides. Peptides are protein fragments that have been produced from various plants, including the *Moringa oleifera* leaf. In previous work, 14 *Moringa oleifera* leaf peptides with antioxidant potential were produced by *in vitro* gastrointestinal digestion and identified. However, the spectrophotometric methods used to evaluate its antioxidant activity do not fully reflect its potential. In this work, the antioxidant activity of the peptides was assessed by the ferric reducing antioxidant power (FRAP) and cellular antioxidant activity method on the human colon cancer cell line Caco-2. Also, the antiproliferative activity of the peptides was evaluated. Previously, the maximum concentration at which the peptides did not exert a cytotoxic effect on healthy colon cells CCD-18Co was determined. *Moringa oleifera* leaf peptides exhibited a FRAP activity of 1435 µmol TE/g. At a concentration of 500 µg/ml, the peptides did not show a cytotoxic effect on CCD-18Co and decreased cellular oxidant activity and cellular proliferation by up to 71.51 and 90.20% in Caco-2, respectively. Our findings suggest that *Moringa oleifera* leaf peptides released by *in vitro* gastrointestinal digestion could potentially function as preventive agents for this disease, especially due to its cellular antioxidant effect and as a treatment agent due to its ability to inhibit cell proliferation. Thus, these molecules could be an effective component of functional foods addressed for colorectal carcinoma. However, further *in vivo* experiments are needed.

Keywords: *Moringa oleifera*, peptides, hydrolysates, cytoprotective effect, antiproliferative activity, cellular antioxidant activity

4.2. Introduction

Reactive oxygen species (ROS) are produced as a result of cellular metabolism (Krumova y Cosa, 2016). However, the lifestyle of people can promote its increase mainly due to the exposure to environmental pollution and ultraviolet radiation, tobacco and alcohol consumption, and physical inactivity (Aseervatham *et al*., 2013). When the concentration of free radicals exceeds cellular antioxidant systems, homeostasis is affected, and biological molecules are damaged, promoting the appearance of chronic conditions such as inflammatory processes, cardiovascular diseases, neural degeneration, diabetes, and cancer (Sharifi-Rad *et al*., 2020). As for the latter, ROS react with the genetic material of cells inducing somatic mutations that, when accumulated, promote the appearance of malignant cells, so they have been linked to the onset and development of several types of cancer. In this regard, ROS also promotes the underexpression of tumor-suppressor genes and the overexpression of oncogenes (Reza *et al*., 2021). Furthermore, much evidence suggests that the consumption of antioxidants prevents the neoplastic initiation stage, so these molecules have been targeted as an alternative for treating and preventing this disease (Griffiths *et al*., 2016). On the other hand, antioxidants study has been encouraged since medications traditionally used to treat various types of cancer are highly toxic, with documented side effects affecting the quality of life. Also, some cancer cells have acquired resistance to some of these drugs and existing treatments, not being effective enough to reduce the high mortality rate associated with cancer (Nurgali et al., 2018). Regarding the use of antioxidant molecules, natural ones are preferred over chemically synthesized ones. For example, they are easier to obtain, exert low or non-toxic effects, and have built-in chirality, which is difficult to achieve when synthesizing molecules (Loizzo *et al*., 2009). Moreover, it has been widely reported that antioxidants of natural origin modulate cell proliferation and death processes (George y Abrahamse, 2020). Natural antioxidants are obtained from cereals, legumes, fruits, vegetables, etc. and have been classified as phenols, flavonols, tannins, alkaloids, and peptides, among others (Masisi *et al*., 2016).

Peptides are protein fragments containing 2 to 20 amino acids. They are released from proteins by using digestive enzymes or other proteases. Peptides' specific bioactivity depends on their amino acid sequence and length (Karami y Akbari-adergani, 2019). As for its antioxidant activity, they may act as reducing agents, metal chelators, singlet oxygen quenchers, hydrogen donors, etc., and inhibit free radical production enzymes (Pan *et al*., 2020). According to what has been reported in

various studies, peptides with anticancer potential are mainly those that have antioxidant and antiproliferative activity (Yaghoubzadeh *et al*., 2020). The main advantage of these natural antioxidants is that they can directly attack cancer cells without affecting normal ones (Thundimadathil, 2012). Many bioactive peptides with antioxidant and anticancer potential have been produced from various plant materials. For example, *Moringa oleifera* (MO). MO tree is native to the Himalayas and is now widely distributed throughout the world being the African continent the one that most takes advantage of its benefits (Devkota y Bhusal, 2020). MO leaf peptides have shown antidiabetic, antihypertensive, antibacterial, and antifungal potential (Dahot, 1998; Kini *et al*., 2017; Ma *et al*., 2021; Paula *et al*., 2017). The antioxidant effect of MO peptides has also been previously evaluated by DPPH, ABTS, and ORAC (Avilés-Gaxiola *et al*., 2021). However, more methodologies can be used to evaluate antioxidant potential, like the cellular antioxidant activity, which is a biologically representative method (Li *et al*., 2015). This test has been used to predict the antioxidant activity of various molecules of plant origin, but little for hydrolysates. As for the anticancer activity of peptides from MO leaves, nothing is known. The present study is a continuation of previous investigations in which 14 MO leaf peptides were produced, extracted, and identified and showed remarkable antioxidant and anti-inflammatory effects. Because it is important to learn more about the mechanism of action of antioxidant molecules such as peptides in cancer cells, the objective of this work was to evaluate more about the biological properties of peptides obtained from MO leaves. The antioxidant activity of the peptides was assessed by the ferric reducing antioxidant power (FRAP) and cellular antioxidant activity method on the human colon cancer cell line Caco-2. The antiproliferative activity of the peptides was also evaluated in Caco-2. Previously, the maximum concentration at which the peptides did not exert a toxic effect on healthy colon cells CCD-18Co was determined.

4.3. Materials and Methods

4.3.1. Materials

Chemical reagents: Protein extraction, hydrolysis, cellular antioxidant assay, FRAP reagents:

tris(hydroxymethyl)aminomethane hydrochloride (TRIS-HCl), NaCl, poly(vinylpolypyrrolidone) (PVPP), phenylmethanesulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), ammonium sulfate, pepsin, HCl, $NaHCO_3$, NaOH, pancreatin, DCFH, APPH, $AcONa•3H_2O$, 2,4,6-Tri-(2-pyridyl)-s-triazine (TPTZ), TROLOX, and $FeSO_4•7H_2O$ as well as LDH and MTT kits were purchased from Sigma-Aldrich (MO, USA). Cell culture reagents were purchased from GIBCO (MA, USA). Human colorectal adenocarcinoma cells (Caco-2) and human colorectal fibroblast cells (CCD-18Co) were purchased from American Type Culture Collection (ATCC, USA).

Plant material: the present investigation was conducted on 20 plants of *Moringa oleifera* Lam. marked from a larger number of plants cultivated on a family-owned Farm in Culiacan, Sinaloa, Mexico (24°51037.0" N / 107°12059,6" W and 86 m above sea level). The farm has sandy-loam soil, irrigated at regular intervals as required. The selected plants were three years old, and after harvest, samples were taken into the laboratory. They were first soaked in gentle commercial detergent for 15 min and washed in running tap water, followed by double-distilled water. Samples were further disinfected with chlorine (150 ppm), and right after, they were double washed in double-distilled water. The fresh young leaves were excised from the plants. After cleaning, leaves were dried at a temperature of 40 °C for 72 h in an Excalibur 3526T food dehydrator (Ca, USA). The dried leaves were milled in a KRUPS Gx41011 coffee grinder (CDMX, Mexico). MO leaves flour was stored at 4 °C in sealed containers until further use.

4.3.2. *Moringa Oleifera* Leaves Protein Extraction

MO leaves flour protein was extracted in 0.05 M Tris-HCl buffer pH 8.0 containing 2% (W/V) PVPP, 0.15 M NaCl, 0.01 M EDTA, and 0.001 M PMSF, 1:5 (w/v). The solution was agitated at 4 ºC for 30 min and subsequently filtered through a cheesecloth layer. The solid retained fraction was discarded, and the filtrate was centrifugated at 10000 rpm and 4 ºC for 30 min. The supernatant was precipitated at 90% saturation of $(NH_4)_2SO_4$ at 4 ºC for 18 h. The precipitate was centrifugated at 10000 rpm and 4 ºC for 30 min. The pellet was recovered and dissolved in double distilled water and dialyzed with a membrane of molecular weight cut-off (MWCO) of 2 kDa against double distilled water at 4 ºC. The dialysate was further lyophilized, and MO leaves protein (MOP) was

stored at -20 ºC until further analyses.

4.3.3. Digestion of *Moringa Oleifera* Leaves Protein

The simulated gastrointestinal digestion of MOP was done based on the modified method from Minekus *et al*. (2014). The protein isolate was suspended in pepsin solution (2000 units per ml) 1:20 (w/v) at 37 ºC and pH 3 during 2 h of continuous stirring to simulate passage through the stomach. Every 30 min, pH was revised and adjusted with 1 M HCl. Afterward, pH was adjusted to 7.0 with 1 M NaOH, and subsequently, $NaHCO_3$ and pancreatin were added at a concentration of 0.1 M and 100 units per ml, respectively, to simulate intestinal fluid. The solution was stirred for 2 h at 37 ºC, and during this time, pH was revised and adjusted with 1 M NaOH every 30 min. It was placed on ice immediately to stop the reaction, and the enzymes were inactivated by heating the hydrolysate for 20 min at 80 ºC. Later, MOP hydrolysate (MOPH) was centrifuged at 4000 rpm and 37 ºC for 20 min, and the supernatant was recovered and ultrafiltered with a 5 kDa MWCO to remove enzymes and undigested protein. The permeated was recovered, lyophilized, and stored at -20 ºC until further analyses.

4.3.4 Antioxidant Activity of *Moringa Oleifera* Leaves Protein and Protein Hydrolysate by Ferric Reducing Antioxidant Power

Antioxidant activity was evaluated by the FRAP method, with some modifications (Benzie y Strain, 1996). The FRAP solution was made by mixing acetate buffer (400 mM, pH 3.6), TPTZ (20 mM in concentrated HCl) and ferric chloride (60 mM) (10:1:1 v/v/v ratio). For the test, 1 mg of MOP or MOPH was diluted in 1 ml of bi-distilled water. 30 µl of these solutions were placed in each well of the microplate and mixed with 120 µl of the FRAP solution. The microplate was incubated for 4 min at room temperature in the absence of light and mixed at medium speed for 1 min in the microplate reader. The Trolox standard was used, and the reading was made at 593 nm.

Results were expressed as mmol TE/g.

4.3.5. Cytotoxic Effect of *Moringa Oleifera* Leaves Protein and Protein Hydrolysates on Human Colon Fibroblasts

Cell culture: CCD-18Co were grown in DMEM supplemented with 5% heat-inactivated FBS. CCD-18Co were seeded in 75 cm^2 flasks and incubated in a humidified atmosphere (5% CO_2 at 37 ºC).

Cytotoxic effect: The LDH kit, where the amount of lactate dehydrogenase is evaluated, was used. The recommendations of Sigma-Aldrich (MO, USA) were followed. For this assay, CCD-18Co was seeded in a 96-well sterile microplate. Five thousand cells were seeded in each well and were treated with 215, 500, 750, and 1000 µg/ml of MOP or MOPH and incubated for 23 h at 37 ºC and 5% CO_2.

4.3.6. In vitro Antioxidant Activity of *Moringa Oleifera* Leaves Protein and Protein Hydrolysate by Cellular Antioxidant Activity

Cell culture: Caco-2 cells were grown in Dulbecco's Modified Eagle's medium (DMEM)/F12 supplemented with 7.5% heat-inactivated fetal bovine serum (FBS). Caco-2 cells were seeded in 75 cm^2 flasks and incubated in a humidified atmosphere (5% CO_2 at 37 ºC).

Determination of intracellular ROS production: to evaluate the cellular antioxidant activity of MOP and MOPH, the method reported by López-Barrios *et al*. (2016) was performed using Caco-2. 24 h before performing the assay, previously cultured Caco-2 were seeded in a clear-bottom and black-walled 96-well microplate. Sixty thousand cells were seeded in each well. Subsequently, cells were treated with MOP or MOPH at concentrations of 215, 500, 750, and 1000 µg/ml containing DCFH -DA 20 µM. Afterward, cells were incubated for 20 min at 37 ºC and 5% CO_2. The solution was removed, and cells were washed once with PBS. Finally, 100 µl of APPH 500 µM was added to

each well except for negative control and blank. Fluorescence was emitted at 538 nm upon excitation at 485 nm. It was measured every 2 min for 90 min at 37 ºC using a microplate reader. CAA values were calculated using the following equation:

CAA Unit =1-(∫SA/∫CA)

∫SA is the integrated area under the sample fluorescence versus time curve, and ∫CA is the integrated area from the control curve.

4.3.7. Antiproliferative Effect of *Moringa oleifera* Leaves Protein and Protein Hydrolysates on Human Colorectal Adenocarcinoma Cells

Cell culture: Caco-2 cells were grown in Dulbecco's Modified Eagle's medium (DMEM)/F12 supplemented with 7.5% heat-inactivated fetal bovine serum (FBS). Caco-2 cells were seeded in 75 cm^2 flasks and incubated in a humidified atmosphere (5% CO_2 at 37 ºC).

Antiproliferative activity: it was measured by MTT assay according to the supplier's recommendation (Sigma-Aldrich, USA). Caco-2 cells were plated in 96-well sterile microplate at a density of 20 000 cells per well. Concentrations of 250, 500, 750, and 1000 µg/ml of MOP or MOPH were tested on Caco-2 for 24 h at 37 ºC with 5% CO_2. In addition, 250 µM 5-fluorasil was used as the reference drug to compare the effect of the treated cells. The percentage of cell viability was calculated based on the percentage of succinate dehydrogenase activity (SDA) and calculated as follows:

%SDA=[(absorbance control- absorbance sample)/absorbance control]x100

Control was cells and DMEM.

4.3.8. Statistical Analysis

Each experiment was conducted in triplicate, and the standard deviations were calculated. Data were reported as mean ± standard deviations. Data were subjected to analysis of variance

(ANOVA) using the statistical software Minitab from MiniTab Inc. (PA, USA). Means were compared using Tukey's multiple comparison test at $p \leq 0.05$.

4.4. Results

4.4.1. Antioxidant Activity of *Moringa Oleifera* Leaves Protein and Protein Hydrolysate by Ferric Reducing Antioxidant Power

The values of the antioxidant potential of MOP and MOPH determined by FRAP are presented in Table 1. MOP was taken into account in this experiment and the following ones since it is considered a positive control. It aims to determine if the hydrolysis process that generates the peptides is responsible for the bioactivity.

4.4.2 Cytotoxic Effect of *Moringa Oleifera* Leaves Protein and Protein Hydrolysates on Human Colon Fibroblasts

The toxicity of MOP and MOPH was tested on CCD-18Co to determine a concentration non-toxic to healthy cells. For this purpose, the CCD18-Co cell line was used (Fig. 1). Fig. 1 shows the mortality of CCD18-Co cells expressed as a percentage. Triton x-100 (TX-100) was used as a positive control, considering that it promotes the maximum release of the enzyme lactate dehydrogenase and, therefore, maximum death rate. On the other hand, CCD18-Co cells grown under the same conditions but without any stimulus (DMEM) were used as a negative control. The percentage of death associated with it is considered the rate of normal cell death. The mortality values of MOPH tested doses 250, 500, 750, and 1000 µg/ml in CCD18-Co were 20.32 ± 0.69, 19.35 ± 0.46, 36.44 ± 3.32, and 75.63 ± 0.11 %, respectively. The effect of the first two doses was not statistically different from the negative control. Based on this, it is established that these

concentrations have no adverse effect on healthy cells viability. On the other hand, all MOP tested doses had no adverse effect on CCD18-Co cells viability.

4.4.3. In Vitro Antioxidant Activity of *Moringa Oleifera* Leaves Protein and Protein Hydrolysate by Cellular Antioxidant Activity

The cellular oxidant activity of MOP and MOPH is presented in Fig. 2. When using MOP, the cellular oxidant activity was reduced concentration-dependent. The 250 µg/ml concentration did not reduce the oxidant activity of Caco-2 cells; nevertheless, 500 µg/ml reduced it significantly by 35.10 ± 0 %. On the other hand, MOPH reduced Caco-2 oxidative activity to a greater extent, reducing it by 62.35 ± 0.87 and 71.50 ± .1.77 % at 200 and 500 µg/ml, respectively. Although the change between the doses was not statistically significant, a tendency is observed that as the dose increases, the oxidative activity decreases.

4.4.4. Antiproliferative Effect of *Moringa Oleifera* Leaves Protein and Protein Hydrolysates on Human Colorectal Adenocarcinoma Cells

Cells were treated with 5-FU since it is the most widely used drug for the treatment of colon cancer, and it is known to be an apoptosis inducer. At the tested concentrations, MOP did not affect Caco-2 proliferation. 5-F reduced Caco-2 cellular proliferation by 23.10 ± 11.61 %. MOPH reduced Caco-2 cellular proliferation to a greater extent, reducing it by 78.19 ± 5.43 and 90.20 ± 1.09 % at 200 and 500 µg/ml doses, respectively. Although the change between the doses was not statistically significant, a tendency is observed that as the dose increases, the cellular proliferation decreases.

4.5.1. Antioxidant Activity of *Moringa Oleifera* Leaves Protein and Protein Hydrolysate by Ferric Reducing Antioxidant Power

According to what is reported in Table 1, there is a significant difference between the antioxidant activity of MOP and MOPH, being increased by 57% after the hydrolyzing process. The reason may be that when peptide chains are released from complex proteins, amino acid residues are exposed and can interact with free radicals (Borawska *et al*., 2016). In the particular case of FRAP, this assay measures the ability of molecules to serve as reducing agents by donating electrons to the Fe^{3+}-2,4,6-tripyridyl-S-triazine complex, converting it to a more stable ion, Fe^{2+} (Nwachukwu y Aluko, 2018). It has been established that the amino acids that promote high antioxidant capacity by FRAP are the presence of sulfur-containing amino acids, such as cysteine and methionine. On the other hand, the presence of hydrophobic amino acids in peptide structures, such as isoleucine, proline, glycine, and methionine, contribute to their high electronic density (Nwachukwu y Aluko, 2019).

There is not much information in the literature on determining the antioxidant activity of vegetable hydrolysates using FRAP. Among what has been published, hydrolysates from soybean and rice have antioxidant activities of 15.32 and 18.78 TE µmol/g, respectively (dos Santos Aguilar *et al*., 2018; Figueiredo *et al*., 2019). Compared to these values, the antioxidant activity of MOPH is higher. However, it falls within the values reported for protein hydrolysates of animal origin. As for them, more varied values have been reported ranging from 26.96 for whey protein hydrolysates to 4200 µm TE/g for *Cyprinus carpius* skin hydrolysates (Corrochano *et al*., 2019; Dey y Dora, 2014; Tkaczewska et al., 2020). Nevertheless, the values with greater similarity to those reported here for MOPH are those of catfish by-products with values of up to 906.90 µmol TE/g (Sandoval-Gallardo *et al*., 2020; Vo *et al*., 2017). Based on the above, this methodology confirms the antioxidant activity of the peptides obtained from *Moringa oleifera* leaves and shows another mechanism by which they act.

4.5.2. Cytotoxic Effect of *Moringa Oleifera* Leaves Protein and Protein Hydrolysates on Human Colon Fibroblasts

Conventional drugs used for cancer treatment often have adverse effects on cancer cells and healthy ones (Nurgali *et al*., 2018). Therefore, new molecules should be studied to prevent and treat this disease, causing the least possible damage to the healthy tissues of the human body. In this sense, one of the advantages of peptides is their low toxicity, using a high amount of them. The main reason is that its degradation products are amino acids that can be used as nutrients by cells (Avilés-Gaxiola *et al*., 2020). Also, these peptides are small molecules and have no immunogenicity (Lei *et al*., 2019). The effect of plant-derived protein and peptides has not been widely evaluated in the CCD-18co cell line. Regarding peptides, various concentrations have been reported that have been considered non-toxic for these cells. These concentrations range between 10 and 800 µg/ml for hydrolysates from plants such as *Phaseolus vulgaris* L. and soybean (Garcia-Mora *et al*., 2015; Rayaprolu *et al*., 2017). As for MOPH, it falls within this range (Fig. 1). It has been determined that the toxicity of peptides in healthy mammalian cells depends on the amount that manages to enter them. When peptides are found in high concentrations, they promote the loss of mitochondria membrane potential, which fragments. This phenomenon can also occur with the cell membrane (Slaninová *et al*., 2012). As for this, the number of peptides that enter a cell depends on their composition. The main characteristic is the proportion of hydrophobic amino acids in the peptides since when this is > 30%, their entry into the cells is facilitated (Ma *et al*., 2015). Concerning the peptides identified and reported in our previous research (Avilés-Gaxiola *et al*., 2021), 11 of the 14 sequences meet this characteristic. Regarding proteins, it is established that they are not toxic at high concentrations because their entry into the cells is limited by their size, which slows down the passive diffusion across the plasma membrane, which may be the reason why MOP did not cause CCD-18co toxicity at any of the concentrations tested (Tripathi *et al*., 2018).

4.5.3. Determination of Intracellular ROS Production in Human Colorectal Adenocarcinoma Cells

Chemical assays are often used to determine the antioxidant activity of various molecules. However, these sometimes do not consider highly important aspects such as cellular absorption. In this regard, cell assays are of great significance (Shahidi y Zhong, 2015). As mentioned later, 500 µg/ml of MOP reduced oxidative stress in Caco-2 by 35.10 ± 0 %. At the same amount, MOPH reduced it by 71.50 ± .1.77 %, demonstrating that the hydrolysis process is responsible for this bioactivity. Similar values regarding the reduction of oxidation in Caco-2 have been reported. As for amaranth hydrolysate, 1160 µg/ml led to significant inhibition of ROS, keeping cells in the basal state (Fillería y Tironi, 2021). Other investigations have yielded important but lower values: 1000 µg/ml of soybean hydrolysate decreased ROS level in H_2O_2 stimulated Caco-2 by 22.74%, and 2500 µg/ml of *Phaseolus vulgaris* L. hydrolysates reduced fluorescence to 27% in Caco-2 treated with the free radical generator ABAP (Carrasco-Castilla *et al*., 2012; Zhang *et al*., 2018)

The antioxidant activity of peptides depends on their sequence. The amino acids most closely related to a peptide's antioxidant activity are glutamic acid, glycine, alanine, leucine, and phenylalanine. Glutamic acid is a negative amino acid due to an excess of electrons and has quenching activity on free radicals. Due to this property, it also inhibits metal-mediated oxidation (He *et al*., 2012; Torres-Fuentes *et al*., 2015). Regarding hydrophobic amino acids such as leucine, alanine, and glycine, as mentioned above, they promote the passage of peptides into cells. On the other hand, they contain imidazole rings, which are proton donors. (Zou *et al*., 2016). In particular, leucine reduces Fe^{3+}, and it has a long aliphatic side chain that interacts with acyl chains of fatty acids (Chunkao *et al*., 2020). As for phenylalanine, its aromatic ring functions as a proton donor. Tyrosine phenolic hydroxyl group also acts as a hydrogen donor, being able to quench radicals. This amino acid also scavenges the peroxyl radicals generated during the AAPH assay and has also been associated with high CAA values mainly since it has a higher ability to remove peroxyl radicals compared to other amino acids (Jin *et al*., 2016; Sun *et al*., 2019). It has been reported that peptides containing tyrosine have twice antioxidant activity compared to those not having it in their structure (Yang *et al*., 2018). A total of 3 of the 14 peptides previously identified have this amino acid within their structure (Avilés-Gaxiola *et al*., 2021).

4.5.4. Antiproliferative Effect of *Moringa Oleifera* Leaves Protein and Protein Hydrolysates on Human Colorectal Adenocarcinoma Cells

Plant-derived peptides are among the most recently proposed molecules as an alternative for the treatment or prevention of cancer. These molecules offer various advantages, such as a wide number of sources, few or no side effects, and high specificity and efficiency (Avilés-Gaxiola *et al*., 2020; Guzmán-Rodríguez *et al*., 2015). As for MOPH, this is the first research that reports its effect on cancer cells.

250 µM 5-F reduced Caco-2 cellular proliferation by 23.10 ± 11.61 %. This value is low compared to what has already been reported, mainly because the action of 5-F is time-dependent and begins to affect cell proliferation more strongly after 48 h (Cheah *et al*., 2014). Unlike MOPH, which reduced Caco-2 cellular proliferation to a greater extent, reducing it by 78.19 ± 5.43 and 90.20 ± 1.09 % at 200 and 500 µg/ml doses in 24 h. Chickpea hydrolysate at these concentrations (500 µg/ml) reduced Caco-2 cell proliferation by 45% (Girón-Calle *et al*., 2010). At 300 µg/ml, a soybean peptide reduced by around 70% Caco-2 cell proliferation (Cruz-Huerta *et al*., 2015).

Higher amounts (650 µg/ml) were required for walnut hydrolysates to exert a similar antiproliferative effect on Caco-2 (Ma *et al*., 2015).

Regarding the antiproliferative activity of MOPH, it has been established that this may be due to the size of the peptides that allow them to penetrate cell membranes and interact with oncogenic proteins inside cells and with surface receptors, which can promote the arrest of the cellular cycle (Fosgerau y Hoffmann, 2015). A type of amino acid that influences the antiproliferative capacity of peptides in cancer cells is one with a positive charge. For example, lysine and arginine. Peptides with a net positive charge bind to the membranes of cancer cells (which have a net negative charge), generating pores that affect cell integrity (Wodlej *et al*., 2019). Three of the 14 identified peptides have a net positive charge (Avilés-Gaxiola *et al*., 2021). Other amino acids important in peptides with anticancer activity are proline, histidine, and tryptophan. As for proline, it increases peptides flexibility (Kannan *et al*., 2010). Histidine gives anticancer peptides the possibility to induce cancer cytotoxicity by membrane permeability. As for tryptophan, it has been found to enter cancer cells by endocytic pathway and bind to the major groove of nuclear DNA (Chiangjong *et al*., 2020). 11 of the 14 identified peptides have at least one of these amino acids (Avilés-Gaxiola *et al*., 2021).

MOP did not affect Caco-2 proliferation, which may be associated with protein structures finding it hard to pass through cell membranes, mainly due to their size and polarity. Due to the above, little or no amount manages to reach the interior of the cell (Yang y Hinner, 2015).

4.6. Conclusions

Our findings show that peptides obtained from the protein extracted from *Moringa oleifera* leaves have antioxidant potential that is limited to chemical assays and those involving human cells, specifically cancer cells. In addition, for the first time, their antiproliferative potential in cancer cells is becoming evident, thus opening a space for the investigation of these molecules as preventive or treatment agents in a disease with a great impact on the global mortality rate. The results presented in this research paper are very promising and provide useful information. These molecules could be studied together or separately in different cancer models. On the other hand, they could be included as part of the formulation of different foods to improve the consumer's quality of life. More assays, especially *in vivo* evaluations, are essential to confirm safety and efficacy.

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4.9. Conflict of Interest

The authors declare that they have no conflict of interest.

4.10. Figures and Tables

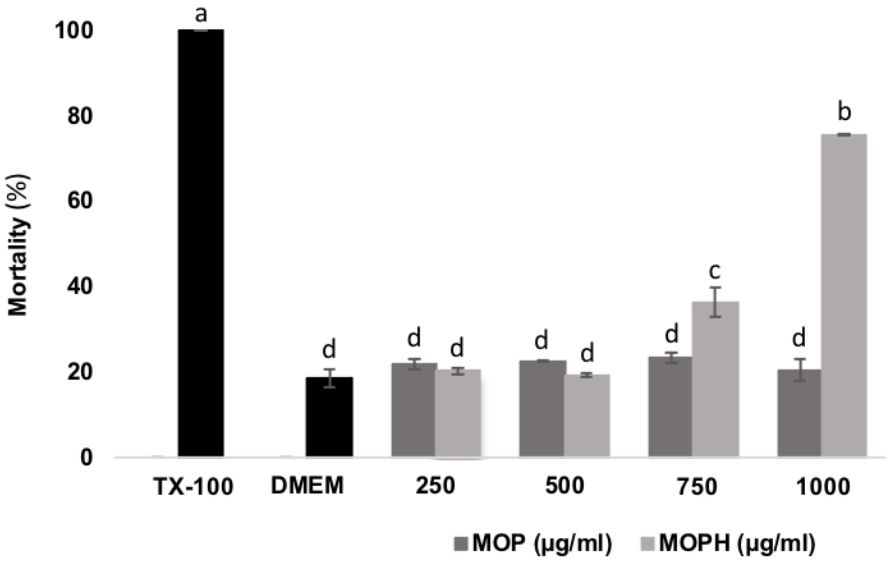

Fig. 1. Cytotoxic effect of *Moringa oleifera* leaf protein (MOP) and protein hydrolysate (MOPH) in human colon fibroblasts (CCD18-Co) expressed as % of mortality. Triton X-100 (TX-100) was used as positive control and cells, and DMEM (DMEM) were CCD18-Co without treatment.

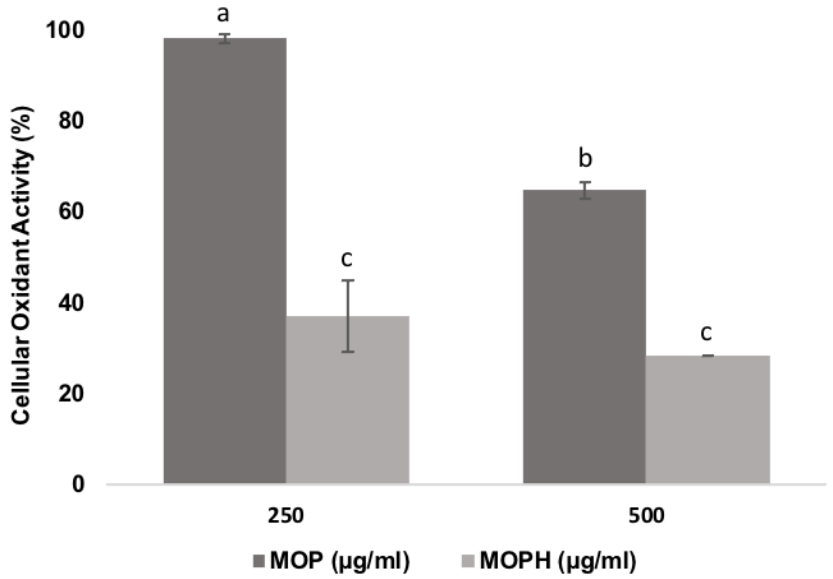

Fig. 2. Cellular oxidant activity as affected by *Moringa oleifera* leaf protein (MOP) and protein hydrolysate (MOPH) in human colorectal adenocarcinoma cells (Caco-2) treated with APPH.

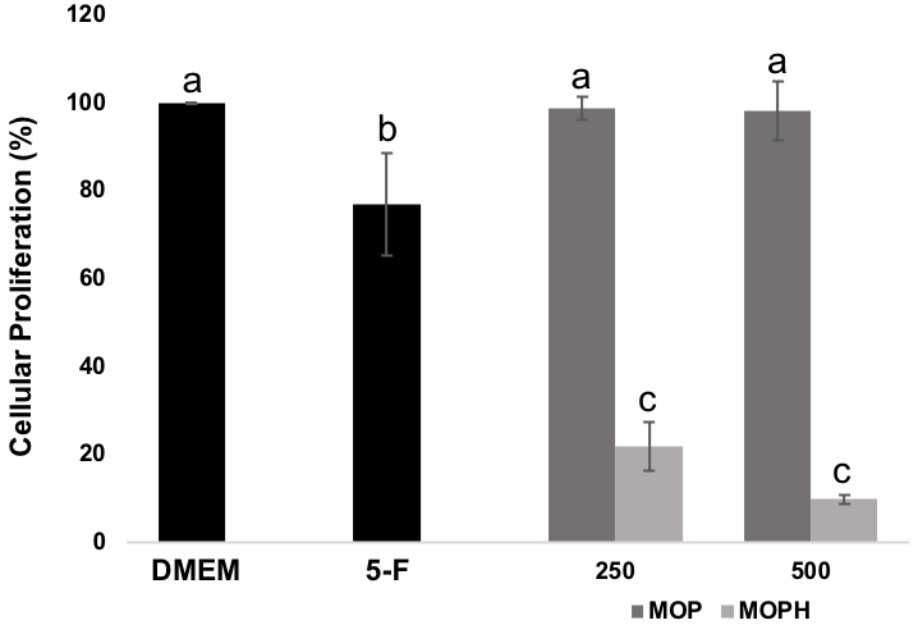

Fig. 3. Determination of the antiproliferative effect of *Moringa oleifera* leaf protein (MOP) and protein hydrolysate (MOPH) in human colorectal adenocarcinoma cells (Caco-2).

Table 1. Antioxidant capacity assessed by the ferric reducing antioxidant power.

Sample	FRAP (μmol TE/g)
MOP	912.5 ± 0.032^{b}
MOPH	1435 ± 0.035^{a}

4.11. References

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5. ANALYZING THE STRUCTURE-FUNCTION RELATIONSHIP OF BIOACTIVE PLANT-PEPTIDES

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Artículo en preparación

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5.1. Abstract

For a long time, different cultures worldwide have attributed particular healing abilities to various plants. After decades of studies, it is now known that the bioactive potential of these plants is associated with the presence of particular molecules. These molecules include short protein fragments, known as peptides. Peptides can be found naturally in plants or generated from plant protein through proteolytic enzymes. In recent years, various biological activities have been associated with plant peptides, and it has been established that there is a relationship between their structure and the particular function they can perform. This review article summarizes the existing information in this regard. In addition, the role of specific amino acids, secondary structure, net charge, and hydrophobicity of plant peptides about their ability to alleviate diseases of global importance is analyzed. Finally, the current scenario and future perspectives of peptide-based drugs are discussed.

Keywords: plant peptides, bioactive peptides, structure, function, diseases, health.

5.2. Introduction

For a long time, it has been established in popular culture that certain foods help alleviate various ailments. However, in recent years the role of specific dietary compounds in preventing these diseases and improving human quality of life has become evident. In recent decades, the search for these molecules has intensified, and the association of their activity to specific mechanisms of action (González, 2020). Peptides are one of the most reported natural compounds with nutraceutical potential. They are linear chains composed by less than 50 amino acids joined by covalent bonds (AA) and released from a more complex protein structure, called the parent protein (Marmiroli y Maestri, 2014). For their size, peptides may be absorbed in the intestine, accessing the bloodstream, allowing these molecules to exert a physiological effect *in vivo*.

They have been found to impact body functions and influence health positively, boosting the

quality of human health, and therefore have been considered bioactive molecules (Singh *et al*., 2014). These molecules have minimal side effects since they are not accumulated in organs, and they have an absence of immunoreactions (Marqus *et al.*, 2017). At the time of writing this review, 4283 bioactive peptides have been reported in the database "Biopep".

Protein rich-food products are ideal for peptide obtaining. As for these, animal-derived foods are the most popular sources. Peptides from foods such as milk, egg, cheese, meat, fish industry by-products, and even bovine blood have been produced (Sánchez y Vázquez, 2017). However, in recent years the study of bioactive peptides has been focused on those coming from protein-rich plants. This is mainly because plant protein production is much more economical and sustainable than animal sources. Also, plants are abundant, and the demand for plant-derived products is increasing, as its consumption is associated with a healthier diet (Marsh *et al*., 2013). Plant peptides have been found to display anti-thrombotic, antimicrobial, antihypertensive, immunomodulatory, antioxidative, and anticancer activities, among others (Sánchez y Vázquez, 2017). In different research articles, it has been established that the activity of these molecules is broadly linked to their conformation. Mainly with its amino acid sequence responsible for many of their properties and, to some extent, with their length, secondary structure, and weight. This review aims to understand the structure-activity relationship of plant peptides based on the current knowledge and analyses carried out by us. This is fundamental in developing therapeutic peptides, representing an innovative, effective, and promising alternative for treating diseases with a high incidence in the current population.

5.3. Plant Peptides Obtaining Methods

While some peptides are naturally occurring, such as lunasin, vglycin, and trypsin inhibitors, others are obtained by chemical or enzymatic hydrolysis and fermentation. Fermentation is currently known to improve the nutraceutical profile of foods by the generation of different compounds, including peptides. Peptides can be produced either by the use of a microorganism or by isolated microorganism proteolytic enzymes (Rajapakse *et al*., 2005). Even though fermentation was an uncontrolled process, these processes have been optimized, being possible for their parameters to

be controlled, including temperature, pH, time, and inoculum size (Lafarga y Hayes, 2017). Lactic acid bacteria are used for plant-based antioxidant peptide production, especially *Lactobacillus planarum*, since it can hydrolyze various proteins producing numerous different oligopeptides (Mechmeche *et al.*, 2017). Soybean is the plant where fermentation is used the most producing peptides by microorganisms such as *Bacillus spp*, *Enterococcus faecium*, *Lactobacillus acidophilus*, *Streptococcus thermophilus*, *Bifidobacterium infantis*, and fungi, mainly *Aspergillus oryzae* (Sanjukta y Rai, 2016).

Proteins enzymatic hydrolysis is the most common and effective way to produce bioactive peptides. There is used an enzyme alone or a combination of enzymes. The most commonly used proteinases are the digestive ones: pepsin, trypsin, chymotrypsin, pancreatic, and pepsin. Enzymes from fungal and bacterial sources are also used, for example, alcalase and plant enzymes such as papain (Bhat *et al*., 2015; Korhonen y Pihlanto, 2006). Each enzyme has a specific cutting site within the protein, and also each one works at a specific temperature and pH conditions. The degree of hydrolysis of each enzyme depends on controllable factors such as hydrolysis time, enzyme concentration, and enzyme combination (Kullman, 2018). Once peptides are produced, it is necessary to separate them from other compounds found in the sample, such as non or poorly hydrolyzed proteins. Many efforts have been made to develop methods for the isolation, purification, and identification of peptides. The main way to separate them is by using ultrafiltration. Whenever the fractionation of very small peptides is needed, chromatography techniques are used and coupled to mass spectrometry when the goal is also to identify them (Ortiz-Martinez *et al*., 2017). Liquid chromatography is one of the most widely used methods for separate and characterizes peptides since it is coupled with a UV detector. Peptide bonds absorb UV light between 210 and 220 nm, while aromatic amino acids do it between 250 to 290 nm (Léonil *et al*., 2000). MS/MS is now considered a routine technique for peptide amino acid sequence identification by producing ions and separating them by mass-to-charge ratio (Taylor y Johnson, 2001). The ionization techniques most used for peptide characterization in MS/MS are electrospray ionization ESI and matrix-assisted laser desorption/ionization (MALDI) (Nadler *et al*., 2017). Time-of-flight, traditionally coupled to MALDI, is the most employed mass analyzer for peptide identification (Chui *et al*., 2015). Peptides with an unknown sequence are analyzed by Edman degradation, capable of determining the sequence of peptides composed of up to 60 amino acids (Restrepo-Pérez *et al*., 2018). The identification of novel bioactive peptides and the association between their structure

and function is thanks to work done to develop these techniques.

5.4. Structure-Function Relationship of Bioactive Plant-Peptides

5.4.1. Relation Between Plant-Peptides Structure and its Antioxidant Activity

Relation between plant-peptides structure and its antioxidant activity Oxidative stress is defined as an imbalance between oxidants and antioxidants, being the oxidants in a higher concentration. This phenomenon breaks redox signaling, promoting cellular damage (Sies, 2020). Reactive oxygen such as O_2^-, HO_2, H_2O_2, and OH damage biomolecules such as DNA, protein, and lipids, and when it cannot be repaired, it leads to chronic diseases (Thanan *et al*., 2015). So, in the past few decades, a great interest has been focused on antioxidants, especially those of natural origin (Lourenço *et al*., 2019). Plant peptides are among the most studied natural antioxidant molecules becoming a hot research area. From 2010 to date, 72 research articles evaluating the antioxidant potential of plant-peptides have been published. Among them, 43 have identified their sequences. The main methods used to determine the antioxidant activity of peptides are chemical-based iron chelating activity, ABTS, and DPPH.

Nevertheless, since these methods cannot determine the peptides performance *in vivo*, other methods have been developed, such as the cellular antioxidant activity assay, using various cells (Pisoschi *et al*., 2016). After our analysis, we determine that the amino acids that repeat the most among antioxidant plant peptides are glutamic acid, glycine, alanine, leucine, and phenylalanine. Glutamic acid is a negatively charged amino acid, and since it has an excess of electrons, it has free radical quenching activity. Also, due to their charged residues, it chelates metals and inhibits metal-mediated oxidation (He *et al*., 2012; Torres-Fuentes *et al*., 2015). Glycine, alanine, and leucine are hydrophobic amino acids. Hydrophobic amino acids have been reported before as a key factor for peptides to scavenge radicals since they are the ones that allow the antioxidant peptide to enter cells through interactions with the lipidic membrane. On the other hand, they contain imidazole rings, which are proton donors (Zou *et al*., 2016). Also, not only hydrophobic amino

acid presence is important, but its location within the peptide. When they are in the C-terminal and N-terminal regions, antioxidant activity is greater, especially since it increases the solubility of the peptides in lipid systems, such as the lipidic membrane; hence the peroxyl radical-mediated cell membrane oxidation is prevented. As for the hydrophobic amino acid leucine particularly promotes plant peptides' ability to reduce Fe_3^+, especially when located at the N and C-terminal, which also increases DPPH radical scavenging activity. Leucine also contributes to antioxidant activity with its long aliphatic side chain interacting with acyl chains of fatty acids (Chunkao *et al*., 2020). Regarding to phenylalanine, it has been reported that this aromatic amino acid, provides protons from its benzene group, acting directly as a radical scavenger and promoting high values on the assays ABTS and ORAC (Selamassakul *et al*., 2018). In addition to the amino acids found to be more abundant, others contribute to the antioxidant activity of the peptides. For example, tyrosine. It contains a phenolic hydroxyl group, which acts as a hydrogen atom donor, providing protons to electron-deficient free radicals and quench radicals such as DPPH. Also, the most effective position for tyrosine has been reported in C-terminal. This amino acid also scavenges the peroxyl radicals generated during the AAPH assay and has also been associated with high CAA values mainly since it has a higher ability to remove peroxyl radicals than other amino acids. It has been reported that peptides containing tyrosine, have twice antioxidant activity than those not having it in their structure (Yang *et al*., 2018). Cysteine is another important amino acid since it directly interacts with free radicals because of its thiol group, which protects cellular molecules from oxidation with high TEAC assay values. The sulfur-containing amino acids, methionine, and cysteine have a nucleophilic character and have been found to contribute to peptides scavenging activity by sulfur hydrogen donation (Zhang *et al*., 2014). Nevertheless, since plant protein is deficient in these amino acids, they are not commonly found in their derived peptides (Jiang *et al*., 2018). Another amino acid recognized for its antioxidant activity is histidine, a radical scavenger due to its imidazole group proton-donating ability (Jiang *et al*., 2018). Histidine contribution to peptides antioxidant activity is higher when it is located in the C-terminus position. Finally, valine at the n-terminal has been related to enhancing antioxidant peptides activity in oil systems (Ghribi *et al*., 2015).

Besides particular amino acids interacting with free radicals, some particular peptides may influence gene expression, such as SOD-3, which is the case for the peptide FDPAL obtained from soybean (Ma *et al*., 2016). In addition, other soy peptides were able to activate the Keap1/Nrf2 pathway, also increasing the expression of antioxidant and phase II enzymes such as SOD1, TrxR1,

NQO1, and GR (Tonolo *et al*., 2020).

A relationship between peptides having a secondary structure and their antioxidant activity has been established, being higher with a lower random coil content (Yang *et al*., 2017). Also, lower α-helix content has been reported to be connected with increased antioxidant capacity (Jiang *et al*., 2018). As for the length of antioxidant plant peptides have been reported to have from 2 to 21 amino acids. We found that up to 66% of the identified antioxidant plant peptides have from 2 to 10 amino acids. This is very convenient because smaller peptides can pass through cell membranes more easily. Also, short peptides may pass through the intestinal barrier and exert their effect in the body. Peptides here reported, have been found not to cause damage to healthy cells, cytotoxic and hemolytic assays have found this. Plant peptides should be utilized as medicinal antioxidants and as ingredients for functional foods to increase antioxidant capabilities and prevent food oxidation reactions increasing shelf life, for example, in high lipid food. Antioxidant plant peptides are an alternative to synthetic antioxidants considered harmful, such as BHA, BHT and TBHQ, which affect spleen, lung and liver (Zhao *et al*., 2020).

5.4.2. Relation Between Plant-Peptides Structure and its Antiproliferative Activity

Cancer is defined as a process that involves an uncontrolled division of body´s cells. It is one of the most relevant health problems globally, one of the main causes of death. The short size of peptides allows them to penetrate cell membranes, and due to their high activity, they can interact with oncogenic proteins inside the cell. Also, they are efficacious signaling molecules that bind to specific cell surface receptors, such as ion channels. All of these actions trigger cell cycle arrest, suppression of cancer cell growth, and invasion by different mechanisms such as cytoplasmic membrane disruption. We determined that the amino acids that repeat the most among plant peptides reported with anti-cancer activity are glutamic acid, leucine, serine, phenylalanine, and alanine. Glutamic acid is a negatively charged amino acid. Negatively charged amino acids have been recognized to have antiproliferative activity on tumor cells (Chiangjong *et al*., 2020). Leucine and alanine are hydrophobic amino acids, which improves the cell penetration of peptides (Schaduangrat *et al*., 2019). As for phenylalanine, peptides containing it have more affinity to

cancer cell membrane since phenylalanine contributes to peptides hydrophobicity (Chiangjong *et al*., 2020). Hydrophobic and hydrophilic amino acids in the C or N-terminal promote an amphiphilic character, which has been reported to be beneficial. While the hydrophobic amino acid is necessary to invade cancer cells membranes, the hydrophilic one gives the peptide stability and high interaction possibility inside the cell (Ma *et al*., 2015). Peptides containing hydrophobic amino acids (> 30%) and also amino acids that give the peptide a positive charge (greater than or equal to 1), for example, lysine and arginine, bind to the membranes of cancer cells, which have a net negative charge associated with the presence of the phosphatidylserine phospholipid, which accounts for 9% of the total amount of phospholipids in the human cell membrane. Normally, this phospholipid faces the cytoplasm, but it goes to the outer part of the membrane in cancer cells, giving them a net negative charge. Once attached to the membrane, peptides with these features open pores due to electrostatic interactions, promoting intracellular components leakage, causing necrosis. Regarding non-cationic peptides, and with the presence of hydrophobic amino acids, they have the possibility of entering the interior of the cell and interact with cytoplasmic regulatory proteins. Other amino acids important in peptides with anticancer activity are proline, histidine, tryptophan, and glycine. As for proline, its presence gives more accessibility to solvent and increases peptides flexibility (Kannan *et al*., 2010). Histidine gives anticancer peptides the possibility to induce cancer cytotoxicity by membrane permeability. As for tryptophan it has been found to enter cancer cells by endocytic pathway and bind to the major groove of nuclear DNA (Chiangjong *et al*., 2020). On the other hand, glycine-rich peptides have been found to stimulate NK cells activation, enhancing antitumor activity in both animal and human models, although the exact mechanism has not been established (Xie *et al*., 2020).

Amino acids described in the antioxidant section are also important in the structure of anticancer peptides since they prevent the cells from being in an oxidative stress state and play the role of anti-tumor (Wang *et al*., 2016). As for the secondary structure, β-pleated sheet peptides usually have two or even more disulfide bonds and therefore are more stable than α-helical peptides (Xie *et al*., 2020). As for the length of the identified anticancer plant peptides they are from 3 to 158 amino acids. Nevertheless, we have determined that around 63% of the identified anticancer plant peptides have from 3 to 10 amino acids. Small peptides have greater molecular mobility compared to those of a greater length, and then they have high diffusivity across membranes and higher probability of interacting with cancer cell molecules inside the cells. Peptides with a mass greater

than 20 kD have been found to possess less cytotoxic effect over cancer cells.

Different mechanisms have been associated with plant peptides such as mitochondrial apoptotic pathway (rapeseed, sweet potato, pecan, soybean, and maize peptides) and autophagic cell death (walnut peptides). On the other hand, been peptides specifically affected gene expression on colon cancer cells HCT116 and RKO, mainly they upregulated transcriptionally activated genes that encode for antioxidant enzymes related to NRF-2, associated to cancer prevention. Also it affected genes involved in MAPK signaling, having a role in proliferation and apoptosis (Vital *et al*., 2014). Been peptides also promoted DNA damage by PARP cleavage and cell cycle arrest by nuclear translocation of p53 (Luna-Vital *et al*., 2016). Another reported mechanism is for *Pombalia calceolaria* peptides which inhibited cancer cells migration (Pinto *et al*., 2018).

5.4.3. Relation Between Plant-Peptides Structure and its ACE-Inhibitory Activity

The feasibility of using bioactive peptides as potential hypotensive drugs depends on their bioavailability and bioactivity, both depending on the peptide structure. The structure of bioactive peptides is conditional to their amino acid sequences, referred by some authors as “cryptides”. Plant bioactive peptides can act as hypotensive drugs by known mechanisms. However, one of the most studied is their capacity to inhibit the angiotensin-converting enzyme. The most studied mechanisms of hypotensive activity are the rennin-angiotensin system and the kinin-nitric oxide system. However, the most well studied in bioactive peptides is the renin-angiotensin system, where the angiotensin-converting enzyme is one of the main protagonists (Kaur *et al*., 2021; Udenigwe y Aluko, 2012). The angiotensin-converting-enzyme, a glycosylated membrane-bound zinc metalloprotease is the main protagonist of the rennin-angiotensin system; in normal conditions, its main metabolic function begins after renin acts on angiotensinogen to angiotensin I, an inactive peptide which is later catalyzed by the ACE to angiotensin II and then binds to vascular wall receptors to cause the contraction of the blood vessels. However, during hypertensive conditions, the abnormal conditions cause the rennin-angiotensin system to function excessively, leading to high levels of angiotensin II, thus causing hypertension (Aluko, 2015). Thus, the angiotensin-converting enzyme inhibition can help lower the blood pressure in hypertensive

patients (Wu *et al*., 2006; Wu *et al*., 2019). Moreover, most recent studies have shown that bioinformatic predictions can help to model peptides with specific bioactivities like antihypertensive biopeptides (Gallego *et al*., 2018).

In this subject, previous studies have already elucidated a correlation between the amino acid sequences and their ACE-inhibitory capacity (Udenigwe y Aluko, 2012). In another related subject, the mode of action of bioactive peptides in the ACE-inhibition is by competition of the peptides with ACE substrate for the union with the enzyme's catalytic site. Nonetheless, this is not always the case. For some bioactive peptides like Leu-Trp and Ile-Tyr, a noncompetitive inhibition has been reported, and in the case of the peptides Ile-Trp and Phe-Tyr as uncompetitive inhibitors also indicates that substitution of a single amino acid can also affect their chemical capacity to interact with the ACE's catalytic site. Moreover, hydrophobic and aliphatic amino acids are necessary for the hypotensive activity (Udenigwe y Aluko, 2012).

Also, quantitative structure-activity relationship methods and datasets have been developed to elucidate the different bioactive properties of bioactive peptides dependent on their structure. These datasets explore the possible structure-activity relationship by the hydrophobicity, bulky properties, electronic characteristics at the sites where the factors affect the ACE-inhibitory capacity of peptides (Bo *et al*., 2021). Amino acids with bulky side chains and hydrophobic side chains in dipeptides have shown ACE-inhibition; also, tripeptides with carboxyl residues like aromatic amino acids with positive charge in the middle of the structure and hydrophobic amino acids have ACE-inhibitory capacity (Wei *et al*., 2021; Wu *et al*., 2006).

Moreover, it is important to mention that unless there is sufficient and significant data regarding the pharmacological potential of bioactive peptides from plant origin, absorption, distribution, metabolism, and excretion studies are important. Unfortunately, this approach is often neglected, whether by instrumental or budget restrictions. Also, studies should focus to mimic physiological conditions and concentrations of peptides, because the aleatory use of peptide concentrations in *in vitro* studies might lead to an overestimation of their antihypertensive potential (Foltz *et al*., 2010; Shen y Matsui, 2017). Thus, an experimental design considering the factors mentioned above is needed for more physiologically relevant results.

5.4.4. Relation Between Plant-Peptides Structure and its Hypolipidemic Activity

The structural and conformational diversity of peptides is responsible for the biological activities they can exert (Görgüç *et al*., 2020; Orona-Tamayo *et al*., 2019). For example, peptides isolated from soybean and lupine have been shown to affect 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR), since they act as competitive inhibitors of the enzymes. This effect is due to characteristics such as number, position and type of residues of amino acids, and peptide hydrophobicity. For example, the proline amino acid has been reported as crucial and the inhibitory activity is higher when it is located within the first and the fourth N-terminal positions and when it is flanked by leucine, valine, phenylalanine, alanine and glycine (Lammi *et al*., 2019). By Molecular docking and *in vitro* studies, lupine peptides (Table **4**) were found to inhibit HMGCoAR, low-density lipoprotein receptor (LDLR) and proprotein convertase subtilisin/kexin type-9 (PCSK9).

As for PCSK9 inhibition, it was reported that the amino acid leucine within the peptide LPKHSDAD was inserted in the hydrophobic pocket of PCSK9, forming a stable peptide- PCSK9 union stabilized by hydrogen bonds (Lammi *et al.*, 2021). Plant peptides containing (Yao, 2018) at least 4 hydrophobic amino acids have a hypocholesterolemic effect. Especially if one of them is located at the C- or N-terminal. Hydrophobic amino acids establish hydrophobic interactions with lipids and with the non-polar molecules of bile acids, that although weak, these interactions promote the reduction of blood cholesterol levels (Chatterjee *et al*., 2018; Karami y Akbari-adergani, 2019; Nagaoka, 2019; Orona-Tamayo *et al*., 2019). On the other hand, peptides with cationic amino acids can also capture bile acids because the cationic residues interact with the carboxyl groups of bile acids. Some plant peptide sequences have been modified to increase their hypocholesterolemic activity by adding polar amino acids like serine residues to the C-terminal, improving their solubility in aqueous systems (Chatterjee *et al*., 2018; García *et al*., 2013). We determined that the amino acids that repeat the most among peptides with hypolipidemic activity are lysine, Threonine, valine, glutamic acid, isoleucine. On the other hand, these peptides are mainly made up of 2 to 20 amino acids.

5.4.5. Relation Between Plant-Peptides Structure and its Potential Hypoglycemic Activity

Diabetes is a degenerative pathology that causes chronic hyperglycemia due to apoptosis of the pancreatic β-cells. If it is not controlled, cardiovascular diseases and other health complications can develop (Meena *et al*., 2020; Moreno-Valdespino *et al*., 2020). Peptides with hypoglycemic activity are characterized by inhibiting carbohydrate metabolism enzymes such as α-amylase, α-glucosidase, and DPP-IV. In addition, they can also act by inhibiting the glucose transporter system and acting as insulin-like molecules (Marya *et al*., 2018; Patil *et al*., 2020).

Particularly in the inhibition of α-glucosidase has been observed that the most potent plant peptides correspond to those containing three to six amino acids, like the N-terminal amino acids with hydroxyl groups or a basic chain. Another outstanding characteristic that has been observed is the presence of proline located in the middle of the peptide sequence and alanine or methionine at the C-terminal since apparently these increase the bioactivity against α-glucosidase. In some investigations, it has been documented that the presence of proline and a basic amino acid such as lysine and arginine can increase the bioactivity against α-glucosidase. Regarding their net charge, peptides having net charges of 0 or +1 are the most effective in the inhibition of α-glucosidase (Ibrahim *et al*., 2018; Rivero-Pino *et al*., 2020; Yan *et al*., 2019). Docking simulation has shown that a sequence of glycine-serine-arginine, inhibits α-glucosidase by attaching to the pocket of the enzyme due to van der Waals forces. The enzymes with which the hydrolysis is carried out play an important role in defining the biological activity of the peptides. Plant hydrolysates obtained with alkaline protease show the highest α-glucosidase inhibition rate (Jiang *et al*., 2018). It is worth mentioning that the most studied peptides inhibitors of α-glucosidase are made up of between 5 and 6 amino acids, being the tetrapeptides the most powerful inhibitors of this enzyme (Acquah *et al*., 2020).

Plant peptides containing proline or alanine residues inhibited DPP-IV enzyme through competitive inhibition for their active site (Castañeda-Pérez *et al*., 2019; Liu *et al*., 2019; Nongonierma y FitzGerald, 2019). Regarding the inhibition of DPP-IV, the amino acids alanine and leucine have the greatest potential to interact with the catalytic site through hydrogen bonds and polar and non-polar interactions (Mojica y De Mejía, 2016; Mudgil *et al*., 2020). Also, peptides with high inhibition of DPP-IV contain isoleucine in the N1 position. This may be because

isoleucine favors the formation of α-helix (Cermeño *et al*., 2019).

On the other hand, amino acids such as serine, threonine, and tyrosine, interact with the α-amylase active site due to their hydroxyl group, inhibiting it. Furthermore, higher α-amylase inhibitory activities have been reported when these amino acids are located at the N-terminal position. On the other hand, proline or alanine near or at the C-terminal position promotes the formation of hydrogen bonds and electrostatic interactions of plant peptides with the catalytic site of α-amylase (González-Montoya *et al*., 2018).. Our literature research found that the reports associate the amino acids glycine, alanine, valine, threonine, and proline were the most abundant amino acids present in peptides with hypoglycemic activity.

5.4.6. Relation Between Plant-Peptides Structure and its Antimicrobial Activity

Medicinal plants used in traditional medicine are attractive sources of bioactive proteins and peptides that demonstrate a broad spectrum of activities, including antimicrobial (Panya *et al*., 2020). Antimicrobial peptides (AMPs) are found in almost all living organisms. Plants can synthesize them during any stage of their life and in nearly all its parts. They come in a variety of forms in terms of sequence, length, and structure. Usually, AMPs are broad-spectrum agents that act on bacteria, fungi, metazoans, and other parasites (Galdiero *et al*., 2013). Most AMPs are short protein fragments composed of about 10 to 50 amino acids and with a net positive charge going from +2 to +11; It has been observed that 50% of AMPs contain hydrophobic residues, which allows them to have a strong affinity to net negative charged microbial membranes (Divyashree *et al*., 2020).

The enormous variety of plant AMPs causes difficulty in their classification. Considering the secondary structures, AMPs are classified into "α" family (with α-helical structures), "β" family (containing β-sheet structures stabilized by disulfide bonds), α-hairpinin (with a motif formed by antiparallel α-helices that are stabilized by 2 disulfide bridges) and "αβ" family (having both "α" and "β" structures) (Santos-Silva *et al*., 2020). On the other hand, also been reported peptides with extended/combined conformation (Scocchi *et al*., 2016).

AMPs are also classified considering their similarity to protein sequence, cysteine motifs, and

distinctive patterns of disulfide bonds, determining the peptide folding. Therefore, they are commonly grouped as thionins, defensins, heveins, knottins, lipid transfer proteins, and cyclotides. Thionins are a family of antimicrobial peptides with low molecular weight (about 5 kDa), rich in arginine, lysine, and cysteine residues. Their structure includes two antiparallel α-helices and an antiparallel double-stranded β-sheet with three or four conserved disulfide linkages. They are positively charged with neutral pH. Their toxic effect was postulated to arise from the lysis of the membranes of attaching cells. However, the precise mechanism underlying toxicity remains unknown (Nawrot *et al*., 2014). Plant defensins consist of three antiparallel β-sheets and an α-helix parallel to them. They possess a variety of biological functions, such as inhibiting microbial growth and inhibiting enzyme activity (Li *et al*., 2021). The hevein family consists of peptides of 29 to 45 amino acids, positively charged, with abundant glycine (6) and cysteine (8-10) residues and aromatic residues, they have a coil-β1-β2-coil-β3 structure that occurs by variations with the secondary structural motif in the presence of turns in 2 long coils in the β3 chain. Heveins domains bind to chitin, which is their primary target, usually their action modes include degradation and disruption of the cell wall and plasma membrane due to its hydrolytic action, causing extravasation of plasma particles, so heveins have good antifungal activity (Santos-Silva *et al*., 2020). Plant knottins contain approximately 30 amino acids. Their antimicrobial activity has been attributed to alterations in functional components of the plasma membrane. The typical structure of knottins involves conserved disulfide bonds between multiple cysteine pairs, forming a cystine knot (Li *et al*., 2021). The lipid transfer proteins (LTPs) family are cationic proteins of approximately 70 and 90 amino acids with eight cysteine residues. They share a defining structural feature, a conserved inner hydrophobic cavity surrounded by α-helices. They bind to a wide range of lipids, including fatty acids, phospholipids, prostaglandin B2, lyso-derivatives, and acyl-coenzyme A. Plant LTPs inhibit bacterial and fungal pathogens' growth by promote pathogen membrane permeabilization (Tam *et al*., 2015). Finally, cyclotides are ultra-stable peptides. They are around 30 amino acids in size and are disulfide-rich peptides from plants that have a head-to-tail cyclic backbone and cystine knot arrangement of three conserved disulfide bonds (Craik y Du, 2017).

Huan (2020) proposed that AMPs can also be classified based on amino acid-rich species, proline-, tryptophan-, arginine-, histidine- and glycine-rich peptides (Huan *et al*., 2020). We observe that the most frequent residues were alanine, arginine, glycine, valine, and cysteine, while alanine, leucine, arginine, glycine, and valine were observed in a greater number of peptides. Furthermore,

in our analysis, we observed an average isoelectric point of 8.5 and an average of hydropathy of -0.15, showing the cationic and hydrophilic nature of most AMPs.

Antimicrobial mechanisms of these small amino acid fragments are as heterogeneous as their structure, and some are based on breaking the membrane to cause lysis of bacterial cells (Rashid *et al*., 2016). Cationic AMPs generally exhibit a balance between hydrophobic and positively charged amino acid residues, allowing them to adopt an amphipathic conformation, allowing greater interaction with negatively charged bacterial membranes, which helps promote their insertion (Bechinger y Gorr, 2017). There are four models of interaction between an AMPs and cell membrane. In (I) Barrel stave model, AMPs insert vertically into the plasma membrane to form transmembrane pores. Here, hydrophobic regions of AMPs align with lipid tails. In (II) Carpet model, peptides are adsorbed parallel into the lipid bilayer to cover the cell surface. Here, peptides disrupt the membrane in a detergent-like manner, breaking the lipid bilayer into a set of separate micelles. (III) Toroidal pore model is an intermediate type between the carpet and the barrel. Finally, (IV) in the disordered toroidal pore model, the pore formation is more random and involves fewer peptides, but additional peptides must stabilize the opening (Huan *et al*., 2020; Li *et al*., 2021; Nawrot *et al*., 2014; Shwaiki *et al*., 2021).

5.4.7. Relation Between Plant-Peptides Structure and its Anti-Viral Activity

Recent evidence highlights that some AMPs may also present activity against a broad range of viruses, thus being called antiviral peptides (AVPs). Several properties may influence the antiviral activity of peptides, such as the topology, amino acid composition, charge, and many other chemical and structural characteristics. The overall biochemical features of AVPs are cationic and amphipathic characteristics and positive net charges. It has been observed that AVPs act primarily by directly inhibiting the viral particle, competing for the receptor on the target cell, and blocking its interaction/adsorption. However, they may act at other levels of the viral cycle as well (Vilas *et al*., 2019). Miscellaneous AVPs target various steps in the viral cycle from receptor binding to replication and may be virucidal. In addition, some peptides can also be translocated into the cell cytoplasm and interact with intracellular targets, interfering with physiological and chemical

functions, such as nucleic acid or protein synthesis. Although most of these AVPs are in the initial stages of scientific study, this field impressively grows (Galdiero *et al*., 2013). We performed with the AVPs a bioinformatic analysis. We observe that the most frequent residues in AVPs were cysteine, proline, glycine, isoleucine, and aspartic acid, while asparagine, proline, aspartic acid, isoleucine, and valine were bear in a greater number of peptides. Likewise, we observed an average isoelectric point of 5.18 and an average of hydropathy of -0.23.

5.5. New Plant Sources of Bioactive Peptides

For a long time, legumes and cereals were the main sources to obtain bioactive peptides. The plant source from which the most bioactive peptides have been obtained are legumes. They represent 27% of the world´s primary agriculture production supplying around 15% of the protein worldwide (Zhu *et al*., 2015). However, obtaining peptides from legumes has become a concern since it has been observed that these peptides may conserve the allergenic sequence of various legume proteins (Belsito *et al.*, 2017). As for cereal proteins, the trend of consuming gluten-free products has diminished their demand, even for gluten-free cereals such as maize, buckwheat, rice, millet, quinoa, etc., since it is known that they can be contaminated with gluten during processing, transportation, and handling (Bustamante *et al*., 2017). For this reason, in recent years, peptides have been obtained from sources such as leaves or fruits, representing greater safety for consumers.

5.6. Current Scenario and Future Perspectives of Peptide-Based Drugs

Currently, the bioactive properties of peptides are often evaluated with non-physiological concentrations, which overestimates the antioxidant, anti-inflammatory, hypotensive, hypolipidemic, antimicrobial, and anti-viral activity. In this sense, we recommend using physiologically active concentrations of peptides and considering the incorporation of absorption, distribution, metabolism, and excretion studies to properly assess the bioavailability of these

molecules. Moreover, the market is increasingly asking for plant-based products, which indicates that the demand for plant-based biopeptides is on the rise.

5.7. Conclusion

Currently, many diseases in the world affect the quality of life of a large population group. Therefore, the search for molecules of natural origin that help to treat and prevent them has been promoted. In this sense, peptides are molecules with great potential against communicable and non-communicable diseases, and in the case of peptides of plant origin, these have become the best option due to the great diversity of sources.

The last years of scientific research have made it possible to relate the bioactive potential of these molecules with their structure. This aspect has become so important that peptide purification and identification is almost a must on any new publication analyzing peptide's biological potential. This is a big step in the medical field focused on peptide design. However, there is still a long way to go.

5.8. Conflict of Interests

THE AUTHORS DECLARE NO CONFLICT OF INTEREST, FINANCIAL OR OTHERWISE.

5.9. References

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6. RESULTADOS Y DISCUSIÓN GENERAL

Tras el proceso de recolección y secado de las hojas de *M. oleifera*, así como la elaboración de harina, se determinó su composición proximal. Este análisis mostró como principal componente a la proteína, conformando el 27.77% de la materia prima. En la literatura se han reportado amplios rangos de valores para los diferentes constituyentes de las hojas de esta planta. Estos valores dependen de la radiación solar, la humedad, el tipo de suelo, la época de cosecha y la zona geográfica. Por otro lado, tras la extracción de proteína, el grado de hidrólisis determinado para la misma fue de 3.53%. El grado de hidrólisis por enzimas proteolíticas del tracto gastrointestinal suele variar de 3.26 a 36.41%. El grado de hidrólisis para la proteína de hoja de *M. oleifera* obtenido se considera bajo; esto indica proteína a partir de la cual en su mayoría se generan péptidos de un tamaño superior a tres aminoácidos. Posteriormente, se determinó el perfil electroforético de la proteína para comprobar su integridad, así como el del hidrolizado para verificar la formación de péptidos. El análisis SDS-PAGE mostró bandas de alrededor de 50-55 y 10-15 kDa de tamaño predominantes en la proteína de la hoja. Estas bandas pueden ser el constituyente de RuBisCo, la proteína soluble más abundante en los cloroplastos. RuBisCo está compuesta por ocho subunidades pequeñas de alrededor de 14 kDa y ocho subunidades grandes de alrededor de 56 kDa. El perfil electroforético del hidrolizado muestra la generación de péptidos. Además, los procesos de centrifugación y ultrafiltración eliminaron la proteína no digerida.

Los péptidos presentes en el hidrolizado fueron identificados mediante nanoHPLC-MS/MS obteniéndose las secuencias: 1) LAYKPPG, 2) YHSEVPV, 3) WPPTFEQPK, 4) LLGFDNR, 5) QVWPTPGLK, 6) FTKDDEWSCFPF, 7) VEQNLVPGLK, 8) TMMLMT, 9) VQLPGWRVFP, 10) SYLPPLSAEVTAK, 11) TMKGPPDTLQ, 12) MPWHEQ, 13) LTAPGQATLPT, 14) LLTPEGPKE. El análisis de los péptidos utilizando la base de datos BIOPEP mostró su potencial como inhibidores enzimáticos, como la enzima convertidora de angiotensina I, y principalmente como antioxidantes. Una vez identificados los péptidos presentes en el hidrolizado, se evaluó su potencial antioxidante. Las técnicas de DPPH y ABTS mostraron una actividad de inhibición de estos radicales de 45.70 y 93.09%, respectivamente, a una concentración de 1.33 mg/ml. Valores similares han sido reportados para otras fuentes vegetales por el método de DPPH como los hidrolizados de avena, cebada y tomate, cuya inhibición del radical a la misma concentración fue

de 46.55, 53.2 y 33.25%, respectivamente. En cuanto a ABTS, los hidrolizados de otras fuentes vegetales como el sorgo y la harina de gluten para la misma cantidad de hidrolizado tenían valores de actividad de eliminación del radical de 66 y 93%, respectivamente. La prueba de ORAC mostró una actividad antioxidante de 3.27 mM TE/g. Otras fuentes vegetales como el hidrolizado de harina de maní desgrasada y el hidrolizado de semilla de cáñamo han mostrado valores de 1,35 y 0,7 mM TE/g, respectivamente. Se ha encontrado que los péptidos antioxidantes contienen una alta proporción de aminoácidos hidrofóbicos, como es el caso de la mayoría de los péptidos identificados en este trabajo. Lo anterior debido a que los aminoácidos hidrofóbicos aumentan la solubilidad de los péptidos en ambientes no polares y, por lo tanto, promueven una mejor interacción con los radicales libres. Existen diferentes aminoácidos con conocida actividad antioxidante, como la tirosina y la glicina, que actúan a través de la transferencia de átomos de hidrógeno. ORAC es un método basado en la transferencia de estos átomos. 10 de las secuencias peptídicas identificadas contienen al menos uno de estos aminoácidos. Por otro lado, los aminoácidos cisteína, triptófano, histidina, fenilalanina y tirosina, encontrados en 9 secuencias, actúan principalmente por transferencia de un solo electrón. DPPH y ABTS son métodos basados en este mecanismo. Además de los métodos anteriores, se evaluó la actividad antioxidante por FRAP, donde el hidrolizado exhibió una actividad de 1435 µmol TE/g. Se ha establecido que los aminoácidos que promueven una alta capacidad antioxidante por parte de FRAP son aquellos que contienen azufre, como la cisteína y la metionina. Por otro lado, la presencia de aminoácidos hidrofóbicos en estructuras peptídicas, como la isoleucina, prolina, glicina y metionina, contribuyen a su alta densidad electrónica. Estas metodologías espectrofotométricas, revelan la alta capacidad antioxidante de los péptidos obtenidos a partir de proteína de hoja de *M. oleifera*, con respecto a los valores reportados en la literatura para otras fuentes por lo que la idea de su posible capacidad anticancerígena mediante la inhibición de radicales libres y la prevención del estrés oxidativo, se refuerza. Otra de las bioactividades determinadas, fue el potencial antiinflamatorio del hidrolizado. Los macrófagos estimulados con lipopolisacáridos liberaron óxido nítrico a un nivel de 31.93 µM. El hidrolizado a concentraciones de 50 y 100 µg/ml no fue citotóxico para estas células y redujo significativamente en un 15.68 y un 30.51%, respectivamente, el óxido nítrico resultante de la exposición a lipopolisacáridos. Una molécula que reduce la producción de óxido nítrico puede suprimir la señalización oxidativa y aliviar la respuesta inflamatoria, previniendo el inicio de enfermedades como cáncer de colon. Uno de los aminoácidos

asociados con un efecto inhibidor del óxido nítrico es la arginina, que inhibe óxido nítrico sintasa. Los péptidos 4 y 9 contienen arginina. Otros aminoácidos que se han encontrado en estructuras peptídicas antiinflamatorias comunes son la leucina y la glicina. Al menos uno de estos aminoácidos se encontró en 10 de los péptidos identificados.

Finalmente, se utilizaron células de cáncer colon para evaluar la bioactividad de este hidrolizado. Previamente se determinó la concentración máxima a la que los péptidos no ejercían efecto citotóxico sobre células de colon sanas CCD-18Co que fue de 500 µg/ml. Una vez determinado lo anterior se encontró que el hidrolizado redujo la actividad oxidativa de Caco-2, reduciéndola en 62.35 y 71.50% a las dosis de 200 y 500 µg/ml, respectivamente, y aunque el cambio entre las dosis no fue estadísticamente significativo, se observa una tendencia que a medida que aumenta la dosis disminuye la actividad oxidativa. El grupo hidroxilo fenólico de la tirosina actúa como donante de hidrógeno, pudiendo apagar los radicales. Este aminoácido también elimina los radicales peroxilo y se ha asociado con valores altos de actividad antioxidante celular, principalmente porque tiene una mayor capacidad para eliminar los radicales peroxilo, en comparación con otros. Se ha informado que los péptidos que contienen tirosina tienen el doble de actividad antioxidante en comparación con aquellos que no la tienen en su estructura. Tres de los 14 péptidos previamente identificados tienen este aminoácido dentro de su estructura. Mediante este ensayo, se confirma el potencial antioxidante de los péptidos de hoja de *M. oleifera* y por lo tanto su capacidad preventiva para esta enfermedad.

Por último, el hidrolizado redujo la proliferación celular de Caco-2 en 78.19 y 90.20% a las dosis de 200 y 500 µg/ml en 24 h. A diferencia del 5-fluoracilo, el medicamento utilizado en el tratamiento de esta enfermedad, que en este tiempo solo redujo la proliferación de estas células en 23.10%. Este valor es bajo, principalmente porque la acción del 5-fluoracilo es dependiente del tiempo y comienza a afectar con más fuerza la proliferación celular hasta pasadas las 48 h. En cuanto a la actividad antiproliferativo del hidrolizado, se ha establecido que esta puede deberse al tamaño de los péptidos que les permite penetrar las membranas celulares e interactuar con proteínas oncogénicas en el interior de las células y con receptores de superficie, lo que puede promover la detención del ciclo celular. Un tipo de aminoácido que influye en la capacidad antiproliferativa de los péptidos en las células cancerosas son los que tienen carga positiva. Por ejemplo, lisina y arginina. Los péptidos con carga neta positiva se unen a las membranas de las células cancerosas (que tienen carga neta negativa), generando poros que afectan la integridad celular. Tres de los 14

péptidos identificados tienen carga neta positiva. Otros aminoácidos importantes en los péptidos con actividad anticancerígena son la prolina, la histidina y el triptófano. En cuanto a la prolina, aumenta la flexibilidad de los péptidos. La histidina otorga a los péptidos anticancerígenos la posibilidad de permear la membrana celular. En cuanto al triptófano, se ha descubierto que ingresa a las células cancerosas por vía endocítica y se une al surco principal del ADN nuclear. 11 de los 14 péptidos identificados tienen al menos uno de estos aminoácidos.

7. CONCLUSIONES GENERALES

El árbol de *M. oleifera* ha sido utilizado en diferentes culturas para la prevención y el alivio de un sinfín de padecimientos, en especial la hoja del mismo. Entre una de las enfermedades en las que su potencial se ha estudiado, se encuentra el cáncer. A la hoja se le ha considerado como un agente anticancerígeno en diversos modelos como de colon, hígado y riñón. Sin embargo, hasta el momento el estudio se había enfocado en el efecto de compuestos fenólicos y no todos los componentes de la hoja habían sido considerados. En este sentido, los péptidos, o hidrolizados proteícos, han sido de gran interés en los útlimos años. Para esto, se extrajo proteína de alta pureza y a partir de ella se generaron péptidos mediante un proceso de digestión gastro intestinal *in vitro*. De este proyecto se logró elucidar características importantes de la proteína y el hidrolizado, como su patrón electroforético, el grado de hidrólisis y la secuencia de 14 péptidos identificados y reportados por primera vez. De igual forma, se analizó su potencial bioactivo mediante el uso de diversas bases de datos. En este proyecto se logró determinar que el hidrolizado en conjunto ejerce una actividad antioxidante tanto por ensayos químicos como por ensayo celular. En relación con eso, se observó que el potencial antioxidante es considerado alto comparado con lo reportado en la literatura para otros hidrolizados. Posteriormente se evaluó su efecto anti-inflamatorio descubriéndose que el hidrolizado es capaz de disminuir la concentración de óxido nítrico, radical que actúa de forma perjudicial en los tejidos. En este aspecto, tanto las moléculas antioxidantes como anti-inflamatorias han sido consideradas como compuestos anticancerígenos de gran importancia para la prevención del cáncer de colon. Posteriormente, se evaluó el potencial anti-proliferativo de este hidrolizado en células de cáncer de colon, encontrándose una alta actividad comparado con lo reportado para otros péptidos. Este proyecto muestra que las hojas de *M. oleifera* son una fuente de compuestos bioactivos y se establece por primera vez un estudio que demuestra su actividad anticancerígena mediante la evaluación de las capacicades antioxidante, anti-inflamatoria y anti-proliferativa de los péptidos en un modelo de cáncer de colon. Esto es importante debido a que es necesario validar el verdadero potencial de este árbol considerado como "el árbol de la vida". Con la información reportada en este documento, se puede concluir que el hidrolizado obtenido mediante el uso de enzimas digestivas a partir de hoja de *M. oleifera*, puede utilizarse potencialmente como alimentos/nutracéuticos y en última instancia fármacos para

mejorar la salud y para el tratamiento de enfermedades crónico degenerativas como el cáncer, abriendo así un espacio para la investigación de estas moléculas como agentes preventivos o de tratamiento en una enfermedad con un gran impacto en la tasa de mortalidad global.

8. RECOMENDACIONES

Para futuros proyectos se recomienda evaluar a fondo los mecanismos de acción asociados a la actividad anti proliferativa de los péptidos obtenidos mediante simulación gastrointestinal *in vitro* a partir de proteína de hoja de *M. oleifera*. De igual forma, sería de gran relevancia estudiar de manera individual cada uno de los péptidos presentes en el hidrolizado para su mejor aprovechamiento.