UNIVERSIDAD DE SONORA

DIVISIÓN DE CIENCIAS BIOLÓGICAS Y DE LA SALUD

Departamento de Investigación y Posgrado en Alimentos

Programa de Posgrado en Ciencias y Tecnología de Alimentos

"El saber de mis hijos hará mi grandeza"

ñEstudio de compuestos quimioprotectores de la fracción lipídica del camarón blanco (*Litopenaeus vannamei*): aislamiento, identificación e intervención en el ciclo celular.ò

TESIS

como requisito parcial para obtener el grado de:

DOCTOR EN CIENCIAS DE LOS ALIMENTOS

Presenta:

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Hermosillo, Sonora **Enero de 2015**

APROBACIÓN

ñEstudio de compuestos quimioprotectores de la fracción lipídica del camarón blanco (Litopenaeus vannamei): aislamiento, identificación e intervención en el ciclo celular.ò

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DERECHOS DE AUTOR

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AGRADECIMIENTOS

"Ser agradecido con la vida y con quienes nos ayudan es edificante y cultiva el amor por la vida"
Abel Pérez Rojas. Educador mexicano.

Agradezco infinitamente a la **Universidad de Sonora**, mi casa de estudios por tercera ocasión, siempre seré orgullosamente búho.

A **CONACYT** por la ayuda económica al proyecto de investigación de ciencia básica 107102 del cual se financió este trabajo, así como la ayuda otorgada a través de mi beca.

Al **Departamento de Investigación y Posgrado en Alimentos**, mi segundo hogar por mucho tiempo, muchas gracias a todas las personas que laboran dentro de este departamento por toda la ayuda brindada, tanto académicos como administrativos, especialmente a **Coty**, ayuda incondicional en todo momento; un agradecimiento especial a las personas laborando en los laboratorios de Microbiología, Análisis Generales y Marinos, quienes lograron con su ayuda a que mi trabajo fuera más llevadero.

A mi director de tesis **Dr. Armando Burgos Hernández**, por toda la ayuda y apoyo brindados durante el desarrollo de este trabajo de tesis, que a pesar de los altibajos que se presentaron, las recompensas que se han obtenido han sido invaluables.

A mi comité de tesis integrado por **Dra. Maribel Robles**, apoyo incondicional en momentos difíciles, **Dra. Maribel Plascencia**, ejemplo de trabajo y perseverancia, **Dra. Lorena Machi**, siempre dispuesta a colaborar y **Dr. Carlos Velázquez**, muchas gracias por todos los aportes que hicieron a este trabajo.

Al **Departamento de Ciencias Químico-Biológicas**, en especial al **Dr. Víctor Ocaño** y al **Q.B César Otero,** del laboratorio de investigación en alimentos por toda la ayuda brindada en los momentos más difíciles, así como al laboratorio de **Inmunología y Biología Celular** y de **Productos Naturales**, por facilitar sus instalaciones para sacar adelante este proyecto.

Al laboratorio **SARA** de la **Universidad Veracruzana**, en especial al **Dr. Javier Hernández** por aceptarme para la estancia cuyos resultados son invaluables para este trabajo de investigación.

A mi Hermanita académica **cDr. Miroslava Suárez**, quien me ayudó en todo momento no sólo académicamente sino de manera personal, te admiro mucho y deseo siempre lo mejor para ti.

Un agradecimiento especial a **Dr. Wilfrido Torres**, **Dr. Enrique Márquez** y **Dr. Francisco Rodríguez** por estar siempre dispuestos a ayudar durante los momentos difíciles así como celebrar los triunfos de la investigación.

Un agradecimiento a todas las personas que estuvieron conmigo durante el desarrollo de esta tesis, mi familia académica siempre dispuesta a ayudarme en todo momento. Un agradecimiento a mis compañeros de generación **Joe**, **Néstor**, **José Luis**, **Estrellita**, **Irene**, **Yaeel**, **Anayansi**, juntos pasamos muchos momentos tanto agradables como desagradables, pero cada momento vivido nos ayuda a ser mejores personas y a tratar de salir de las adversidades. Agradezco a mis compañeros del laboratorio de Microbiología, en especial a mi hermanita **Susana** y a **Ana Karenth** por estar siempre a mi lado. Y siempre llevarán un lugar especial en mi corazón los compañeros del laboratorio de análisis de alimentos **Machuy**, ejemplo de actitud positiva, juntas pasamos muchas cosas, pero ella siempre con una sonrisa me ayudó a superar las adversidades. A **Emma**, quien siempre estuvo dispuesta a ayudarme en todo momento, y a **Carretas** y **Nataly** por la ayuda brindada.

Agradezco también a los compañeros del laboratorio de Inmunología celular, **Heriberto**, **Wenceslao**, **Ana Laura**, **Sergio**, **Mario**, por toda la ayuda y consejos brindados, gracias a ustedes salió adelante este trabajo.

Un agradecimiento también a la **Dra. Ana Gloria Villalba** que me acompañó durante el desarrollo de la tesis, dando consejos oportunos en todo momento. Gracias por brindarme tu amistad.

Y por último quiero agradecer a todos mis amigos, que gracias a mi Dios son muchos; mil gracias por ayudarme con su apoyo a salir adelante, a mis **ELMOS**, a los jovenazos del **CBTIS 11**, las chicas **IQôs**, todos mis amiguis **DIPA**. Gracias también a **la Saizada**, que desde lejos siempre me han hecho sentir su amor y su apoyo.

DEDICATORIA

*A **Dios**, mi fuerza y fortaleza*

*A mi Esposo **Ricardo Rendón**, apoyo incondicional durante esta aventura.*

*A mis Padres **María Elena** y **Pedro** y Hermanos **Pedro Alonso** y **Laura Elena**, por ser un ejemplo de vida y los pilares que me sostienen.*

*A mis sobrinas **Jessica** y **Emma**, fuente de luz y de alegría.*

*A mi amiga **María Esther** por siempre estar a mi lado.*

RESUMEN

El camarón es uno de los alimentos de orígen marino más populares a nivel mundial. Se ha reportado que este organismo es una fuente de compuestos quimiopreventivos incluyendo compuestos con actividad antioxidante, antiinflamatoria, antimutagénica, y antiproliferativa. La búsqueda de esta clase de compuestos se ha convertido en una prioridad a nivel mundial ya que el cáncer es la primera causa de muerte. En este estudio, los lípidos del camarón fueron separados por partición en solventes y después fraccionados por medio de HPLC de fase reversa y finalmente por cromatografía de columna abierta con el fin de aislar compuestos antimutagénicos y antiproliferativos.

La actividad antiproliferativa fue establecida midiendo la inhibición del crecimiento de la línea celular murina M12.C3F6 utilizando el ensayo de MTT (3-(4,5-dimetil-2-tiazol)-2,5-difenil-2-H-bromuro tetrazolio). La fracción metanólica mostró la actividad antiproliferativa más alta; esta fracción fue separada en 15 sub-fracciones (M1-M15). Las fracciones M8, M9, M10, M12 y M13 fueron antiproliferativas a una concentración de 100 μg/mL y fueron probadas a concentraciones más bajas. Las fracciones M12 y M13 presentaron la mayor inhibición con IC_{50} de 19.5 ± 8.6 y 34.9 ± 7.3 μg/mL, respectivamente. La fracción M12 fue dividida en tres subfracciones M12a, M12b y M12c. La fracción M12a fue identificada como el compuesto *di*-etil-hexil-ftalato, la fracción M12b como un triglicérido substituído por al menos dos ácidos grasos (predominantemente ácido oléico acompañado con ácido

eicosapentaenóico) y la fracción M12c como otro triglicérido substituído con ácido eicosapentaenóico y ácidos grasos saturados. El triglicérido bioactivo presente en M12c ejerció la actividad antiproliferativa más elevada con una IC_{50} de 11.3 ± 5.6 μg/mL.

La actividad antimutagénica fue evaluada por la inhibición del efecto de aflatoxina B_1 (AFB_1) en las cepas de *Salmonella typhimurium* TA98 y TA100 utilizando la prueba Ames. La fracción metanólica ejerció la actividad más elevada (95.6 y 95.9% para TA98 y TA100, respectivamente); y fue separada en 15 subfracciones (M1-M15). La fracción M8 ejerció la actividad más elevada (96.5 y 101.6% para TA98 y TA100, respectivamente) y esta fracción fue nuevamente dividida en las fracciones nombradas M8a, M8b, M8c y M8d. De estas fracciones, M8a presentó la actividad más elevada con inhibición de 87.8 y 94.1% de la mutación de AFB_1 para las cepas de TA98 y TA100, respectivamente

La actividad biológica en camarón había sido previamente atribuída a astaxantina; en este estudio, se demostró que los ácidos grasos poliinsaturados esterificados en triglicéridos son los principales compuestos responsables de la actividad antiproliferativa, mientras que compuestos con anillos aromáticos son los responsables de la actividad antimutagénica.

ABSTRACT

Shrimp is one of the most popular seafood worldwide, and has been reported as a source of chemopreventive compounds, including compounds with biological activities such as antioxidant, antiinflamatory, antimutagenic, and antiproliferative. The search of chemopreventive compound to prevent cancer has become a priority globally since cancer is the leading cause of death worldwide. In this study, shrimp lipids were separated by solvent partition and further fractionated by semi-preparative RP-HPLC and finally by open column chromatography in order to obtain isolated antimutagenic and antiproliferative compounds

Antiproliferative activity was assessed by inhibition of M12.C3.F6 murine cell growth using the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) assay. The methanolic fraction showed the highest antiproliferative activity; this fraction was separated into 15 different sub-fractions (M1ï M15). Fractions M8, M9, M10, M12, and M13 had antiproliferative effects at 100 ε g/mL and they were further tested at lower concentrations. Fractions M12 and M13 exerted the highest growth inhibition with an IC_{50} of 19.5 ± 8.6 and 34.9 ± 7.3 ε g/mL, respectively. Fraction M12 was further fractionated in three sub-fractions M12a, M12b, and M12c. Fraction M12a was identified as di-ethyl-hexyl-phthalate, fraction M12b as a triglyceride substituted by at least two fatty acids (predominantly oleic acid accompanied with eicosapentaenoic acid) and fraction M12c as another triglyceride substituted with

eicosapentaenoic acid and saturated fatty acids. Bioactive triglyceride contained in M12c exerted the highest antiproliferative activity with an IC_{50} of 11.33 ± 5.6 εg/mL.

The antimutagenic activity was assessed by inhibition of aflatoxin B_1 (AFB_1) effect on TA98 and TA100 *Salmonella* tester strains using the Ames test. Methanolic fraction showed the highest antimutagenic activity (95.6 and 95.9% for TA98 and TA100, respectively); and was further separated into 15 different sub-fractions (M1-M15). Fraction M8 exerted the highest inhibition of AFB_1 mutation (96.5 and 101.6% for TA98 and TA100, respectively) and was further fractionated in four sub-fractions M8a, M8b, M8c, and M8d. Of these, M8a exerted the highest activity with inhibition of 87.84 and 94.18% of AFB_1 mutation for TA98 and TA100, respectively.

Biological activity in shrimp had been previously attributed to astaxanthin; this study demonstrated that polyunsaturated fatty acids esterified to triglycerides are the main compounds responsible for antiproliferative activity, while apocarotenoid compounds are responsible for the antimutagenic activity.

CONTENIDO

INTRODUCCIÓN

El cáncer es una enfermedad crónico-degenerativa que consiste en un proceso de diseminación incontrolado de células, ocupa los primeros lugares de mortalidad y constituye un verdadero problema de salud a nivel mundial (World Health Organization, 2014). En México, en el año del 2012, el 13% de las muertes registradas fueron a causa del cáncer (INEGI, 2014b), lo que lo colocó como el tercer lugar en las causas de mortalidad en nuestro país y en el mismo año; en Sonora esta enfermedad representó el 15.9% del total de decesos, lo cual la coloca la segunda causa de muerte en el estado (INEGI, 2014a).

Siempre se ha pensado que la herencia genética es de los factores determinantes en el desarrollo del cáncer; sin embargo, ésta no marca el destino de una persona, nos puede dar información útil acerca de un riesgo a una enfermedad, pero en la mayoría de los casos no determina que sea la responsable de desarrollarla; de hecho, solo del 5 al 10% de los casos de cáncer se deben a defectos en la genética y el 90 a 95% restante de los casos son debido al ambiente y al estilo de vida (Anand et al., 2008); por ejemplo, se ha estimado que del 30 al 35% de las muertes por cáncer en los Estados Unidos están ligadas a los hábitos alimenticios de las personas. Es por eso que se debe considerar mejorar las dietas y el estilo de vida.

Cada vez está creciendo el interés de enfocarse en componentes de los alimentos que ayuden a la población a reducir el riesgo de desarrollar cáncer (Bhattacharjee, Das, Rana, & Sengupta, 2007). Las nuevas tecnologías e ingeniería genética han creado posibilidades sin límites en el área de los descubrimientos científicos, los desarrollos logrados han aumentado en un número potencial los productos de beneficio médico y para la salud. Entre los componentes más destacados están la fibra dietética, azúcares de baja energía, aminoácidos, ácidos grasos poliinsaturados, fitoesteroles, vitaminas, minerales, antioxidantes y otras sustancias (alimentos funcionales). Dentro de esta inmensa cantidad de compuestos, se localiza un grupo en particular, los quimioprotectores, que son una serie de compuestos y elementos que en conjunto o por sí solos tienen actividad biológica en el humano, específicamente sobre el cáncer (Pelayo-Zaldivar, 2003).

La quimioprevención, como fue definida originalmente por Sporn en 1976, es el uso de agentes químicos naturales, sintéticos o biológicos para revertir, suprimir o prevenir la progresión de cáncer (Tsao, Kim, & Hong, 2004). Se ha demostrado el poder quimioprotector en muchos alimentos como en frutas (Phapale & Misra-Thakur, 2010; Rodríguez-Muñoz, Herrera-Ruiz, Pedraza-Aboytes, & Loarca-Piña, 2009; Zahin, Ahmad, & Aqil, 2010), vegetales (Bhattacharjee et al., 2007; Bárta et al., 2006; J.-H. Kim, Kang, & Jeong, 2003), aceites (Baser, 2008), entre otros. La naturaleza es una fuente atractiva de nuevos compuestos terapéuticos con una gran diversidad de especies de plantas, animales, organismos marinos y microorganismos. El ambiente marino es una fuente prometedora de compuestos

biológicamente activos (Stankevicins, Aiub, Maria, Lobo-Hajdu, & Felzenszwalb, 2008); de hecho, ya se ha demostrado actividad quimioprotectora en músculos de animales marinos como jurel, lisa, cazón (Burgos-Hernandez, Peña-Sarmiento, & Moreno-Ochoa, 2002), pulpo (Moreno-Félix et al., 2013), camarón (Wilson-Sánchez et al. 2010)

Por lo anterior, se planteó en esta investigación aislar compuestos provenientes del músculo de camarón que presenten actividad biológica e identificar sus estructuras químicas.

REVISIÓN BIBLIOGRÁFICA

Características Biológicas y Composición del Camarón

El camarón es uno de los crustáceos más populares a nivel mundial y forma parte tradicional de las dietas en muchos países (Okzus, Ozylmaz, Aktas, Gercek, & Motte, 2009).

Composición Química del Camarón

El camarón blanco (*Litopenaeus vannamei*) es un alimento rico en proteína de alta calidad y minerales beneficiosos para el cuerpo humano; además, su aporte calórico es bajo debido al bajo contenido de grasas (Okzus et al., 2009); sin embargo, su composición puede verse afectada por factores ambientales como la temporada, la profundidad y la localización geográfica de captura (Ezquerra-Brauer, Brignas-Alvarado, Burgos-Hernández, & Rouzaud-Sández, 2004).

El camarón es bajo en el contenido total de grasa (media de 10.9 mg/g de la porción comestible) y muy bajo en ácidos grasos saturados (2.89mg/g de porción comestible) y con una alta cantidad de ácidos grasos ω-3 (3.35 mg/g), de los cuales, los más importantes son el eicosapentaenóico (EPA) (20:5) y el docosahexaenóico (DHA) (22:6) (Gonçalves Abreu et al., 2010).

Actividad Biológica de los Compuestos del Camarón

De los compuestos presentes tanto en el músculo como en el exoesqueleto del camarón, existen algunos componentes que son capaces de intervenir en procesos biológicos, lo cual sugiere la presencia de actividad biológica (Jackson, Esnouf, Winzor, & Duewer, 2007). Dentro de éstos, se ha reportado que existen compuestos en el extracto etanólico del músculo de camarón que presentan actividad antioxidante, esta actividad se ha sido detectada tanto en el camarón crudo como cocido y fue atribuida a un derivado polihidroxilado de un aminoácido aromático (De Rosenzweig-Pasquel & Babbitt, 1991). También se ha reportado actividad biológica en los extractos lipídicos del músculo de camarón (De Rosenzweig-Pasquel & Babbitt, 1991; Wilson-Sanchez et al., 2010) así como en los extractos lipídicos de los deshechos del mismo (Sowmya & Sachindra, 2012).

Por otra parte, en algunos estudios de carcinogénesis, se ha demostrado que las dietas ricas en aceites poliinsaturados, como los presentes en la fracción lipídica del camarón, han tenido un efecto protector contra cáncer de colon inducido químicamente (Boudreau et al., 2001; Kelley et al., 2000). Estos compuestos también suprimen el crecimiento tumoral *in vitro* e *in vivo* por mecanismos aún desconocidos. De acuerdo a estudios epidemiológicos se ha demostrado también una asociación inversa entre el consumo de productos marinos y cáncer de colon, así como muchos otros tipos de cáncer (de Deckere, 1999; Terry, Rohan, & Wolk, 2003).

Por lo tanto, el camarón podría ser considerado como un organismo capaz de aportar compuestos con actividad biológica, los cuáles se sumarían a los compuestos

aislados de fuentes marinas, ya que este ambiente es considerado como una fuente prometedora de compuestos biológicamente activos (Stankevicins et al., 2008).

Cáncer: Características, Estadísticas y Prevención

La participación de compuestos químicos en procesos biológicos, como aquella que se ha observado en compuestos químicos aislados de camarón, al igual que en el resto de aquellos provenientes de otras fuentes marinas, se ha relacionado con la prevención de cáncer.

Definición y Estadísticas de Morbilidad y Mortalidad del cáncer

El cáncer es una enfermedad crónico-degenerativa que consiste en un proceso de diseminación incontrolado de células anormales; y si esta diseminación no es controlada, puede provocar la muerte (American Cancer Society, 2014). Las células cancerosas son capaces de infiltrar y destruir las estructuras adyacentes y esparcirse a lugares distantes (metástasis) (Robbins, Kumar, & Cotran, 2004).

El cáncer ocupa el primer lugar de mortalidad a nivel mundial y constituye un verdadero problema de salud (World Health Organization, 2014). Éste no es solo una enfermedad, sino cientos de ellas, que tienen en común alteraciones genéticas, muchas de las cuales contribuyen a la transformación neoplásica. Dichas alteraciones causan la pérdida de la función correcta de genes encargados del

mantenimiento y control de la división celular; se activan los oncogenes y se pierden aquellos que son supresores de tumores (Brenner & Duggan, 2005).

Características de las Células Cancerígenas

Se deben considerar los genes relacionados con el cáncer en el contexto de seis variaciones fundamentales de la fisiología celular que, en conjunto, son las que determinan el fenotipo maligno (Robbins et al., 2004). Estas características son: Autosuficiencia con respecto a las señales de crecimiento; insensibilidad a las señales inhibidoras del crecimiento; escape de la apoptosis; potencial de multiplicación ilimitado: angiogenia mantenida y la capacidad para infiltrar y metastatizar (Herrera-Fernandez et al., 2009).

Prevención de Cáncer

La prevención del cáncer debe ser una estrategia de salud pública y de prevención que involucra tanto cambios en el estilo de vida como un enfoque médico y esto representaría la primera línea de defensa en la reducción del número de muertes por cáncer (Greenwald, 2005).

La Sociedad Americana del Cáncer (2014) ha declarado que parte de las muertes debidas al desarrollo del cáncer se podrían prevenir realizando esfuerzos

sistemáticos en la reducción del uso de tabaco, mejorando las dietas y la actividad física, reduciendo la obesidad y aumentando las pruebas de detección temprana de la enfermedad (American Cancer Society, 2014).

La mayor parte de lo que se sabe de la prevención del cáncer relacionada con la dieta proviene de estudios epidemiológicos de población acoplado a investigación de componentes alimenticios en animales de laboratorio y cultivo celular (American Cancer Society, 2014). Las recomendaciones para una dieta incluye una con bajo contenido de grasas, específicamente de grasas saturadas, que sea relativamente alta en almidón y en fibra (con énfasis en productos de plantas relativamente poco procesados), y además baja en azúcares. En general, los reportes de los componentes de la dieta son similares a la prevención de otras enfermedades crónicas, además hay que considerar que el consumo de cereales poco procesados, vegetales, frutas, verduras y leguminosas así como alimentos de origen animal, se esperaría que provean los micronutrientes que están relacionados con la prevención del cáncer (World Cancer Research Fund / American Institute for Cancer Research, 2007). Incluyendo esta dieta y específicamente hablando desde un enfoque médico, la prevención del cáncer estaría integrada por un programa de quimioprevención que combine los resultados de investigación básica, genética, ciencia nutrimental, desarrollo de marcadores y ensayos clínicos para determinar aquellos individuos en riesgo de cáncer y desarrollar programas individualizados de prevención. El uso de agentes quimiopreventivos en combinación con una dieta específica y modificaciones

en el estilo de vida proveerá un nivel de individualización que actualmente no es posible (Greenwald, 2005).

Quimioprevención

La quimioprevención se considera parte importante de las estrategias de prevención del cáncer, ya que el proceso de desarrollo de esta enfermedad involucra muchos factores complejos, con una progresión en etapas que lleva a la metástasis, un esparcimiento descontrolado y crecimiento de células cancerosas a través de todo el cuerpo (Pan & Ho, 2008).

Estudios epidemiológicos han proporcionado evidencia convincente de que los extractos o compuestos bioactivos que se encuentran en la naturaleza podrían beneficiar la salud humana a través de la inhibición de procesos carcinogénicos y mecanismos de muerte celular.

Tipos de Actividades Quimioprotectoras

Los compuestos quimioprotectores pueden ser divididos de acuerdo con los efectos benéficos que ofrecen a la salud humana, algunos de ellos son los que ofrecen las actividades, antioxidante, antimutagénica, antiproliferativa, antiinflamatoria y antiangiogénica.

Actividad antioxidante

La primera actividad quimiopreventiva es la antioxidante, ésta es otorgada a los compuestos que, en bajas cantidades, pueden prevenir o retrasar la oxidación, ofreciendo protección contra la oxidación debida principalmente a radicales libres (Chipault, 1962); estos últimos son compuestos caracterizados por una alta reactividad debido a los electrones no apareados del orbital externo, y tienen mecanismos de acción que dañan las células, los tejidos, proteínas, lípidos y DNA (Brambilla et al., 2008). En este contexto, los antioxidantes previenen el daño celular por medio de su reacción con radicales libres oxidantes y promocionando su eliminación del organismo. De esta manera, los antioxidante se asocian con la prevención de ciertos tipos de cáncer (Borek, 2004; Lamson & Brignall, 1999) y pueden ser encontrados en varios alimentos como micro y macro nutrientes (de Lorgeril & Salen, 2006). Los antioxidantes son también importantes en las últimas etapas del desarrollo del cáncer ya que hay evidencia de que los procesos oxidativos promueven la carcinogénesis; por lo tanto, esta clase de compuestos pueden promover la regresión de lesiones pre-malignas e inhibir su desarrollo a cáncer (S. K. Kim, Thomas, & Li, 2011).

Actividad antimutagénica

La mayoría de los compuestos carcinogénicos son mutágenos (Loechler, 2001), los cuales son compuestos que alteran la información genética (ADN) de un organismo

(NikoliĹ-VukosavljeviĹ et al., 2004). Una mutación es cualquier cambio heredable y permanente en el DNA (es decir en la secuencia del DNA).

Los compuestos antimutagénicos son capaces de ofrecer protección contra la mutación celular del ADN provocado por agentes mutagénicos (Gebhart, 1974) y hacen más lento el desarrollo de cáncer (Shankel et al., 2000). Puede haber dos clases de compuestos antimutágenicos, aquellos que ejercen su función extracelularmente inactivando a los agentes mutágenicos antes de que afecten el DNA, conocidos como desmutágenos y aquellos que actúan dentro de la célula y participan en la supresión de la mutación después de que el DNA fue dañado, llamados bioantimutágenos (SğpczyĆska, PowroŦnik, PŇkala, & Waszkielewicz, 2014).

Los posibles mecanismos de acción de los compuestos antimutagénicos son diversos e incluyen aquellos con potencial antioxidante, los que inhiben la activación de compuestos mutágenos, los que actúan como agentes de bloqueo, entre otros (SğpczyĆska et al., 2014).

Actividad antiproliferativa

Como se había mencionado previamente, las células cancerosas pierden su ciclo celular normal provocando que desarrollen características y propiedades diferentes a

las células de las que provienen (Robbins et al., 2004), que incluyen una proliferación descontrolada así como la evasión de la apoptosis.

El ciclo celular involucra numerosas proteínas regulatorias que dirigen la célula a través de una específica secuencia de eventos (Schafer, 1998) llamados fases del ciclo, estas fases son G_1, S, G_2, y M. Las células en G_1 pueden, antes de la replicación del DNA, entrar en un estado de latencia llamado G_0, el estado en el cual se encuentran la mayoría de las células del cuerpo humano que no se encuentran en crecimiento ni en proliferación (Vermeulen, Van Bockstaele, & Berneman, 2003). Las fases G_1 y G_2 son ñhuecosò en el ciclo celular que ocurren entre dos puntos obvios, la síntesis del DNA (S) y la mitosis (M); en la fase G_1 la célula se está preparando para la síntesis de DNA y en la fase G_2 la célula se prepara para la mitosis (Schafer, 1998).

La forma en que la división celular ocurre de manera correcta es: la replicación del ácido desoxirribonucleico (ADN), seguido de la segregación de cromosomas (mitosis o fase M) y posteriormente, la división celular (citocinesis); este ciclo cuenta con puntos de control que impiden la progresión a través del ciclo en G1 → S, G2 →M y la transición de la metafase a la anafase (Novák, Sible, & Tyson, 2002). La activación de éstos inducen el arresto del ciclo celular por medio de la modulación de las quinasas dependientes de ciclina (CDK); esto permite que los defectos celulares se reparen de manera adecuada y, de esta manera, se previene la transmisión de las

alteraciones a las células hijas. Los puntos de control protegen del daño al ADN de los constantes ataques de agentes genotóxicos (Malumbres & Barbacid, 2009).

Por otra parte, la apoptosis es la muerte celular programada, la cual está caracterizada por la condensación nuclear y citoplasmática seguidas por la disociación de la célula en fragmentos (McKenna, McGowan, & Cotter, 1998). Aquellos compuestos que son capaces de intervenir en la proliferación y provocar la apoptosis previenen la división incontrolada que se presenta en el cáncer.

Por todo lo anterior, y teniendo como base los trabajos realizados por nuestro grupo de investigación, donde se han encontrado fracciones y compuestos con diversas actividades biológicas, incluyendo antimutagénica y antiproliferativa, en diversos organismos marinos, como pulpo (Moreno-Félix et al., 2013), jurel, lisa, cazon (Burgos-Hernandez et al., 2002) y en particular el camarón (Wilson-Sanchez et al., 2010), se planteó la necesidad de aislar los compuestos que son responsables de las actividaes biológicas, así como elucidar sus estructuras químicas en el extracto lipídico de camarón; esta información es muy valiosa ya que puede ayudar a comprender los mecanismos por los cuales los compuestos son capaces de intervenir en los procesos biológicos, dándole importancia a este trabajo. La información que se obtiene nos ayuda a responder a las preguntas de investigación ¿cuál o cuáles son las moléculas capaces de intervenir en los procesos biológicos relacionados con las actividades antimutagénica antiproliferativa? ¿Que efectos pueden presentar estos compuestos dentro del ciclo celular de células cancerosas?

Todo esto con el fin de contribuir a avanzar en el conocimiento de compuestos con actividad quimiopreventiva para ayudar a prevenir el desarrollo del cáncer, enfermedad que, conforme han pasado los años, se ha convertido en la primera causa de muerte a nivel mundial.

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HIPÓTESIS

La fracción lipídica de camarón posee compuestos con actividad antimutagénica cuya estructura química es diferente a la de astaxantina; y además posee compuestos con actividad antiproliferativa, con estructura química presenta ácidos grasos poliinsaturados omega-3 los cuales son capaces de intervenir en la apoptosis celular.

OBJETIVO GENERAL

Caracterizar bioquímica y estructuralmente los compuestos extraídos de la fracción lipídica de camarón con actividad antimutagénica y antiproliferativa y estudiar su capacidad de inducir la apoptosis de células cancerosas.

Objetivos Específicos

1. Aislar los compuestos con mayor actividad antimutagénica *in-vitro* de extractos lipídicos de camarón.
2. Aislar los compuestos con mayor actividad antiproliferativa *in-vitro* de extractos lipídicos de camarón.
3. Caracterizar las estructuras químicas de los compuestos aislados con mayor actividad quimioprotectora.
4. Determinar in vitro, la intervención de los compuestos bioactivos en el ciclo celular de células cancerosas.

DESARROLLO DEL TRABAJO DE INVESTIGACIÓN

El trabajo de investigación está dividido en 4 capítulos, cada uno de ellos representa un artículo publicado o enviado para su publicación en revista indizada en el Journal Citation Reports (JCR) del Institute of Scientific Information de la base de datos Thomson-Reuters

Descripción del Capítulo I

Este capítulo corresponde a un artículo de revisión publicado en la revista Marine Drugs (Factor de impacto de 3.512) que lleva por título: ñ*Shrimp Lipids: A Source of Chemopreventive Compounds*ò

El capítulo contiene la información referente a los antecedentes que se necesitaron para sentar las bases del trabajo experimental. Se inicia proveiendo de información básica acerca del camarón blanco, materia prima utilizada para la realización de este trabajo, la composición química básica del mismo así, como también las actividades quimiopreventivas/quimioprotectoras que se han reportado en compuestos aislados de este organismo. Además, se describen los mecanismos de acción de los compuestos presentes en el músculo donde se ha reportado la presencia de actividades biológicas, centrándose principalmente en los compuestos carotenoides y los ácidos grasos poliinsaturados y las actividades quimiopreventivas descritas son antioxidante, antimutagénica, antiproliferativa, antiangiogénica, antiinflamatoria y la

forma en que mejora la respuesta inmune, concluyéndose que el músculo de camarón podría ser una buena fuente de compuestos con actividad biológica.

Descripción del Capítulo II

Este capítulo corresponde a un artículo original que se enviará a la revista Natural Product Research (Factor de impacto de 1.225) que lleva por título: ñ*Isolation and structural elucidation of antimutagenic lipidic compounds from white shrimp (Litopenaeus vannamei)*ò

En este capítulo se describe el proceso de aislamiento biodirigido de compuestos con actividad antimutagénica, utilizando la prueba de Ames como base y aflatoxina B_1 como mutágeno control. También se describe la elucidación estructural de los compuestos con mayor actividad; con esta información se cumple el objetivo 1 y parte del objetivo 3 planteados para este trabajo de investigación.

Descripción del Capítulo III

Este capítulo corresponde a un artículo original publicado en la revista International Journal of Molecular Sciences (Factor de impacto de 2.339) que lleva por título: ñ*Isolation and Structural Elucidaton of Antiproliferative Compounds of Lipidic Fractions from White Shrimp Muscle (Litopenaeus vannamei)*ò

En este capítulo se describe el proceso de aislamiento biodirigido de compuestos con actividad antiproliferativa, utilizando la técnica de MTT como base y la línea celular cancerosa M12.C3F6. También se describe la elucidación estructural de los compuestos con mayor actividad; con esta información se cumplen los objetivos específicos 2 y 3 planteados para éste trabajo de investigación.

Descripción del Capítulo IV

Este capítulo corresponde a un artículo original que tiene como título tentativo: ñ*Apoptosis induction in M12.C3F6 cells by bioactive triglycerides isolated from white shrimp muscle (Litopenaeus vannamei)*ò y que será enviado a la revista Evidence-based Complementary and Alternative Medicine, la cual tiene un factor de impacto de 2.175.

En este capítulo se describe la activación de las vías de apoptosis celular de los compuestos aislados con mayor actividad antiproliferativa. Con esta información se cumple el objetivo específico 4 planteado para ésta investigación

CAPÍTULO I

Shrimp Lipids: A Source of Chemopreventive Compounds

Artículo publicado en: Marine Drugs
Año: 2013
Mar. Drugs. (2013) 11, 3926-3950

Mar. Drugs **2013**, *11*, 3926-3950; doi:10.3390/md11103926

OPEN ACCESS

marine drugs

ISSN 1660-3397

www.mdpi.com/journal/marinedrugs

Review

Shrimp Lipids: A Source of Cancer Chemopreventive Compounds

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Received: 15 August 2013; in revised form: 22 September 2013 / Accepted: 27 September 2013 / Published: 16 October 2013

Abstract: Shrimp is one of the most popular seafoods worldwide, and its lipids have been studied for biological activity in both, muscle and exoskeleton. Free fatty acids, triglycerides, carotenoids, and other lipids integrate this fraction, and some of these compounds have been reported with cancer chemopreventive activities. Carotenoids and polyunsaturated fatty acids have been extensively studied for chemopreventive properties, in both *in vivo* and *in vitro* studies. Their mechanisms of action depend on the lipid chemical structure and include antioxidant, anti-proliferative, anti-mutagenic, and anti-inflammatory activities, among others. The purpose of this review is to lay groundwork for future research about the properties of the lipid fraction of shrimp.

Keywords: shrimp; chemoprevention; fatty acids; carotenoids; cancer

1. Introduction

Shrimp is one of the most popular seafoods of traditional diets [1] worldwide, and the top ten shrimp-producing nations include some of the richest and poorest nations in the world [2]. World shrimp production has increased in the last few years, from 2.85 up to 3.12 million tons (2002 and 2008, respectively) [3]. Shrimp muscle is rich in high quality proteins and minerals, and is low in fat

content [1,4]; in addition, its lipids exhibit chemopreventive and chemoprotective activities, which are important biological properties in thin product.

Several biological activities have been reported for methanolic and lipidic extracts from shrimp muscle [5,6] and waste [7]. These activities, which are capable of modifying biological processes [8], have been related to cancer prevention throughout mechanisms grouped in a term called chemoprevention, a term that is used to describe the use of natural or synthetic substances to prevent cancer development [9]. Cancer, the leading cause of death in economically developed countries and second in developing countries [10], affects approximately one of three individuals in Europe and in the United States of America, appearing as one of one hundred different kinds of this disease, with a mortality rate of approximately 20% [11]. By the year 2020, world population is expected to increase up to 7.5 billion, and approximately 17 million new cancer cases will be diagnosed [12].

In addition to socioeconomic status [13,14], geographic variability [13,15], age [16], and physical activity [13,17], nutrition is one of the factors that may influence the development of cancer and other human diseases. Nowadays, changes in the life style that include the consumption of chemopreventive compounds, such as those found in a great variety of foods, are highly recommended. In this review, the mechanisms of action of compounds that are found in a very popular seafood such as shrimp, especially in its lipidic fraction, will be discussed.

2. Chemoprevention

2.1. Definition of Chemoprevention

Epidemiological studies have provided convincing evidence that naturally occurring bioactive extracts or isolated compounds may benefit human health through the inhibition of carcinogenic processes and cell death mechanisms [18,19]. New technologies and genetic engineering have accounted for unlimited possibilities in scientific discoveries, which have raised a potential for a number of health beneficial products such as chemopreventive compounds [20]; this constitutes an area of research in disease prevention [21]. Chemoprevention was originally defined by Sporn (1976) as the use of natural, synthetic, or biologic chemical agents, in order to reverse, suppress, or prevent cancer progression [9]. Chemoprevention strategies address four goals: inhibition of carcinogens, logical intervention in persons at genetic risk, treatment of pre-malignant lesions, and translation of leads from dietary epidemiology to intervention strategies. Agents that may be useful to achieve at least one of these goals are broadly classified into three categories: blocking agents, suppressing agents, and those that reduce tissue vulnerability to carcinogenesis [22,23].

2.2. Types of Chemopreventive Activities

Chemopreventive compounds can be subdivided according to the benefit they offer to human health; among those are antioxidant, antimutagenic, antiproliferative, antiinflamatory, and antiangiogenic.

Antioxidant chemopreventive compounds prevent or delay oxidation at low concentrations, offering protection against oxidation mainly due to free radicals [24], molecules that are characterized by high reactivity due to non-paired electrons of external orbitals in some of their atoms. Free radicals have mechanisms of action that harm cells and body tissues, damage proteins, DNA, and lipids [25].

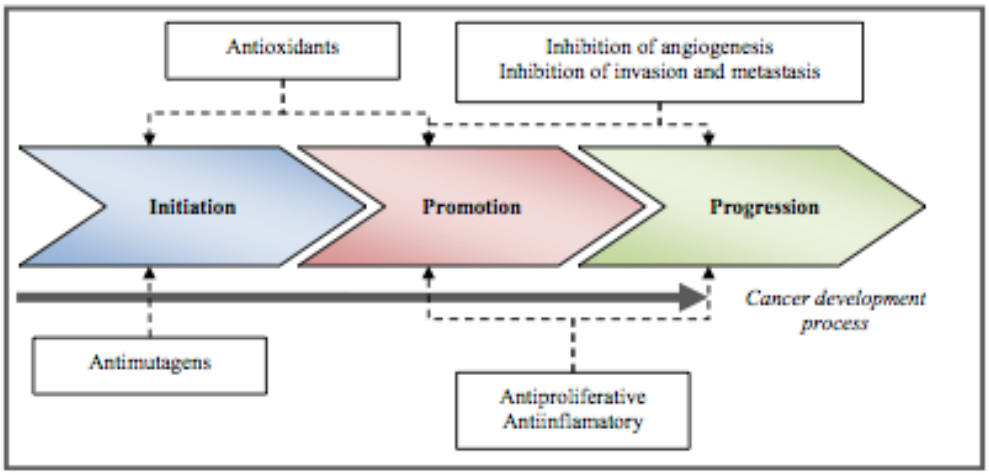
Antioxidants prevent cellular damage by reacting with oxidizing free radicals and promoting their elimination from the organism; these free radicals may be present in the cell at an oxidative stress event or during an event induced by an external source such as chemical compounds or ionizing radiation (including singlet oxygen, hydroxyl radical, peroxyl radicals, superoxide anion, hydrogen peroxide, nitric oxide, among others). Antioxidants can be found in foods as micro and macronutrients and may be able to promote the regression of premalignant lesions and inhibit their development into a cancer [26].

Antimutagenic chemopreventive compounds offer protection against cell DNA mutation caused by mutagenic agents (that alters the DNA) and slow cancer initiation [27], while antiproliferative compounds interfere in the cell cycle preventing and/or slowing down uncontrolled cancer cell division.

Inflammation promotes cell proliferation and is linked to carcinogenesis. Although proliferation alone does not cause cancer, a sustained proliferation in an environment rich in inflammatory cells, growth factor, activated stroma, and DNA-damage-promoting agents, potentiates and/or increases neoplastic risk [28]. Anti-inflammatory compounds might help to prevent the development of suitable environments for tumors [21]. Finally, antiangiogenic compounds prevent proliferation of cancerous cells by reducing the amount of blood nutrients in the tumor environment. Angiogenesis, described as the formation of new blood vessels that allow sustained tumor growth [29], is the result of loss balance between pro- and anti-angiogenic factors.

Molecules with these activities are directed to one or more cancer stages, including anti-initiation, anti-promotional, and anti-progression strategies (Figure 1). Nature is a vast source of novel therapeutic compounds with diverse origins in plants, animals, and marine species, as well as from microorganisms that have been also reported as chemopreventive. Most chemopreventive compounds have been found in fruits and vegetables [30,31]; however, the marine environment, due to its extensive biodiversity, is a rich source of biological active compounds, such as lipids, sterols, proteins, polysaccharides, among others [32–34] yet to be discovered and studied.

Figure 1. Stages during cancer development where chemopreventive compounds exert their activity.



3. Chemopreventive Compounds in the Lipidic Fraction of Shrimp

More than 15,000 natural compounds and extracts have been obtained from marine organisms [35] and have been tested for biological activities. These include compounds such as peptides and depsipeptides, extracted from tunicates, sponges, and mollusks [36]; shark's cartilage [37], and squalamine [38], obtained from the squalidae family; lipidic extracts, isolated from yellowtail fish [39], shrimp [5], and octopus [40]; marine pigments, among others. Some of these, such as carotenoids, appear to fit the criteria for the development of functional food ingredients [34]. Contribution of the marine environment to therapeutic approaches for cancer and other chronic-degenerative diseases are expected to increase in the future [41]. Shrimp is a marine organism in which chemopreventive molecules have been detected, such as lipids and lipophylic compounds. Wilson-Sanchez *et al*. (2010) [5] demonstrated that several compounds in the lipidic fraction of shrimp muscle have antimutagenic and antiproliferative activities. Also, Sindhu and Sherief (2011) [42] proved antioxidant and antiinflamatory activities in carotenoids and lipids obtained from shrimp shell. Different compounds integrate the lipidic fraction of shrimp and their mechanisms of action are diverse, which mainly depend on their chemical structures; these issues will be discussed in the subsequent sections.

3.1. Lipidic Content of Shrimp Muscle

The lipid fraction represents the 1%–2% [43] of shrimp muscle weight (dry basis) and it is integrated by carotenoids, phospholipids, neutral lipids (including cholesterol, triglycerides, free fatty acids, diglycerides, and monoglycerides) and glycolipids. Carotenoids have been studied for chemopreventive properties and they constitute the main group of pigments found in aquatic animals [44] producing colors from yellow to dark red [45]. The main chain of their chemical structure is 40 carbon long, highly unsaturated, inflexible, and easily oxidizable [46], most of them symmetrical around the central carbon atom. These pigments are lipophylic and those with polar hydroxyl and keto functionalities have increased affinities for lipid/water interfaces [47,48]. To date, more than 750 carotenoids have been identified in nature and over 250 of those are from marine origin [49]; nevertheless only 24 have been identified in human tissues.

Fatty acids, known as any aliphatic monocarboxylic acid that may be released by hydrolysis of natural fat [50], have a carboxyl group at the head end and a methyl group at the tail end [51], and constitute the starting point in many lipid structures [52]. Fatty acids can be classified as saturated, monounsaturated, and polyunsaturated, according to the number of double bounds in their structure. The polyunsaturated compounds are characteristic in marine animals. Their mechanisms of action involved in cancer chemoprevention are discussed in the next section.

3.2. Carotenoids

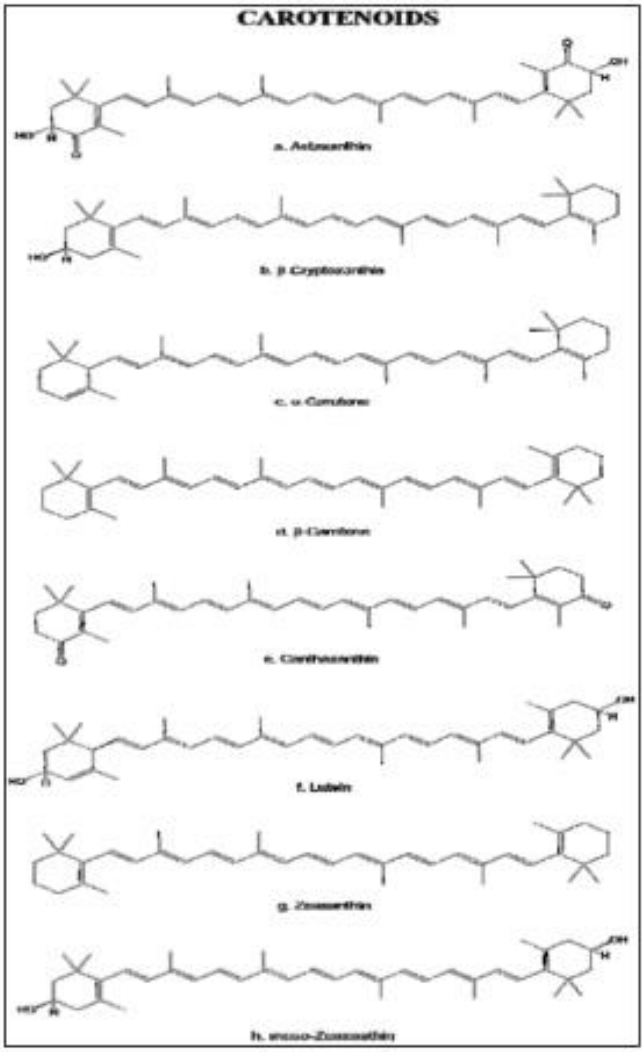
Carotenoids are synthesized by members of the plant kingdom and they are transferred to animals through the food chain [48]. The industrial use of these compounds for animal feed supplementation has been suggested in order to enhance the pigmentation of fish skin and flesh, and also as a human nutraceutical [53]. Since various natural carotenoids (such as zeaxanthin, lycopene, β-cryptoxanthin, fucoxanthin, astaxanthin, capsanthin, crocetin, and phytoene), have proven to have anticarcinogenic

activity, they have been proposed for cancer treatment and bio-chemoprevention [54] at concentrations obtained from food supply. Higher concentrations have been related to adverse effects on cellular function, and even augment cancer risk [55].

β-Carotene and cantaxanthin have proved chemopreventive activity in induced skin cancer in female Swiss albino mice [56]; these carotenoids have a protective effect against indirectly-induced skin and breast cancer, and also against directly-induced gastric carcinogenesis [57]. Thus, many studies have been focused on proving that dietary carotenoids are in fact chemopreventive agents, highly valued information that could be used for the benefit of general population.

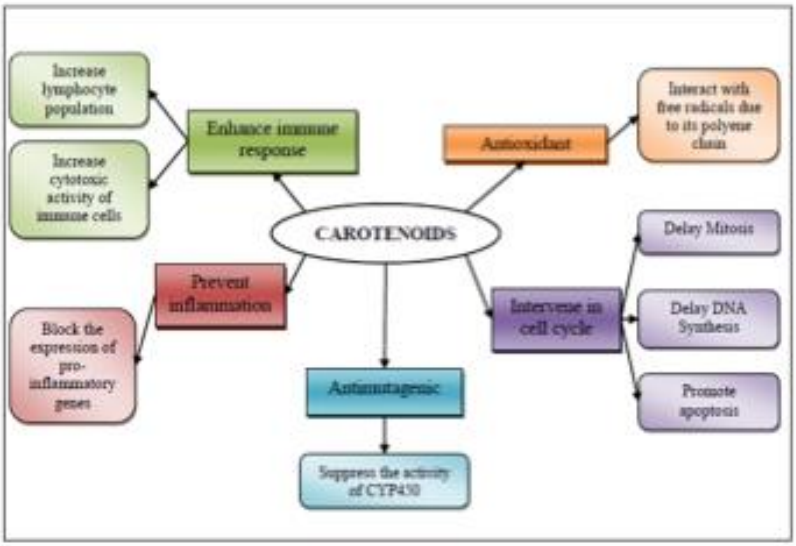
Shrimp is a source of different carotenoids; the main one found in the Penaeidae family is astaxanthin, a keto-oxycarotenoid that contains oxygen functional groups on the lateral rings, which classifies it among the xanthophylls. This carotenoid is found in high amounts in the exoskeleton of crustaceans, in the flesh of salmon and trout, and in other marine organisms as well [58]. Astaxhantin esters [59,60], β-criptoxanthin, α-carotene, β-carotene [61], meso-zeaxanthin [62], canthaxanthin, lutein, zeaxanthin, and crustacyanin [63] can also be found in this organisms at lower concentrations (Figure 2).

Figure 2. Chemical structure of the main carotenoids found in shrimp.



Carotenoids have been associated to cancer prevention, which may undergo by five mechanisms of action: (1) membrane antioxidant [64]; (2) involvement in the control of cell differentiation and proliferation [65]; (3) antimutagenic effect; (4) as anti-inflammatory agents; and (5) their ability to produce an immune response in cancer (Figure 3).

Figure 3. Mechanism of action for chemopreventive/chemoprotective activity of shrimp's carotenoids.



3.2.1. Antioxidant Mechanism

A number of studies have shown that carotenoids act as antioxidants by quenching singlet oxygen and free radicals [66]; this antioxidant activity directly emerges from the molecular structure of carotenoids [64], specifically due to the vibration of the polyene C=C and C–C bonds, where the energy of the singlet state oxygen is transferred to the carotenoid by direct contact [67,68].

The quenching activity of the different carotenoids mainly depends on the number of conjugated double bonds of their molecule and is influenced to a lesser extent by the functional end groups or by the nature of substituent in carotenoids containing cyclic groups [69]. In fact, carotenoids containing nine or more conjugated double bounds in their structure are potent singlet oxygen quenchers, a biological function which is independent of the provitamin A activity [70].

Astaxanthin and its esters, the main carotenoids present in the lipidic fraction of shrimp, have reported strong antioxidant activity in *in vitro* assays, as well as in membrane model system using phospholipid liposomes [7]. This carotenoid has a higher antioxidant activity than α-tocopherol, α-carotene, lutein, β-carotene, lycopene [71], and even higher than trolox [72], a synthetic antioxidant with a known high antioxidant activity. Martínez *et al.* (2008) [73] proposed the creation of a donor-acceptor map by measuring the electro-donating and the electron-accepting power; using this method, astaxanthin had the most effective antiradical effect in terms of its electro-acceptor capacity compared to other dietary carotenoids. This efficient antioxidant capacity is attributed to the unique structure of the terminal ring moiety. Due to these characteristics, astaxanthin may inhibit the production of lipid peroxides [74], as

well as maintain the mitochondria in a reduced state even under oxidative challenge [75]. Liang *et al.* (2009) [48] suggested that astaxanthin scavenges radicals in the water/lipid interface and transfers an electron from a non-polar and more reducing carotenoid in the membrane. In cell cultures, astaxanthin has been able to act as an antioxidant even at high concentrations, when cells are exposed to oxidative stress [76]; however, other studies have reported pro-oxidant behavior in the same concentrations [77–79] this effect is reported when the experimental conditions include a low α-tocopherol diet, this compound usually helps carotenes to be regenerated; if there is an absence it can induce the formation of peroxyl radicals and or augment the rate of carotenoid auto-oxidation.

In *in vivo* studies, astaxanthin attenuates the promotion of hepatic metastasis induced by restraint stress in mice, throughout inhibition of the stress-induced lipid peroxidation [80]. In another study, when rats were exposed to mercuric chloride (a nephrotoxic compound that increases reactive oxygen species by decreasing glutathione levels due to its affinity to SH groups) and astaxanthin, the xantophyl was able prevent the increase of lipid and protein oxidation and attenuated histopathological changes [81].

Although these studies have proved the antioxidant activity of carotenoids, these compounds may shift into a pro-oxidant effect, depending on different factors such as oxygen tension or concentration. Mixtures of carotenoids alone or in association with others antioxidants may increase their activity against lipid peroxidation [69].

3.2.2. Intervention in Cell Cycle

The cell cycle, a sequence of events by which a growing cell duplicates and divides into two identical daughter cells [82], involves numerous regulatory proteins that drive the cell throughout a sequence of specific events called cycle phases: G_1, S, G_2, and M [83]. Cells in G_1 phase can, before commitment to DNA replication, enter into a resisting state called G_0, the state where most non-growing and non-proliferating cells are in human body [84]. G_1 and G_2 phases are the "gaps" in the cell cycle that occur between the two landmarks, DNA synthesis (S) and mitosis (M); during G_1 and G_2 phases, the cell is preparing for DNA synthesis, and for mitosis, respectively [83].

Each of the cell cycle phases involves a number of proteins that regulate cell's progression such as cyclin-dependent kinases (CDKs) and cyclin proteins [83,85]. Cancer cells are frequently associated with genetic or epigenetic alteration and these proteins help cells to sustain proliferation independent of external mitogenic or anti-mitogenic signals [84]; therefore, the regulation of the cell cycle may constitute a strategy to inhibit proliferation of cancer cells. It has been proposed that carotenoids affect differentiation and proliferation of cancerous cells. Different carotenoids (such as α-tocopherol, β-carotene, lycopene, and lutein) show different abilities to control cell cycle [86].

β-Carotene has been associated to both, cell apoptosis and inhibition of cell cycle throughout different mechanisms of action. In the cell cycle of normal human fibroblasts, β-carotene has a delaying effect on the G_1 phase [87] which is related to the expression of $p21^{waf1/cip1}$ protein (an inhibitor of CDK) [88]; in colon cancer, the presence of β-carotene has been associated to a reduced expression of cyclin A (regulator of G_2/M progression) [89]; in leukemia cells, the inhibition of cell cycle progression is attributed to the up-regulation of PPARγ signaling pathway and the expression of Nrf2, an important transcription factor in Keap 1-Nnf2/EpRE/ARE signaling pathway [90]; therefore, the *in vitro* effect of β-carotene on the cell cycle depends on the studied cell line. On the other hand, β-carotene influences

and enhances cellular apoptosis by modulating the expression of regulatory genes in cancer cell lines such as colon [89], leukemia [91], melanoma [92], and breast [93]. The mechanism of action is the suppression of apoptosis blocking proteins (specifically the protiens Bcl-2 and Bcl-xL) [91–93].

Astaxanthin has also been related to both, the inhibition of cell growth and apoptosis, in *in vitro* [94] and *in vivo* [95] studies. The apoptosis mechanism in hepatoma cell cancer has been attributed to depletion of GSH levels [94], and in leukemia cells to down-regulation of Bcl-2 protein [96]. The inhibition of cell cycle progression and apoptosis mechanisms are attributed to the up-regulation of PPARγ signaling pathway and the expression of Nrf2, an important transcription factor in Keap 1-Nnf2/EpRE/ARE signaling pathway [90].

Lycopene has been related to the insulin-like growth factor I (IGF-I); a factor that, at high blood levels, is related to breast and prostate cancer. This carotenoid changes the amount or affinity of IGF-I receptor signaling and cell cycle progression; therefore, lycopene has been related to the inhibition of cell progression at the G_1 phase throughout the reduction of cyclin-dependent kinase (cdk4 and cdk2) [97] as well as the decrease in cyclin D1 and D3 [98]. In other *in vivo* studies including α-tocopherol, β-carotene, lycopene, and mixed carotenoids, in which they were used to treat cancer-induced hamsters, these carotenoids acted as suppressors of the cell cycle inhibiting the expressions of proliferating cell nuclear antigen (PCNA) and cyclin D_1 [86].

3.2.3. Antimutagenic Activity

Individual carotenoids such as astaxanthin and its esters, meso-zeaxanthin, β-carotene, zeaxanthin, α-carotene, among others, as well as their mixture, have been tested in the Ames test [99]. Researchers have found that these structures are able to produce an antimutagenic activity [99,100]. The inhibition of mutagenicity depends on both, the structure of the carotenoid and the mutagenic agent used.

Using sodium azide, ethidium bromide, and hydroxyl amine as control mutagens, astaxanthin and its esters, have shown high antimutagenic activity followed by lutein, β-carotene, violaxanthin, zeaxanthin, and α-carotene; however, a mixture of different carotenoids has shown higher inhibition of induced mutation in *Salmonella typhimurium* tester strains [99]. These results are supported by the study by González de Mejía *et al*. (1998) [100]; they assert that carotenoids have synergistic effect when the tester strains are exposed to 1-6-dinitropyrene (1,6-DNP) and 1,8-dinitropyrene (1,8-DNP) as control mutagens. However, not only those carotenoids have antimutagenic activity, meso-zeaxanthin has also proved biological activity when tested with sodium azide, 4-nitro-*O*-phenylenediane, and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, as control mutagens. Meso-zeaxanthin showed 41 to 93% of mutagenesis inhibition in all *Salmonella* tester strains used [62]. β-Carotene also showed positive results when tested against 1-methyl-3-nitro-1-nitrosoguanidine and benzo[a]pyrene as control mutagens in *Salmonella typhimurium* TA100 tester strain [101].

Canthaxanthin and commercial carotene have been able to inhibit neoplastic transformation in cell culture exposed to the carcinogen 3-methylcloranthrene; this inhibition stopped after the removal of the carotenoid treatment [102].

In *in vivo* studies, the antimutagenic activity can be described as the ability of a compound to inhibit the mutagenic effect of a known mutagen in an animal model. In this sense, the mechanism of action for meso-zeaxanthin is the inhibition of CYP450 enzymes, which was demonstrated in a rat model,

where even low concentrations of meso-zeaxanthin showed inhibitory effect towards various isoforms of CYP450 [63]. β-carotene has also proved *in vivo* activity in a Haffkine Swiss mouse tumor model [101], as well as in a Fisher 344 rats model [103] in which cancer was induced with *N*-ethyl-*N*-nitrosourea; the authors attributed the effect the unmetabolized β-carotene molecule, they concluded that this compound is absorbed, stored, and exerted antimutagenic effects against the chemical carcinogen without being transformed in the gastrointestinal tract.

3.2.4. Anti-Inflammatory Mechanism and Tumor Immunity

The mechanism by which carotenoids enhance the immune system is still unclear. Studies have revealed that astaxanthin can prevent inflammatory processes by blocking the expression of pro-inflammatory genes, as a consequence of suppressing the nuclear factor kappaB (NF-κB) activation; moreover, carotenoids inhibits the production of nitric oxide and prostaglandin E2, and the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-R), as well as the interleukin-1 beta (IL-1β) [104]; this cytokines are molecules that mediate cell-to-cell interactions [105].

The immuno-regulatory action of carotenoids have been demonstrated through their role in tumor immunity [105]. These carotenoids enhance lymphocyte blastogenesis, increase the population of specific lymphocyte subsets, increase lymphocyte cytotoxic activity, and stimulate the production of various cytokines [106].

Wang *et al.* (1989) [107] demonstrated the inhibitory effect of carotenoids (beta-carotene, lycopene, and crocetin) on the growth and development of the C-6 glioma cells inoculated in rats, cells whose growth was inhibited in 57%–67% after 7 weeks without any observable hepatotoxic effect.

Lutein decreases H_2O_2 accumulation by scavenging superoxide and H_2O_2, and the NF-κB regulates inflammatory genes, iNOS, TNF-α, IL-1β, and cyclooxygenase-2, in lipopolysaccharide (LPS)-stimulated macrophages [108].

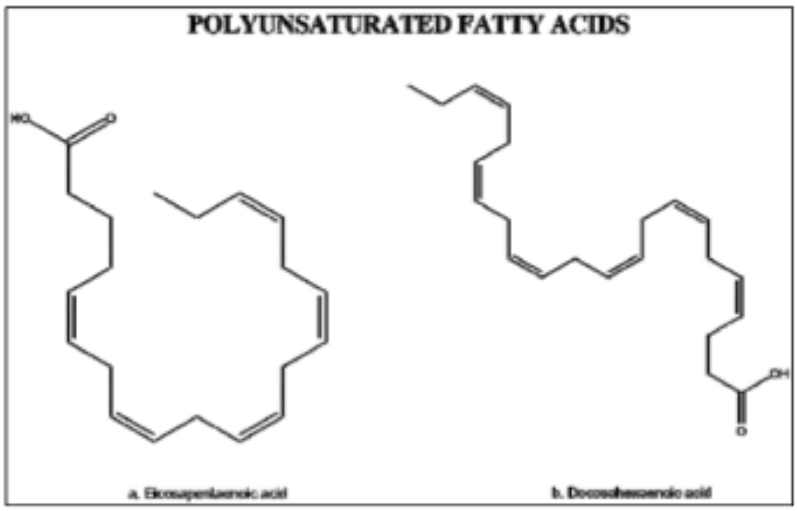
In other *in vivo* studies, dietary astaxanthin heightened immune response in domestic cats, which was attributed to the enhanced delayed-type of hypersensitivity response, peripheral blood mononuclear cell proliferation, natural killer cell cytotoxic activity, and increased T cell population [109]. In a similar way, dietary astaxanthin in dogs enhances lymphocyte proliferation, and natural killer (NK) cell cytotoxic activity; it also increases concentrations of immunoglobulin G and M (IgG and IgM), and B cell population. Therefore, dietary astaxanthin heightened cell-mediated and humoral immune response, and reduced DNA damage and inflammation in dogs [58,71]. In rats, astaxanthin was able to modulate the expression of NFkB, COX-2, MMPs-2/9, and ERK-2; therefore, it had an anti-inflammatory effect [95].

3.3. Polyunsaturated Fatty Acids

Polyunsaturated fatty acids (PUFAs) in shrimp account for about 40% of the total fatty acid content [110] with about 15% occurring in the form of ω-3 and ω-6 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Figure 4). Therefore, the quality of the fatty acid profile is similar to that of most of the marine fish species in terms of EPA and DHA content [1]. Both, fat and unsaturated fatty acids contents in shrimp muscle vary with diet [44], species [1], and maturity

stage [111]. PUFAs are a subclass of bioactive components divided into two groups ω-6 and ω-3 fatty acids, both studied for cancer chemoprevention [112].

Figure 4. Chemical structure of polyunsaturated acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

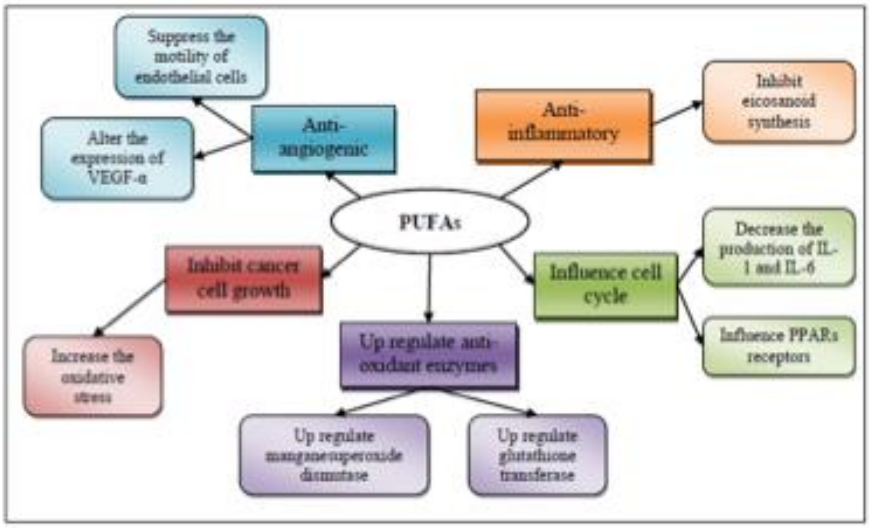


The polyunsaturated fatty acids have been identified to have a role in ameliorating various human diseases [113]. The pioneering epidemiological work on Greenland Inuit [114,115] suggested a possible link between the low incidence of heart diseases and the high consumption of seafood. Recently, the Women's Intervention Nutrition Study (WINS) provided evidence that dietary lipids may influence local or distant recurrences, and in turn influence survivorship of woman in breast cancer treatment [116]. PUFAs uptake has also been inversely related to prostate [117], breast [118], and colorectal [119,120] cancer. Nevertheless, the association between fatty acid consumption and the reduction of cancer is still controversial. Some studies have related it with no effect [121,122] or even an increase in the risk for cancer [123], whereas clinical trials on the effect of polyunsaturated fatty acids are currently being conducted [124]. The inconsistent association observed in epidemiologic studies has been attributed mainly to three reasons [125,126], (1) the intake of the long-chain fatty acids used in the studies was too low to produce a protective effect; (2) in observational research, there has been weakness in estimating PUFAs consumption, mainly due to the difference in oil content between the different species of fish; and (3) the inclusion of low variability within-population in the intake of fish or ω-3 fatty acid.

Zhang *et al*. (2010) [127] proved three different oils diets containing ω-3, ω-6, and ω-9, suggesting that diets rich in ω-3 fatty acid oil attenuates the neoplastic effect of acrylamide-induced neoplasia in mice, while diets rich in ω-6 and ω-9 oils seemed to promote this activity.

The mechanisms of action for the chemopreventive properties of ω-3 fatty acids are presumably five [125]: (1) suppression of arachidonic acid-derived eicosanoid biosynthesis[128]; (2) influence on transcription factor activity [129]; (3) increased or decreased production of free radicals and radical oxygen species; (4) mechanisms involving insulin sensitivity and membrane fluidity; and (5) antiangiogenic potential (Figure 5).

Figure 5. Mechanisms of action for the chemopreventive/chemoprotective activity of polyunsaturated fatty acids (PUFAs).



3.3.1. Anti-Inflammatory Effect of Polyunsaturated Fatty Acids

As mentioned above, PUFAs have been associated to cancer chemoprevention through the inhibition of the arachidonic acid-derived eicosanoids formation. These compounds, characterized by 20 carbon long lipophilic molecules derived from arachidonic acid, are powerful regulators of cell function.

The generation process of eicosanoid compounds consists in a series of steps beginning with the mobilization of arachidonic acid from the cellular membrane glycerolipid pools by phospholipase A_2 (PLA_2). Then, arachidonic acid can be oxidized by three different enzymes: (1) the cyclooxigenases (COX-1 and COX-2) to form prostanglandins, protacyclin or tromboxanes; (2) lypoxigenase (LOX) to form 5(S)-, 8(S)-, 12(S)-, and 15(S)-hydroxyeicosatetraenic acid (HETE) leukotrienes, lipoxins, and hepoxilins; and (3) P450 epoxygenase (EOX) to form HETEs and epoxyeicosatrienoic acid (EET) [130].

The overexpression of eicosanoid-forming enzymes [COX, LOX, and prostaglandin E synthase (PGE)] has been related to cancer development in a wide variety of human and animal tumors [131]. These enzymes promote tumor proliferation and angiogenesis, and inhibit apoptosis [132]; for example, COX2 is normally absent in most cells, however, is highly induced in early stages of tumor progression [133].

Some studies have proved that PUFAs enhance the anti-inflammatory response in people with other conditions such as rheumatoid arthritis [134,135], and it has even been concluded that fatty acids can even be used as non-steroidal anti-inflammatory drug (NSAID) in patients with this pathology [136]. This type of drugs has been reported to be beneficial, since they reduce the risk of developing solid tumors in breast, colon, lung, and prostate cancers [137,138].

Gogos (1998) [139] carried out a randomized prospective study with patients with solid tumors who received fish oil supplementation; patients treated with ω-3 PUFA had an increased immunomodulating effect and prolonged survival.

The anti-inflammatory effect of PUFAs may also be attributed to their action on macrophages [140]; they decrease the production of IL-1, IL-6, and the tumor necrosis factor-α (TNF-α) when feeding ω-3 PUFA containing oil to rats [141].

3.3.2. Influence in Transcription Factor Activity

According to Larsson *et al*. (2004) [125], one of the chemopreventive mechanisms of PUFAs is the modification of gene expression and signal transduction involved in the cell cycle. One of the transcription factor regulated by fatty acids is the peroxisome proliferator-activated receptors (PPARs), which are members of the nuclear hormone receptor family, the largest family of transcription factors [142]. Three PPAR have been identified including PPARα, PPARβ/δ, and PPARγ [143], all of them can be activated by naturally occurring fatty acids or fatty acid derivatives [144]. Their functions underlie a multitude of cellular and physiological processes by directly modulating target gene expression and indirectly modulating other transcription factors [142,143].

The effect of activating PPARβ/δ in cancer models and cancer cell lines depends on the cell line. Colon cancer is the most studied type of cancer and one of the proposed mechanisms of regulation is throughout the up-regulation of the adenomatous polyposis coli (APC)/β-CATENIN/transcription factor 4 (TCF4), pathway in which PPARβ/δ is activated by COX2-derived ligands (such as prostacyclins), leading to the expression of target genes that increase cell proliferation and promote tumor growth. Another proposed mechanism is the ligand activation of PPARβ/δ, which mediates terminal differentiation of colonocytes and inhibits cell proliferation [145]. Most fatty acids are considered PPARs agonist; nevertheless DHA suppresses its activation. This statement was demonstrated by Lee and Hwang (2002) [146] in colon tumor cells (HCT116).

3.3.3. Increased or Decreased Production of Free Radicals and Radical Oxygen Species

Free radicals and reactive oxygen species (ROS) produced in cells seems to attack fatty acids present in the cell, in order to form a variety of by-products from lipid oxidation, including more free radicals and reactive aldehydes [50]. These metabolites potentially generate pro-mutagenic compounds, which eventually can lead to cancer development [147]. Nevertheless, highly polyunsaturated fatty acids, specifically EPA and DHA present in fish oil, have been proved to help up-regulate some antioxidant enzymes such as glutathione transferases and manganese superoxide dismutase in an *in vivo* study with mice [148].

The metabolites from the oxidation of *n*-3 PUFAs inhibit breast [149] and colon [150] cancer cells. This effect, observed in cell culture studies *in vitro*, was related to the formation of lipid peroxidation products, but the inhibitory effect is lost when they are exposed to vitamins that have antioxidant activity (specifically vitamin C and E [149]). Nevertheless, clinical trials have demonstrated that the DNA damage decreases with the intake of vitamin E when a high intake of PUFAs is taken [151].

3.3.4. Antiangiogenic Potential

A high rate in neovascularization in solid tumors has been associated with a negative prognosis for cancer patients [152,153], since cancerous cells need the nutrients from the vascular system. Therefore, antiangiogenic agents may be helpful in cancer.

EPA [154] has proved *in vitro* antiangiogenic activity with a dose-dependent response for inhibition. PUFAs has also been used in an *in vivo* study, to prove an enhanced response of docetaxel (a drug used

for antiangiogenic purposes in chemotherapy) with the aid of DHA [155], where both, micro and macrovascularization in the Sprague-Dawley rat model, were inhibited.

Two mechanisms have been suggested for the anti-angiogenic potential of PUFAs: the suppression of motility of endothelial cells [156], and alterations in the expression of the pro-angiogenic vascular endothelial growth factor (VEGF)-α [157].

3.4. ω-3 Fatty Acids as a Co-Treatment during Chemotherapy

In *in vitro* studies, DHA and/or EPA have been found to improve the cytotoxic effects of several anticancer drugs toward cell lines such as breast, colon, bladder, neuroblastoma, and glioblastoma [158,159]. This observation has also been made in animal models in different types of cancer such as lung, colon, mammary, and prostate [51]. The proposed mechanism of action for this beneficial effects is attributed to the change of the phospholipids in the cell membrane to more polyunsaturated fatty acids, mainly DHA and EPA; this alters the physical properties of the plasma membrane, resulting in an increase in membrane fluidity, enhancing the uptake of the chemotherapy drugs [160]. However, they can only be used at a maximum dose of 0.3 g/kg, according to a phase I clinical trial where adverse effects, mainly diarrhea and vomit, were observed [161].

Xenographic studies have been carried out to explain the benefits of a diet rich in PUFA's in chemotherapy treatment. Atkinson *et al*. (1997) [162] inoculated fibrosarcoma tumor cells into Fisher 344 rats and fed them with diets containing different proportions of safflower oil and DHA oil, and treated them with arabinosylcytosine (AraC) or saline. Authors concluded that consumption of a diet rich in DHA could slow tumor growth, prevent hyperlipidemia, and enhance bone marrow cellularity, compared to a moderate-fat diet rich in ω-6 fatty acids. In a similar work, Cha *et al*. (2002) [163] investigated the effect of dietary supplementation with DHA in combination with AraC chemotherapy and found that DHA diet (1.8 g DHA/kg/day) can be associated with a longer life span and no incidence of death due to drug toxicity; nevertheless, the overconsumption of DHA (up to 4.5 g DHA/kg/day) shorten survival time, increases circulating tumor cell burden, and reduces red blood cell concentration.

The increased permeability of the small intestine of mice caused by methotrexate has been reported. Horie *et al*. (1998) [164] proved that oral administration of DHA protects the small intestine from the effects of methotrexate by reducing the permeability. Gomez de Segura *et al*. (2004) [165] studied the effect of DHA in male rats treated with 5-fluorouracil (5-FU), an antitumoral drug, and concluded that enriching diet with DHA protects the intestine from lesions produced by 5-FU.

In dogs with lymphoma, treated with doxorubicin chemotherapy in combination with PUFAs and arginine, Ogilvie *et al*. (2000) [166] observed that subjects with higher DHA plasma levels had better diet tolerance, and increased disease free interval and survival time.

A very specific example of the beneficial effects of ω-3 fatty acids was reported by Pardini *et al*. (2005) [167]. They reported that an old man diagnosed with malignant fibrous histiocytoma of the lungs, declined the conventional chemotherapy and elected nutritional intervention by changing his diet to a high ω-3 and low ω-6 supplementation. This study demonstrated that the size of the tumors was reduced, which was attributed to the intake of DHA, specifically to the ω-6/ω-3 ratio.

A proposed mechanism for the effect of the ω-3 fatty acids in chemotherapy is through the inhibition of the NF-κB transduction way, which suggests ω-3 PUFAs may be used during chemotherapy in cancer treatment [168].

4. Conclusions

There is an extensive research effort aimed to obtain efficient chemopreventive compounds in nature, mostly from vegetable sources. However, since the number of cancer cases is constantly increasing, the search, isolation, and study of chemopreventive compounds, has become an important area of research. Many of this research has focused on land organism; however, the great biodiversity that characterizes the marine environment, makes the search for bioactive compounds in this ecosystem a topic of great interest.

The lipidic fraction in shrimp is a source of chemopreventive compounds because its' component, mainly attributed to carotenoids and PUFAs, have proved biological activity in both, *in vivo* and *in vitro* studies, as well as in xenographic research. Carotenoids exert their chemopreventive/chemoprotective activity mainly by four mechanisms: antioxidation, antiproliferation, antimutagenisis, and anti-inflammatory action, and these activities are mainly attributed to their chemical structure. On the other hand, PUFAs exert their chemopreventive potential mainly throughout four mechanisms: antiinflamatory and antiangiogenic activities, the ability to influence the transcription factor activity and the increased or decreased production of free radicals.

PUFAs could also be used as a co-treatment in cancer patients in order to enhance chemotherapy treatment as well as a chemopreventive agent without adverse toxic effects.

Based on the above, the lipidic fraction of shrimp represents an important commodity with high potential for the search of chemopreventive agents. However, in order to select the appropriate compound to be proposed as chemotherapeutic agent, a good knowledge is required concerning the pathways that each type of compound may modulate.

Acknowledgments

The authors wish to thank Mexico's National Council of Science and Technology (CONACyT), for financing grant No. 107102, and for the graduated student scholarships granted to López-Saiz Carmen-María and Suárez-Jiménez Guadalupe-Miroslava.

Conflicts of Interest

The authors declare no conflict of interest.

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CAPÍTULO II

Isolation and Structural Elucidaton of Antimutagenic Lipidic Compounds from White Shrimp (*Litopenaeus vannamei*)

Manuscrito preparado para: Natural product research

Isolation and Structural Elucidation of antimutagenic Lipidic Compounds from White Shrimp (*Litopenaeus vannamei*)

Natural Product Research

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Acknowledgements

The authors wish to acknowledge the National Council for Science and Technology (CONACyT) of México for financing grant proposal 107102 and graduated scholarship granted to Carmen-MaríaLópez-Saiz.

Isolation and Structural Elucidation of antimutagenic Lipidic Compounds from White Shrimp (*Litopenaeus vannamei*)

Abstract

Cancer is the leading cause of death worldwide and the search of chemopreventive compound to prevent it has become a priority globally; shrimp has been reported as a source of these type of compounds. In this study, shrimp lipids were extracted and separated in order to obtain isolated antimutagenic compounds. The antimutagenic activity was assessed by inhibition of aflatoxin B1 effect on TA98 and TA100 *Salmonella* tester strains using the Maron and Ames assay. Methanolic fraction showed the highest antimutagenic activity (95.62 and 95.96% for TA98 and TA100 respectively); and was further separated into 15 different sub-fractions (M1-M15). Fraction M8 exerted the highest inhibition of AFB1 mutation (96.5 and 101.6% for TA98 and TA100 respectively) and was further fractionated in four sub-fractions M8a, M8b, M8c and M8d. Of these, M8a exerted the highest activity with inhibition of 87.84 and 94.18% of AFB1 mutation for TA98 and TA100 respectively.

Keywords: chemoprevention; antimutagenic activity; shrimp.

1. Introduction

Cancer, the leading cause of death in the economically developed countries and second in developing countries (Jemal and others 2011), is a preventable disease (Anand and others 2008), mainly by making changes in life style that include the consumption of chemopreventive and chemoprotective compounds. Chemopreventive agents are able to reverse, suppress or prevent cancer progression (Tsao and others 2004), and epidemiological studies have provided convincing evidence that some naturally occurring bioactive extracts or isolated compounds may benefit human health through the inhibition of carcinogenic processes (Nerurkar and Ray 2010; Wang and others 2010). In the search of these kind of compounds, more than 15,000 natural compounds and extracts have been isolated from

different marine organisms (Munro and others 1999) and have been tested for biological activities.

The muscle of shrimp is rich in high quality proteins and low in fat content (Oksuz and others 2009; Silva and others 1996); however, there is evidence that this lipidic fraction may exhibit chemopreventive and chemoprotective activities, including antimutagenic compounds which are capable of prevent mutations in cells.

The lipidic fraction of shrimp muscle is composed of several different lipidic compounds including carotenoids, phospholipids, neutral lipids (such as cholesterol, triglycerides, free fatty acids, diglycerides, and monoglycerides) and glycolipids. This fraction represents around 1-2% of the muscle weight (dry weight) (Ezquerra-Brauer and others 2004). Several carotenoids including astaxanthin and its esters, meso-zeaxanthin, β-carotene, zeaxanthin, Ŭ-carotene, among others, as well as their mixture have been tested in the Ames test (Bhagavathy and others 2011) founding inhibition of known carcinogenic compounds (such as sodium azide, ethidium bromide, and hydroxyl amine). The aim of this study is to isolate compounds found in muscle of shrimp which are responsible for the antimutagenic activity as well as identify the chemical structure of those with the highest activity.

2. Results and Discussion

2.1. Lipidic extraction and partition

The chloroformic extraction of shrimp muscle had a yield of 1.860 ±0.004 % (dry basis). Lipids in shrimp muscle usually represents 1-2% (Ezquerra-Brauer and others 2004) of its weight (dry basis).

Antimutagenic activity was measured by the Maron and Ames assay, using aflatoxin B1 (AFB1) as control mutagen. Raw extract exerted the inhibition of AFB1 mutagenic potential in 94.6 ± 1.10 and 95.36 ± 2.41 % for TA98 and TA100 tester strains, respectively; this can be considered as a high inhibition of a mutagen (Ikken

and others 1999). Antimutagenic activity had previously been reported for shrimp flesh, using sodium azide and potassium permanganate as control mutagens (Mehrabian and Shirkhodaei 2006) and also AFB1 (Wilson-Sanchez and others 2010). In the search for compounds with biological activity, raw extract was partitioned in methanol and hexane. Polar compounds were solubilized by methanolic fraction with a 58% yield while the hexanic, where the less polar compounds were solubilized had a 42% yield. Both fractions were tested for antimutagenic activities.

2.1.1. Antimutagenic activity of fractions obtained by partition

Hexanic fraction inhibited the mutagenic effect of AFB1 in a very low percentage while methanolic fraction exerted the highest activity (95.6 ± 0.68 and 95.96 ± 1.99 % for TA98 and TA100, respectively), comparable to that of the raw extract (Table1). Methanolic fraction is composed by a large variety of compounds, where at least one of them had to be responsible for the biological activity, for this reason, methanolic fraction was selected for further fractioning.

2.2 *Analysis of lipidic composition by RP-HPLC*

Methanolic fraction was analyzed and sub-fractionated by semi-preparative RP-HPLC. The highest absorbance of methanolic fraction was at 450 nm (Figure 1); these signals can be attributed to the diversity of carotenoid compounds that can be found in muscle of shrimp (Chew and others 2011), these compounds include astaxanthin (Chew and others 2011), and in lower amounts astaxanthin esters (Arredondo-Figueroa and others 2003; Sánchez-Camargo and others 2011); ɓ-criptoxanthin, Ŭ-carotene, ɓ-carotene (Mezzomo and others 2011), canthaxanthin, lutein, and zeaxanthin (Babu and others 2008) have also been reported as carotenoids isolated from shrimp muscle. Even though the highest signals were detected in the visible spectra (with the highest absorption detected at 450 nm), other signals in the near and middle ultraviolet spectrum were observed.

2.2.1. Antimutagenic activity of methanolic sub-fractions obtained by semi-preparative RP-HPLC

The methanolic fraction was further fractionated by RP-HPLC into 15 different sub-fractions using elution time (fractions were obtained every 1.33 min) and each one was tested at a concentration of 4 mg/plate using AFB1 as control mutagen in the Ames test, in order to identify the bioactive fractions with the highest activity (Table 2). Five sub-fractions where selected for further analysis including M2, M8, M12, M14, and M15 since all showed high antimutagenic activity in both tester strains (higher than 60% of mutation inhibition) (Ikken and others 1999) without a significant difference among them. These five sub-fractions are very different in polarity as well as in chemical nature, as we can deduce from retention time. M2, M14 and M15 were obtained as colorless fractions, while M8 exhibited an intense orange color.and M12 a pale yellow color. The effect of the five sub-fractions were evaluated at lower concentrations (seriated dilutions from 4 to 0.04 mg/ plate) (Figure 2); All five sub-fractions exhibited a dose-response behavior, and sub-fraction M8 was selected for further analysis since it was the one that exhibited the highest activity on both tester strains.

2.3 Fractioning by open column chromatography

In order to continue with the isolation of the bioactive compounds, fraction M8 was subjected to an open column chromatographic procedure. Four fractions were obtained from this chromatographic step and they were coded as M8a, M8b, M8c and M8d.

2.3.1. Antimutagenic activity of methanolic sub-fractions isolated by open column chromatography.

All of the M8 sub-fractions were highly antimutagenic and exerted a dose-response behavior (Figure 3). M8a was the fraction that exerted the highest antimutagenic activity in

both tester strains (87.94 ± 3.45 and 94.18 ± 1.26 % for TA98 and TA100 tester strains, respectively), for this reason, M8a was analyzed for its chemical structure.

2.3.2. Chemical/structural characterization of M8a fraction.

Previous studies have reported the presence of bioactive compounds in shrimp, most of these were not extracted from shrimp muscle. Antioxidant activity was previously been reported in crude extracts obtained from several shrimp by-products such as head (Mao and others 2013; Sowmya and Sachindra) and shell (Sindhu and Sherief 2011). Another biological activity previously described for shrimp extracts is anti-inflammatory (Sindhu and Sherief 2011).

Antimutagenic activity was detected in shrimp muscle but also using a crude extract (Mehrabian and Shirkhodaei 2006). In all these reports, the bioactivities have been attributed to compounds of carotenoid nature. Nevertheless, authors studied crude extracts only and they reported the absorbance at visible spectra wavelength, attributing the biological activity to carotenoids; however, they did not purify the extract in order to identify the chemical structure of the compound responsible for the activity. In our laboratory, we have reported antimutagenic compounds present in fractions obtained after a serial of thin layer chromatography procedures (Wilson-Sanchez and others 2010),

3. Experimental

3.1. Testing species

Shrimp (Litopenaeus vannamei) was obtained from a local market in Hermosillo, Sonora, México, and transported in ice to the University of Sonora Seafood Laboratory. Edible portions of shrimp were separated, packed, and stored at ï 20 °C until analysis. The extraction of the lipidic fraction of shrimp was obtained according

to the methodology proposed by (Burgos-Hernandez and others 2002). A 100 g shrimp muscle sample was homogenized with five parts of CHCl3 (w/w) in a blender at high speed for 1 min, the resulting mixture was poured into a flask and agitated for 40 min with the aid of a Wrist Action Burrel Shaker (Burrel Corporation, Pittsburg, PA, U.S.). The mixture was filtered through Whatman No. 1 filter paper under vacuum and the filtrate was evaporated to dryness under reduced pressure at 40 °C. The lipidic extract was then re-dissolved in methanol-hexane (1:1 v/v), agitated for 30 min and filtrated again through Whatman No. 1 filter paper under vacuum. The immiscible phases were separated in a separating funnel and concentrated under reduced pressure at 40 °C, re-dissolved in chloroform and dried under N_2 stream. All the process was performed in the darkness.

3.2 Analysis of lipidic composition by RP-HPLC

Fractionation of lipidic fractions was carried out by semi-preparative HPLC using an Agilent Technologies HPLC station in conjunction with a Zorbax Eclipse XDB-C18 semi preparative column (250 × 9.4 mm i.d.; 5 µm particle size; Agilent Technologies, USA). A guard column made of the same material was also used. Aliquots of 100 εL from each extract were injected into the column according to the modified procedure of (Weber and others 2007). Elution of components was performed using a flow rate of 2 mL min-1 and continuously monitored at 450 nm. Column temperature was maintained at 20° C. Solvents used for elution were water (A), acetone (B), and hexane (C). Lipids were eluted from the column using a linear gradient from 70 % A, 30 % B to 100% B in 5 min, and then a linear gradient from 100% B to 70% B, 30% C up to minute 20 with a 3 min re-equilibration period at the initial conditions before application of the next sample. For identification of lipidic peaks, fractions were collected using an Agillent Technologies fraction collector with a flow delay of 30 sec. The collected fractions were individually tested in antiproliferative activitiy.

3.3 Isolation of active component by column chromatography

The sub-fraction obtained by HPLC with the highest antiproliferative activity was subjected to open column chromatography under gravity on silica gel (2.5cm x 40cm using silica, Sigma 60-120 mesh). Sample was poured into the column and then eluted using 1L of solvent mixture of hexane: ethyl acetate (50:50), followed by 0.5L of acetone and finally 0.5 L of methanol. The eluent was monitored using TLC and iodide. The fractions containing similar signals were combined. Schematic representation of the isolation procedure is presented in Figure 4.

3.4 Bacterial cultures

Salmonella typhimurium TA98 and TA100 were used. Fresh overnight tester strain cultures, were stored at ï 80 °C. Tester strains were checked routinely to confirm genetic characteristics using the procedure described by Maron and Ames 1983 (Maron and Ames 1983).

3.5 Antimutagenicity test

The Ames test (Maron and Ames 1983) was used to test the antimutagenic activity of the dry extracts obtained from preparative HPLC and fractionationing were reconstituted with acetone to a final concentration of 1.5 mg/mL., and then serially diluted 1:10 with acetone and were spiked with pure aflatoxin AFB1 (AFB_1) as control mutagen (Sigma-Aldrich, St. Louis, MO, U.S.) to a final concentration of 500 ng of AFB1/100 ε L. Metabolic Activation System S9 mix (Aroclor 1254-induced, Sprague-Dawley male rat liver in 0.154 M KCl solution), purchased from (Molecular Toxicology, Inc.; Annapolis, MD, U.S.) was used to bioactivate the AFB_1 and fractions. Residual mutagenicity of AFB_1 was assayed using the standard plate incorporation procedure described by Maron and Ames. Different AFB_1 concentrations were used as a control for both tester strains. All assays were performed in triplicate.

3.6 ^{1}H NMR Analysis

Measurements were performed using an Agilent Technologies equipment operating at 400MHz. Each fraction was dissolved in CDCl3 (500µL, Sigma-Aldrich, Saint Louis, Missouri, USA) with a small amount of tetramethylsilane (TMS) as internal standard

and the resulting mixture was placed into a 5 mm diameter ultra-precision NMR sample tubes. Chemical shifts were recorded in ppm, using TMS proton signal as internal standard.

4. Conclusions

The lipidic extract of white shrimp muscle is a source of antimutagenic compouds, and even though, this activity has been previously attributed to carotenoid compounds, this study demonstrates that compounds responsible for the highest activity are apocarotenoids.

Acknowledgements

The authors wish to acknowledge the National Council for Science and Technology (CONACyT) of México for financing grant proposal 107102 and graduated scholarship granted to Carmen-María López-Saiz.

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Table 1. Percentage of Inhibition of AFB1 mutation by the effect of raw extract of white shrimp muscle and methanolic and hexanic fractions.

Dose (mg/plate)	Raw extract	Methanolic fraction	Hexanic fraction
TA98			
5	94.6± 1.10 [a]	95.6 ± 0.68 [a]	67.85± 1.17 [b]
0.5	12.63± 12.64	31.31± 13.87	54.71± 14.37
0.05	10.27± 12.40	10.27± 4.24	-10.27± 9.81
TA 100			
5	95.36± 2.41 [A]	95.96± 1.99 [A]	32.70± 8.03 [B]
0.5	2.5± 14.65	11.53± 6.02	0.24± 11.13
0.05	-14.51± 17.28	-8.64± 12.81	-34.96± 9.18

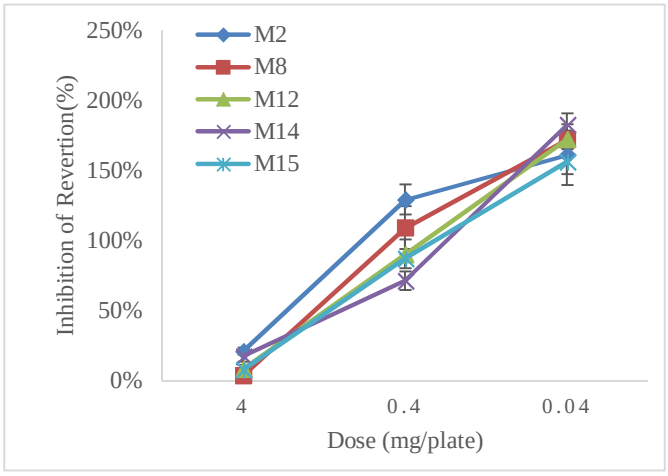
The results are represented as percentage of inhibition of AFB1 mutation
The results shown are representative from three repetitions.
Different letters in a row represent significant differences ($p < 0.05$).
Spontaneous revertants were 31±2.65 and 117±5.69 and AFB1 control (500 ng) 625±26.16 and 958±27.43 revertants for TA98 and TA100 respectively

Table 2. Scanning for antimutagenic compounds using AFB1 as control mutagen.

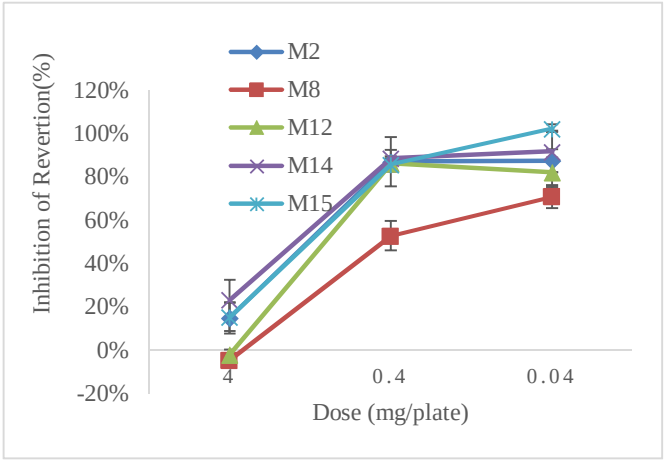
	TA 98	TA 100
M1	22.79 ± 5.72 [a]	27.1 ± 10.24 [ab]
M2	66.54 ± 5.09 [bc]	66.14 ± 5.64 [de]
M3	63.11 ± 10.16 [bc]	17.7 5± 8.57 [a]
M4	58.63 ± 10.73 [abc]	72.48 ± 7.29 [de]
M5	70.19 ± 3.61 [bc]	31.02 ± 10.75 [abc]
M6	66.48 ± 1.26 [bc]	42.85 ± 2.14 [abcd]
M7	41.16 ± 11.71 [ab]	30.96 ± 7.44 [abc]
M8	80.09 ± 7.06 [c]	63.73 ± 4.65 [cde]
M9	40.82 ± 11.69 [ab]	52.99 ± 9.33 [bcde]
M10	45.01 ± 11.43 [abc]	46.59 ± 6.88 [abcde]
M11	48.67 ± 9.67 [abc]	56.25 ± 11.63 [bcde]
M12	68.04 ± 5.52 [bc]	79.67 ± 4.35 [e]
M13	52.66 ± 11.43 [abc]	53.35 ± 2.90 [bcde]
M14	74.78 ± 7.41 [bc]	59.26 ± 7.23 [bcde]
M15	71.90 ± 4.77 [bc]	70.98 ± 7.11 [de]

The results are represented as percentage of inhibition of AFB1 mutation (500 ng)
The results shown are representative from three repetitions.
Different letters in a column represent significant differences ($p < 0.05$).
Spontaneous revertants were 31±2.65 and 117±5.69 and AFB1 control (500 ng) 625±26.16 and 958±27.43 revertants for TA98 and TA100 respectively

Figure 1. Antimutagenic activity by the effect of methanolic subfractions M2, M8, M12, M14 and M15 at different concentrations. Results are represented as percentage of inhibition of AFB1 mutation (500 ng) in *Salmonella* (A) TA98 and (B) TA100 tester strains. Results shown are representative of three repetitions. Spontaneous revertants were 33±4.04 and 120±7.64 and AFB1 control (500 ng) 493±37.17 and 724±21.50 revertants for TA98 and TA100 respectively.

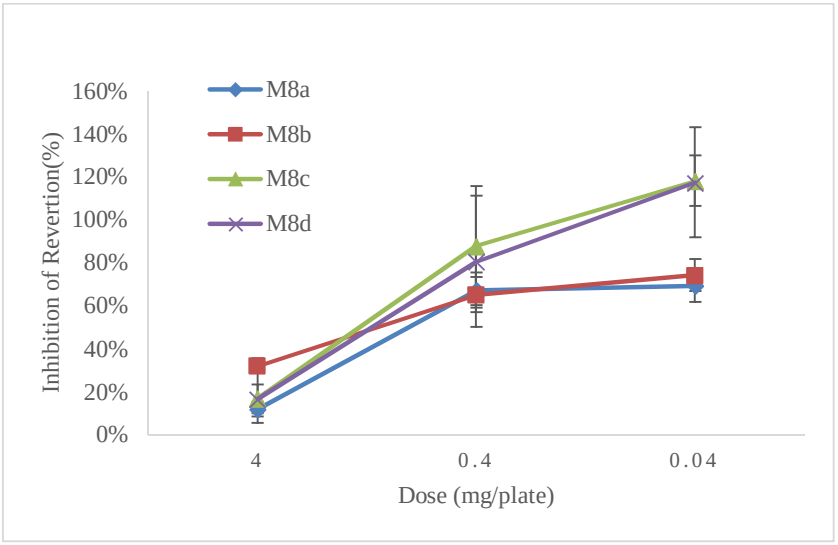


(A)

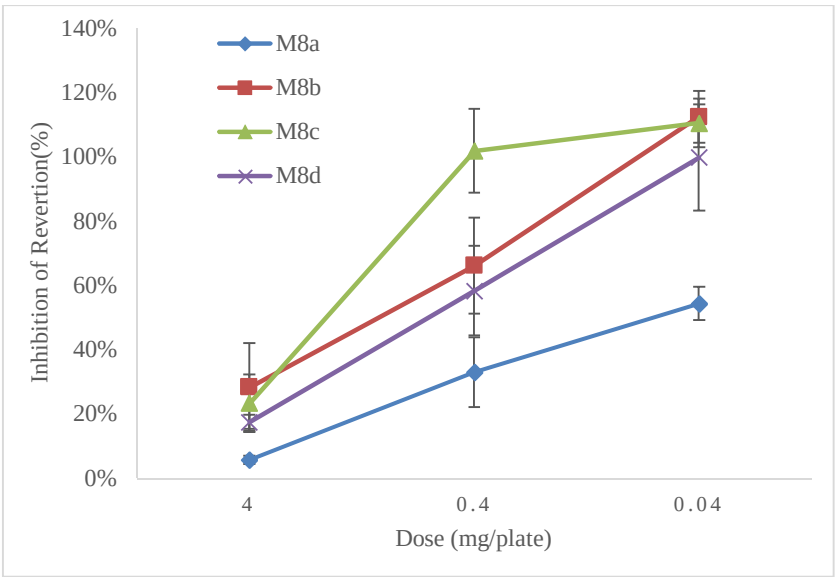


(B)

Figure 2. Antimutagenic activity by the effect of methanolic sub-fractions M8a, M8b, M8c and M8d at different concentrations. Results are represented as percentage of inhibition of AFB1 mutation (500 ng) in *Salmonella* (A) TA98 and (B) TA100 tester strains. Results shown are representative of three repetitions. Spontaneous revertants were 33±4.04 and 120±7.64 and AFB1 control (500 ng) 493±37.17 and 724±21.50 revertants for TA98 and TA100 respectively.



(A)



(B)

CAPÍTULO III

Isolation and Structural Elucidaton of Antiproliferative Compounds of Lipidic Fractions from White Shrimp Muscle (*Litopenaeus vannamei*)

Artículo publicado en: International Journal of Molecular Sciences
Año: 2014
Int. J. Mol. Sci. (2014) 15, 23555-23570

Int. J. Mol. Sci. **2014**, *15*, 23555-23570; doi:10.3390/ijms151223555

OPEN ACCESS
International Journal of
Molecular Sciences
ISSN 1422-0067
www.mdpi.com/journal/ijms

Article

Isolation and Structural Elucidation of Antiproliferative Compounds of Lipidic Fractions from White Shrimp Muscle (*Litopenaeus vannamei*)

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External Editor: Vladimír Křen

Received: 27 October 2014; in revised form: 5 December 2014 / Accepted: 5 December 2014 / Published: 17 December 2014

Abstract: Shrimp is one of the most popular seafood items worldwide, and has been reported as a source of chemopreventive compounds. In this study, shrimp lipids were separated by solvent partition and further fractionated by semi-preparative RP-HPLC and finally by open column chromatography in order to obtain isolated antiproliferative compounds. Antiproliferative activity was assessed by inhibition of M12.C3.F6 murine cell growth using the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide)

assay. The methanolic fraction showed the highest antiproliferative activity; this fraction was separated into 15 different sub-fractions (M1–M15). Fractions M8, M9, M10, M12, and M13 were antiproliferative at 100 µg/mL and they were further tested at lower concentrations. Fractions M12 and M13 exerted the highest growth inhibition with an IC_{50} of 19.5 ± 8.6 and 34.9 ± 7.3 µg/mL, respectively. Fraction M12 was further fractionated in three sub-fractions M12a, M12b, and M12c. Fraction M12a was identified as *di*-ethyl-hexyl-phthalate, fraction M12b as a triglyceride substituted by at least two fatty acids (predominantly oleic acid accompanied with eicosapentaenoic acid) and fraction M12c as another triglyceride substituted with eicosapentaenoic acid and saturated fatty acids. Bioactive triglyceride contained in M12c exerted the highest antiproliferative activity with an IC_{50} of 11.33 ± 5.6 µg/mL. Biological activity in shrimp had been previously attributed to astaxanthin; this study demonstrated that polyunsaturated fatty acids are the main compounds responsible for antiproliferative activity.

Keywords: shrimp; chemoprevention; antiproliferative activity

1. Introduction

Naturally occurring bioactive extracts may benefit human health through inhibition of carcinogenic processes and cell death mechanisms [1,2], and compounds with these properties are termed chemopreventives [3]. Chemoprevention was originally defined by Sporn (1976) as the use of natural, synthetic, or biological chemical agents to reverse, suppress, or prevent cancer [4], which is the leading and second cause of death in economically developed and developing countries, respectively [5].

Shrimp's muscle is rich in high quality proteins and low in fat content [6,7]; however, there is evidence that the lipidic fraction may exhibit chemopreventive and chemoprotective activities, including antiproliferative compounds which are capable of interfering in the cell cycle preventing uncontrolled cancer cell division.

The lipidic fraction of shrimp muscle, which is composed of carotenoids, phospholipids, neutral lipids (including cholesterol, triglycerides, free fatty acids, diglycerides, and monoglycerides) and glycolipids, represents 1%–2% (dry weight) [8] of the total weight. Several carotenoids (such as β-carotene, lycopene, and lutein) show different abilities in controlling the cell cycle [9], such as apoptosis [10–13] and inhibition of the cell cycle [10,14,15]. Polyunsaturated fatty acids (PUFAs) can also intervene in the cell cycle. According to Larsson (2004) [16], one of the mechanisms of PUFAs is through the modification of gene expression and signal transduction involved in the cell cycle [17]. Their functions underlie a multitude of cellular and physiological processes by directly modulating target gene expression and indirectly modulating other transcription factors [18]. However based on the above, the aim of the present study is to isolate and identify the compounds responsible for the antiproliferative activity that has been previously reported in shrimp muscle.

2. Results and Discussion

2.1. Lipidic Extraction and Partition

The chloroformic extraction of shrimp muscle had a yield of 1.860% ± 0.004% (dry basis). The lipidic fraction of shrimp muscle usually represents 1%–2% [8] of weight (dry basis) and consists of carotenoids [19], phospholipids [20], neutral lipids (including cholesterol, free fatty acids, mono, di and tryglicerides [21]), and glycolipids [22].

The chloroformic extract was partitioned in methanol-hexane; the yield for the methanolic fraction was 58% while the hexanic fraction was 42%. Both fractions were tested for antiproliferative activity.

Antiproliferative Activity of Fractions Obtained by Partition

Antiproliferative activity was measured by the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide) assay. In order to select the fraction with the highest antiproliferative activity, methanolic and hexanic fractions were tested in M12.C3.F6 murine cell lines at three different concentrations (Table 1).

Table 1. Percentage of proliferation of M12.C3.F6 murine exposed to methanolic and hexanic fractions at different concentrations.

Fraction	400 µg/mL	200 µg/mL	100 µg/mL
Hexanic	80.69 ± 4.62 [a]	86.78 ± 10.13 [a]	96.59 ± 6.94 [a]
Methanolic	23.29 ± 5.61 [b]	33.61 ± 13.8 [b]	90.44 ± 8.27 [a]

The results are represented as percentage of proliferation; the results shown are representative from three independent experiments. Different letters in a column represent significant differences ($p < 0.05$). Control cell cultures were incubated with DMSO (0.5%).

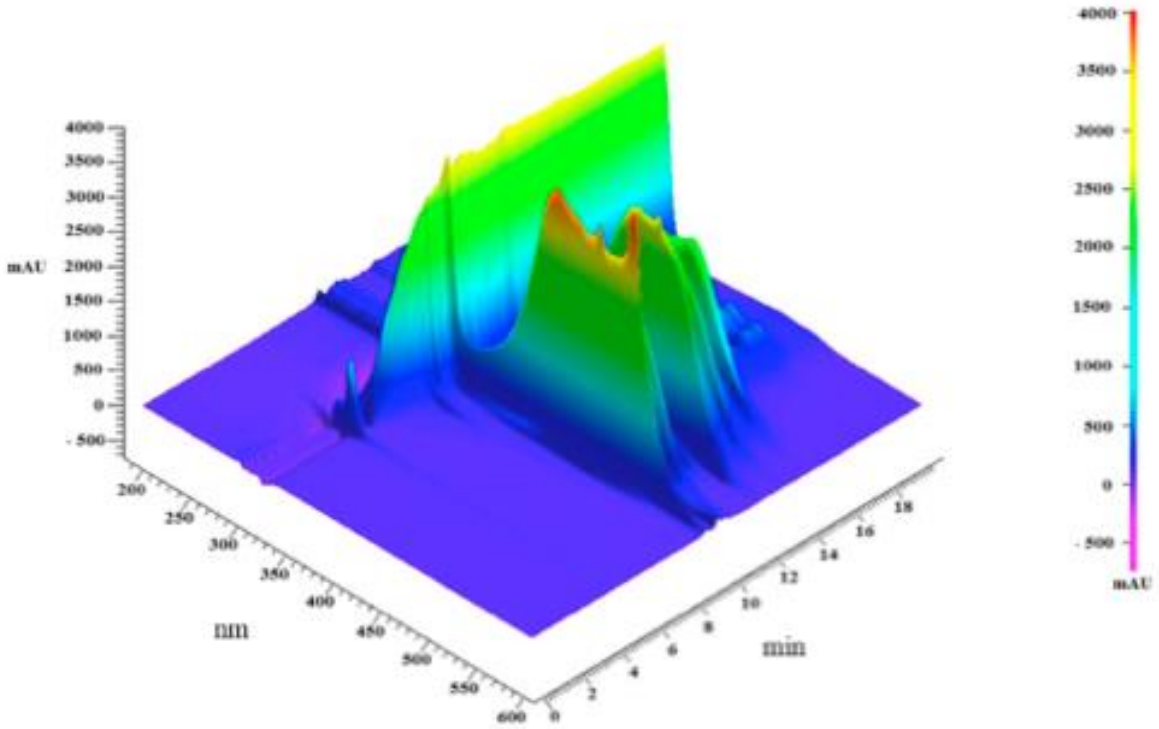
The hexanic fraction only inhibits M12.C3.F6 growth at a very low percentage while the methanolic fraction exerted the highest activity at the lower concentration (200 µg/mL). Even though the used concentration is still high, we had to consider that this fraction is composed of a large variety of compounds, where at least one of them is responsible for the biological activity. The methanolic fraction was selected for further fractioning.

2.2. Analysis of Lipidic Composition by RP-HPLC

In order to establish the absorbance at which the analysis was going to be carried out, the lipidic fraction was scanned from 190 to 600 nm (Figure 1). Signals were detected at various wavelengths; the signal at 275 nm is due to acetone in mobile phase; the highest signals were at 450 nm, these signals might be attributed to carotenoids in shrimp muscle [23]. Methanolic and hexanic fractions were analyzed to study their composition by RP-HPLC (Figure 2) and both were complementary. The more polar compounds were partitioned to the methanolic fraction while the non-polar remained in the hexanic phase. Several carotenoid compounds have been identified on shrimp, including astaxanthin [23], and astaxanthin esters in lower amounts [19,24], as well as β-criptoxanthin, α-carotene, β-carotene [25], canthaxanthin, lutein, zeaxanthin, and crustacyanin [26], which can also be found in this organism.

Even though the highest signals were detected at 450 nm, other signals in the near UV spectrum were observed.

Figure 1. Spectrophotometric scanning of the lipidic extract from shrimp after reversed phase chromatography.



Antiproliferative Activity of Methanolic Sub-Fractions Obtained by Semi-Preparative RP-HPLC

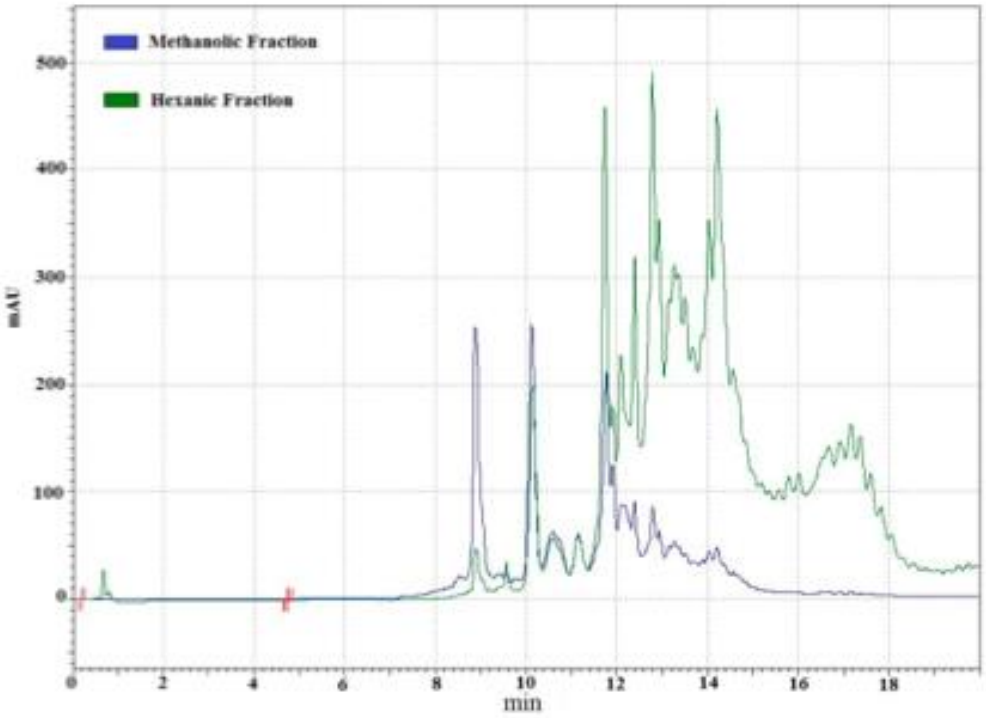
Due to its higher antiproliferative activity, the methanolic fraction from white shrimp lipidic extract was further fractionated by RP-HPLC into 15 different sub-fractions (fractions were obtained every 1.33 min). In order to screen for the compounds with the highest antiproliferative activity, the contents from these fractions were tested against M12.C3.F6 cells at concentrations of 200 and 100 µg/mL (Table 2). Five (M8, M9, M10, M12, and M13) of the tested sub-fractions were selected for further analysis since all showed antiproliferative activity at 100 µg/mL without a significant difference among them. Fractions M8, M9, and M10 showed an orange color while M12 and M13 were colorless; this suggest that the nature of the compounds contained in these two groups of fractions significantly differ from each other.

The antiproliferative activity of the five sub-fractions was evaluated at lower concentrations (Figure 3) and their half inhibitory concentration (IC_{50}) was calculated. Fractions M12 and M13 showed the highest antiproliferative activity at the lowest concentration with IC_{50} of 19.5 ± 8.6 and 34.9 ± 7.3 for M12 and M13 respectively, this concentrations exhibited significant differences ($p < 0.05$). Fraction M12 was selected for further fractioning since it significantly showed the highest antiproliferative activity.

2.3. Fractioning by Open Column Chromatography

In order to continue with the isolation of the bioactive compounds, fraction M12 was subjected to an open column chromatographic procedure. Three fractions were obtained from this chromatographic step and they were coded as M12a, M12b, and M12c. All fractions obtained were analyzed for their chemical structure and antiproliferative activity.

Figure 2. Chromatogram (450 nm) of methanolic and hexanic fractions obtained from lipidic extracts from shrimp.



2.3.1. Chemical/Structural Characterization of M12 Fractions

Fraction M12a was obtained as a colorless liquid. GC-MS spectra exhibited a molecular ion peak *m/z* = 390, corresponding to the molecular condensed formula $C_6H_4(C_8H_{17}COO)_2$ of the compound *di*-(2-ethylhexyl) phthalate, as well as the characteristic fragment ions of this compound with *m/z* values of 278, 166, 149, and 113 (Figure 4); this data was corroborated by the ^{1}H NMR spectra (400 MHz) (Figure 5), where downfield-signals at δ = 7.5–7.75 ppm evidence the presence of hydrogen attached to an aromatic ring in the ortho position. Signals observed at δ = 4.2 and 4.5 ppm may be attributed to protons of adjacent carbons attached to an ester bond (C–O). Finally, chemical shifts at high field (δ = 0.88–1.71 ppm) are attributed to methyl, methylene, and methine protons.

Phthalate derivatives have been previously reported as compounds isolated from various natural sources such as microorganisms [27–29], Aloe vera [30], and from marine organisms such as sponges [31] rhizoid [32], and seahorse [33]. The presence of phthalate in several natural sources is consistent with our findings.

Phthalate derivatives have been reported as bioactive compounds, exerting a variety of biological activities such as antioxidant [31,33], antimicrobial [27,29], cytotoxic [27], anti-leukaemic, and antimutagenic [30] potential. Specifically *di*-(2-ethylhexyl) phthalate, has been previously tested as a compound that can induce cell death in mammalian [34] and leukemic [28] cells, and decreasing cell proliferation in human corneal endothelial cells [35].

Table 2. Percentage of proliferation of M12.C3.F6 murine cells exposed to methanolic fractions at different concentrations.

Fraction	200 µg/mL	100 µg/mL
M1	129.22 ±16.75 ^{a}	113.60 ± 13.22 ^{b}
M2	133.53 ± 11.62 ^{a}	118.22 ± 8.77 ^{b}
M3	129.76 ± 0.51 ^{a}	120.17 ± 10.67 ^{b}
M4	98.06 ± 3.60 ^{b}	117.86 ± 9.05 ^{b}
M5	2.004 ± 2.05 ^{e}	74.61 ± 5.92 ^{c,d}
M6	3.402 ± 3.31 ^{e}	69.14 ± 10.94 ^{c,d}
M7	57.72 ± 6.02 ^{c,d}	171.56 ± 8.20 ^{a}
M8	6.379 ± 4.48 ^{e}	16.16 ± 4.19 ^{e,f}
M9	2.19 ± 0.63 ^{e}	3.28 ± 1.26 ^{f}
M10	6.20 ± 1.48 ^{e}	16.76 ± 4.55 ^{f}
M11	13.00 ± 8.505 ^{e}	44.83 ± 12.21 ^{d,e}
M12	2.67 ± 0.92 ^{e}	2.79 ± 2.84 ^{f}
M13	8.51 ± 3.79 ^{e}	22.84 ± 2.59 ^{e,f}
M14	67.63 ± 8.75 ^{d}	105.95 ± 13.78 ^{b,c}
M15	97.93 ± 5.67 ^{b}	136.19 ± 2.69 ^{a,b}

All values represent mean of triplicate determinations ± standard deviation. Different letters in a column represent significant differences ($p < 0.05$); Control cell cultures were incubated with DMSO (0.5%) and represent 100% proliferation.

However, anthropogenic contamination of marine environments with phthalates has also been reported [36–38], and this kind of compound may be found in both, plant and animal marine species; therefore, the possibility that the antiproliferative phthalate derivative compound isolated from white shrimp in this study might be a product of environmental contamination should be considered.

Regarding fraction M12b, characteristic signals produced by the acyl glycerol type of compound in which glycerol is substituted by unsaturated fatty acids are shown in the ^{1}H NMR spectra (400 MHz) (Figure 6). Downfield-signals at δ = 5.3–5.4 ppm suggest the existence of hydrogens that are characteristically attached to carbon atoms participating in a double-bond; signals at δ = 4.1–4.35 ppm suggest protons of an esterified glycerol; up-field signals at δ = 2.7–2.9 ppm are attributed to *bis*-allylic protons of polyunsaturated fatty acids; however, the characteristic signal of docosahexaenoic acid (DHA) (δ = 2.4 ppm) was not present in the spectra, suggesting that proton signals could be attributed to the presence of eicosapentaenoic acid (EPA) [39]. The other signals may be attributed to oleic acid; both fatty acids have been reported previously as two of the main fatty acids along with DHA and linoleic acid [40].

Figure 3. Percentage of proliferation of M12.C3.F6 murine cells exposed to sub-fractions at different lower concentrations. All values represent mean of triplicate determinations ± standard deviation. Control cell cultures were incubated with DMSO (0.5%).

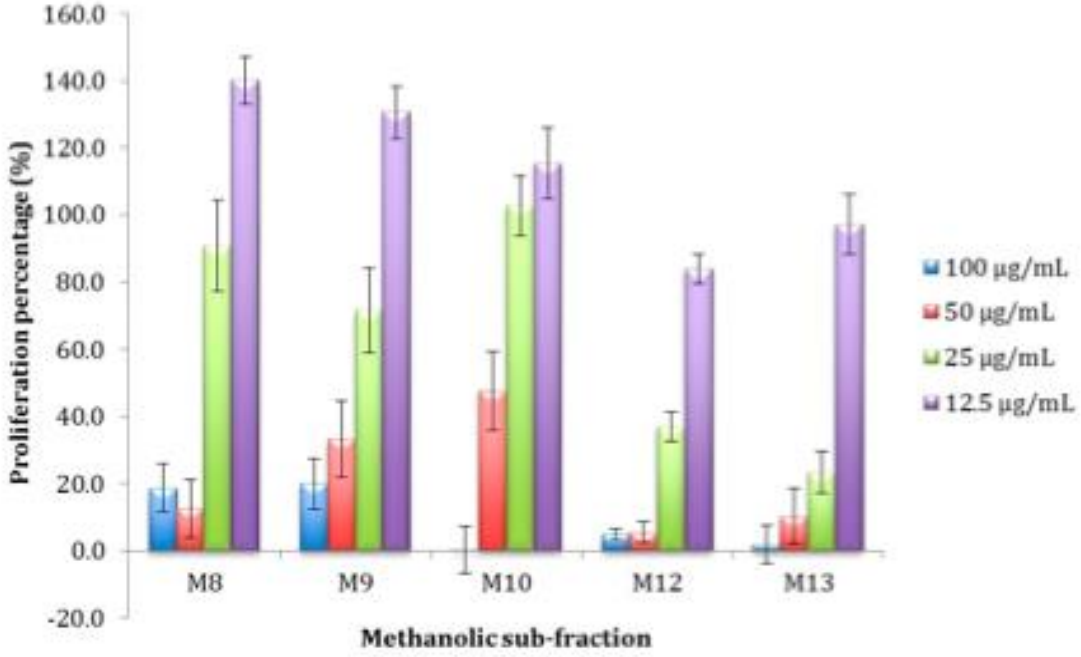


Figure 4. GC-MS spectra of M12a fraction obtained from lipidic extracts of shrimp.

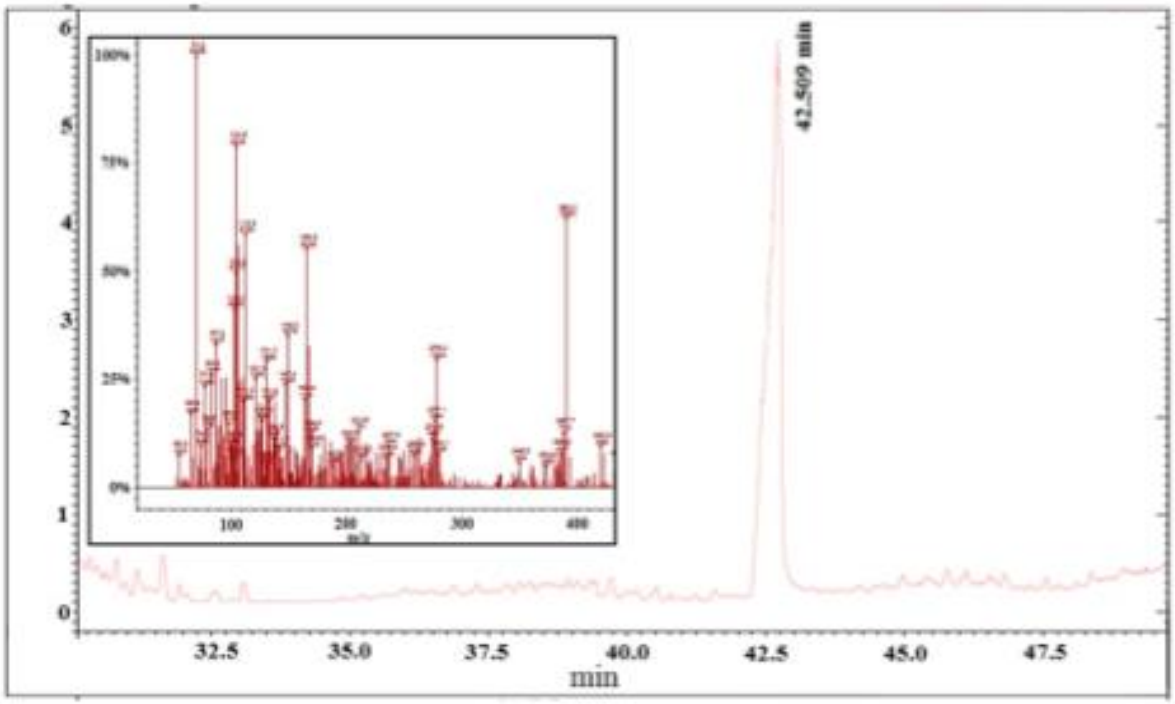


Figure 5. ^{1}H NMR (in $CDCl_3$) spectra of M12a fraction obtained from lipidic extracts from shrimp.

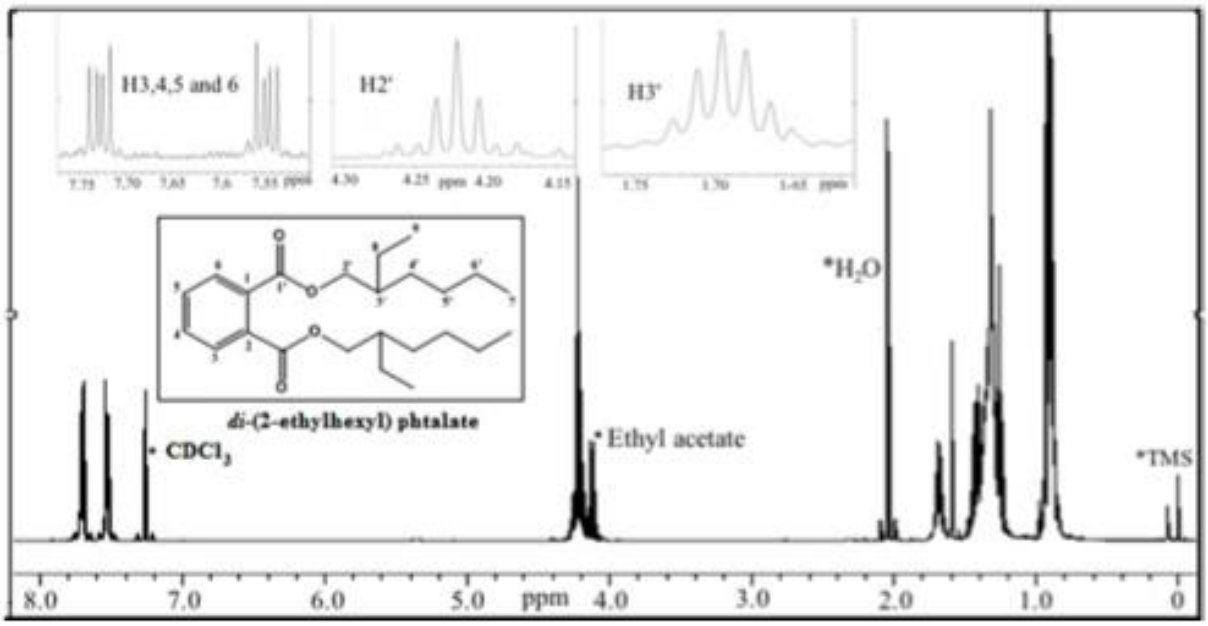
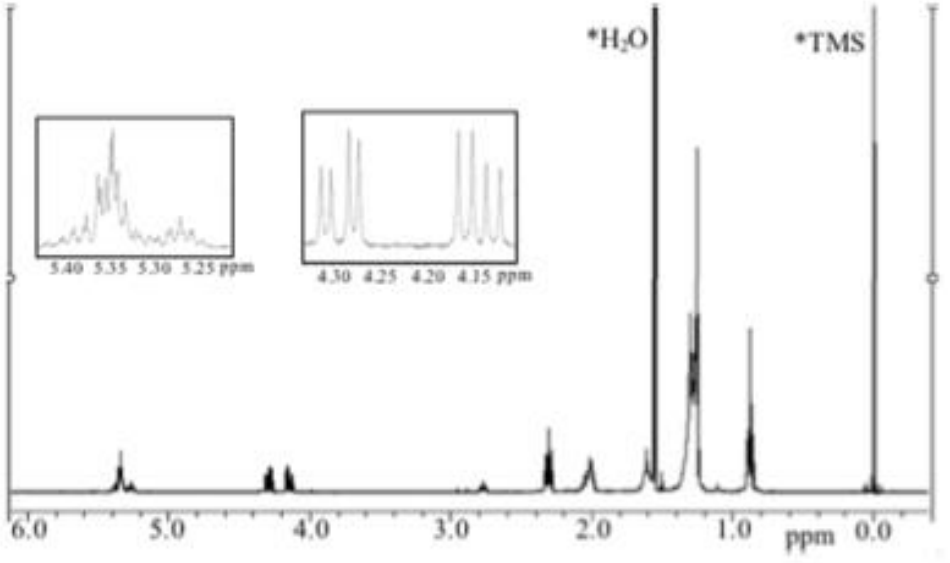
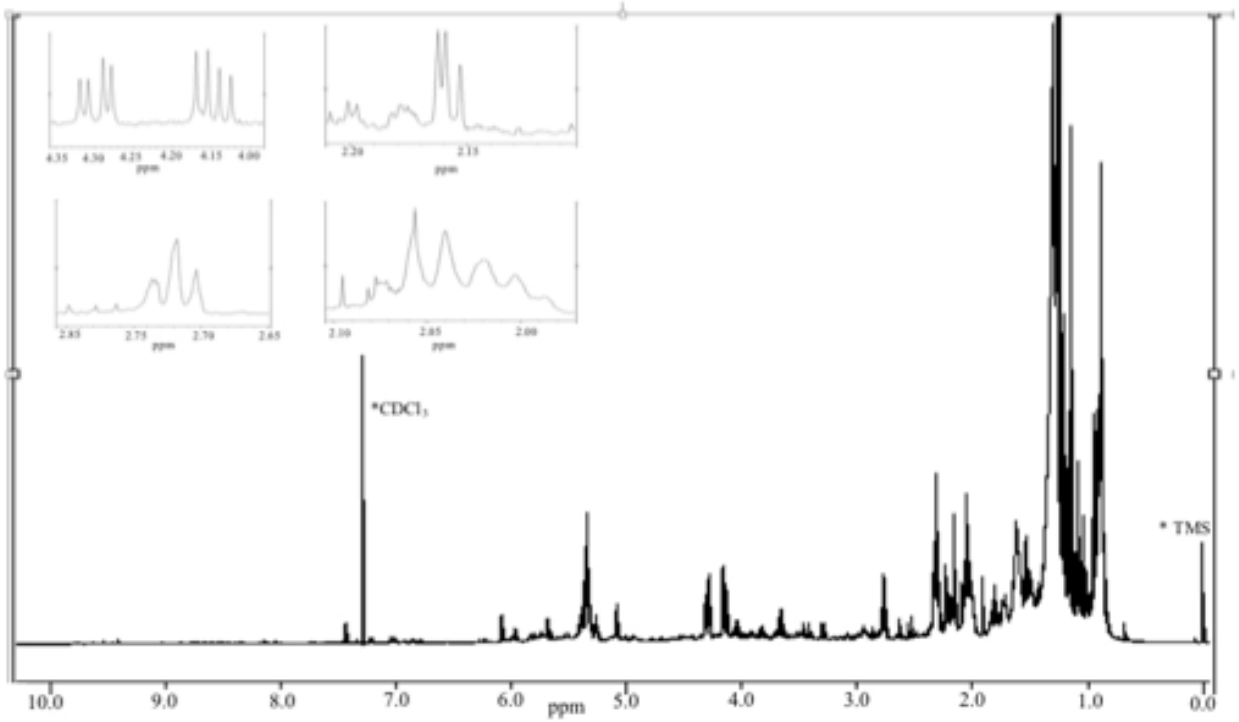


Figure 6. ^{1}H NMR of M12b lipidic fraction in $CDCl_3$.



The ^{1}H NMR spectra (400 MHz) (Figure 7) of fraction M12c also showed characteristic signals of those produced by acyl glycerol type of compounds, in which glycerol is substituted by saturated and polyunsaturated fatty acids. Downfield-signals at δ = 5.3–5.4 ppm, evidence of the existence hydrogen that are characteristically attached to carbon atoms participating in a double-bond, signals at δ = 4.1–4.35 ppm are evidence of protons of an esterified glycerol, up-field, signals at δ = 2.7–2.9 ppm are attributed to *bis*-allylic protons of polyunsaturated fatty acids, and other signals are characteristic of saturated fatty acids such as stearic and palmitic acids.

Figure 7. ^{1}H NMR of M12c lipidic fraction in $CDCl_3$

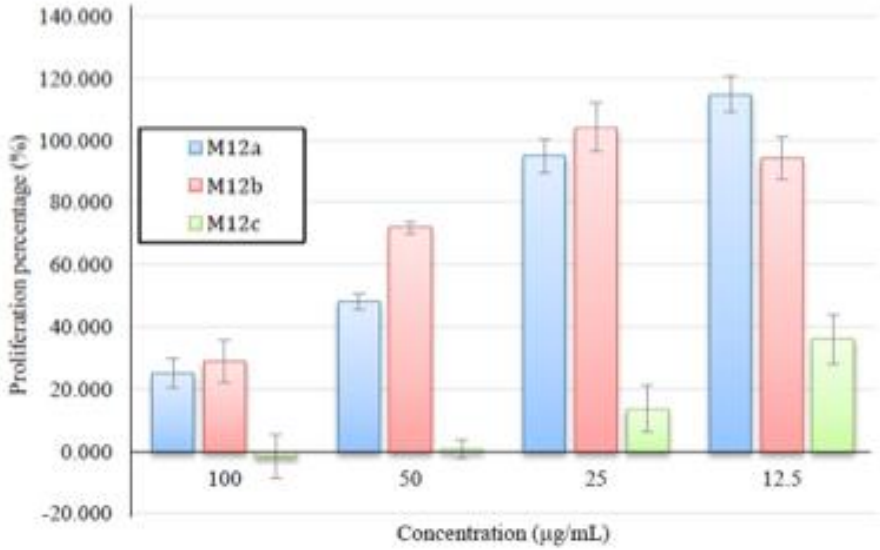


2.3.2. Antiproliferative Activity of M12 Fractions

M12 sub-fractions were further tested in M12.C3.F6 cells with concentrations from 12.5 to 100 µg/mL in order to determine which of the compounds exerted the highest activity (Figure 8). M12c, had the highest activity with an IC_{50} further estimated of 11.33 µg/mL.

Even though previous studies have reported the presence of bioactive compounds in shrimp, most of these were not extracted from shrimp muscle. Antioxidant activity was detected in extracts obtained from several shrimp byproducts such as head [41,42] and shell [43]. Anti-inflammatory properties have been reported in shrimp shell [43] and antimutagenic activity was detected in shrimp muscle using a crude extract [44]. In all these reports, the bioactivities have been attributed to compounds of a carotenoid nature. Nevertheless, only crude extracts were studied and the absorbance at visible spectra wavelength reported, attributing the biological activity to carotenoids; the extracts were not purified in order to identify the chemical structure of the compound responsible for the activity. In our laboratory, we have reported the presence of antiproliferative compounds, initially extracted from shrimp muscle lipidic fraction, which were obtained from fractions after a series of thin-layer chromatography procedures [45]. After an isolation procedure involving solvent partitioning, open-column chromatography and semi-preparative RP-HPLC, the present research work provides evidence that the compound that exerts the highest antiproliferative activity in shrimp are triglycerides esterified with polyunsaturated fatty acids, compounds that have previously been reported as biologically active [46,47].

Figure 8. Antiproliferative activity of M12 sub-fractions. All values represent mean of triplicate determinations ± standard deviation. Control cell cultures were incubated with DMSO (0.5%), which represented 100% proliferation.



3. Experimental Section

3.1. Testing Species

Shrimp (*Litopenaeus vannamei*) was obtained from a local market in Hermosillo, Sonora, Mexico, and transported in ice to the University of Sonora Seafood Laboratory. Edible portions of shrimp were separated, packed, and stored at −20 °C until analysis. The extraction of the shrimp lipidic fraction was carried out according to the methodology reported by [48]. A 100 g shrimp muscle sample was homogenized with five parts of $CHCl_3$ (*w*/*w*) in a blender at high speed for 1 min and the resulting mixture was poured into an Erlenmeyer flask and agitated for 40 min with the aid of a Wrist Action Burrel Shaker (Burrel Corporation, Pittsburg, PA, USA). The mixture was filtered through a Whatman No. 1 filter paper (Whatman, Clifton, NJ, USA) under vacuum and the filtrate was evaporated to dryness under reduced pressure at 40 °C. The lipidic extract was then re-dissolved in methanol-hexane (1:1 *v*/*v*), agitated for 30 min and filtrated again through a Whatman No. 1 filter paper under vacuum. The immiscible phases were separated in a separating funnel and concentrated under reduced pressure at 40 °C, re-dissolved in chloroform, and dried under N_2 stream. All the process was performed in darkness.

3.2. Analysis of Lipidic Composition by RP-HPLC

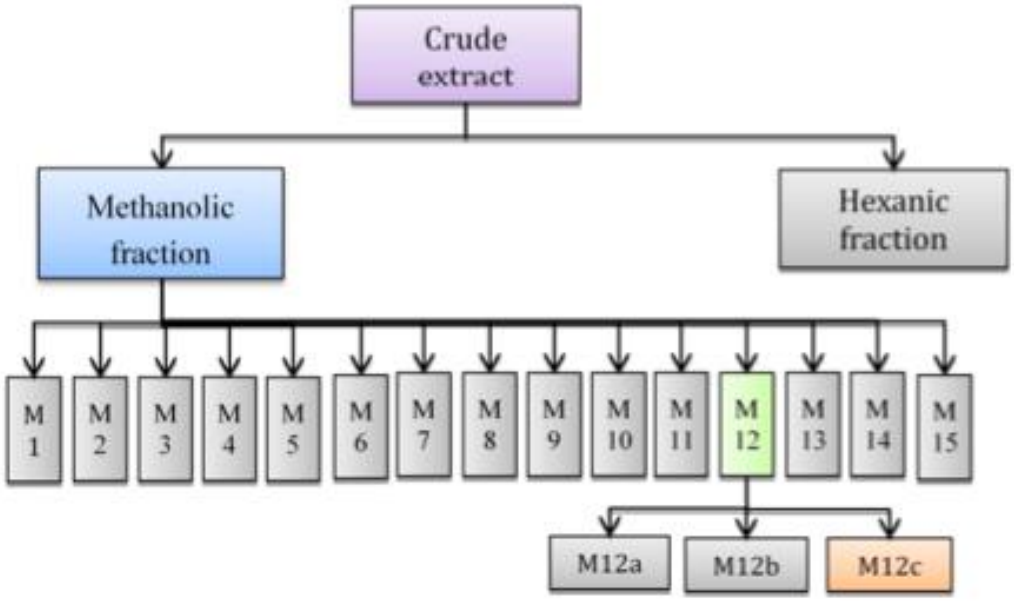
Fractionation of lipidic fractions was carried out by semi-preparative HPLC using an Agilent Technologies HPLC station (Palo Alto, CA, USA) equipped with a Zorbax Eclipse XDB-C18 semi-preparative column (250 mm × 9.4 mm internal diameter; 5 µm particle size; Agilent Technologies). A guard column made of the same material was also used. Aliquots of 100 µL from each extract were injected into the column according to the modified procedure of [49]. Elution of components was performed using a flow rate of 2 mL/min and was continuously monitored by diode

array detector (DAD) (Agilent Technologies) at 450 nm. Column temperature was maintained at 20 °C. Solvents used for elution were water (A), acetone (B), and hexane (C). Lipids were eluted from the column using a linear gradient from 70% A, 30% B to 100% B in 5 min, and then a linear gradient from 100% B to 70% B, 30% C up to minute 20 with a 3-min re-equilibration period at the initial conditions before application of the next sample. Fractions were collected using an Agilent Technologies fraction collector with a flow delay of 30 s, for further chemical characterization. The collected fractions were individually tested for antiproliferative activity.

3.3. Isolation of Bioactive Component by Column Chromatography

The sub-fraction obtained by HPLC with the highest antiproliferative activity was subjected to open column chromatography under gravity on silica gel (3.5 cm × 20 cm using silica, 60–120 mesh, Sigma, St. Louis, MO, USA). Fraction M12 was poured onto the column and eluted using a mobile phase consisting of hexane:ethyl acetate (99:1). The eluents were monitored using TLC (Thin Layer Chromatography) testing plates coated with silica gel and contents were revealed using an iodide solution and observed under *UV* light. The fractions containing similar signals were combined. Schematic representation of the isolation procedure is showed in Figure 9.

Figure 9. Schematic for separation and isolation of antiproliferative fractions from shrimp.



3.4. Cell Lines

Cell line M12.C3.F6 (murine B-cell lymphoma) was kindly provided by Dr. Emil R. Unanue (Department of Pathology and Immunology, Washington University at St. Louis, MO, USA). Cell cultures were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 5% heat inactivated fetal calf serum and grown at 37 °C in an atmosphere of 5% CO_2.

3.5. Antiproliferation Assay

The effect of shrimp lipidic fractions on the proliferation of the M12.C3F6 cell line was determined using the standard MTT assay [50]. Briefly, 10,000 cells (50 µL) were placed in each well of a flat 96-well plate. After 12 h incubation at 37 °C in an atmosphere of 5% CO_2 to allow cell attachment, cell cultures were incubated with 50 µL of medium containing various concentrations of the lipidic fractions and incubated for 48 h. Shrimp lipidic fractions were first re-suspended in DMSO and diluted in supplemented DMEM media. Control cell cultures were incubated with DMSO (final concentrations of DMSO 0.06%–0.5% *v*/*v*). Control cell cultures did not show any evidence of cell damage. Prior to the last 4 h of the cell culture, 10 µL of MTT stock solution (5 mg/mL) were added to each well. Formazan crystals formed were dissolved with acidic isopropanol and the plates were read in an ELISA plate reader (Benchmark Microplate Reader; Bio-Rad, Hercules, CA, USA) using a test wavelength of 570 nm and a reference wavelength of 630 nm. Plates were normally read within 15 min after the addition of isopropanol.

3.6. ^1H NMR Analysis

Measurements were performed using an Agilent Technologies equipment operating at 400 MHz. Each fraction was dissolved in $CDCl_3$ (500 µL; Sigma-Aldrich, Saint Louis, MI, USA) with a small amount of tetramethylsilane (TMS) as internal standard and the resulting mixture was placed into a 5 mm diameter ultra-precision NMR sample tubes. Chemical shifts were recorded in ppm, using TMS proton signal as an internal standard.

3.7. GC-MS Analysis

GC-MS analysis was performed using a Varian 450 chromatograph equipped with a VF-5ms column (30 m × 0.25 mm internal diameter; 0.25 µm film). Aliquots of 3 µL were injected into the column; elution of components was performed using a flow rate of 1 mL/min. Compounds were detected using a Varian 220 mass detector (50–500 *m*/*z*).

3.8. Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) with Tukey-Kramer and Duncan's multiple comparison tests (Number Cruncher Statistical Software (NCSS), Kaysville, UT, USA).

4. Conclusions

The lipidic extract of white shrimp muscle is a source of chemopreventive compounds, and even though the biological activity of shrimp has been previously attributed to the presence of carotenoid compounds, mainly astaxanthin, this study demonstrates that the compounds mainly responsible for the antiproliferative activity are triglycerides substituted with polyunsaturated fatty acids, eicosapentaenoic and saturated fatty acids. While these bioactive triglycerides have shown promising antiproliferative properties against murine tumorous cell lines, further research work is necessary to assess their actual chemotherapeutic potential.

Acknowledgments

The authors wish to acknowledge the National Council for Science and Technology (CONACyT) of Mexico for financing grant proposal 107102 and the graduate scholarship granted to Carmen-María López-Saiz. Thanks to CONACyT Mexico and COVECyT Mexico for FOMIX VER-2009-C03-127523.

Author Contributions

Carmen-María López-Saiz carried out all the experiments, data analysis and their interpretation; Carmen-María López-Saiz and Armando Burgos-Hernández conceived and designed the study, and prepared the draft manuscript; Maribel Plascencia-Jatomea, Maribel Robles-Sánchez and Lorena Machi-Lara critically reviewed the content of this manuscript; and Carlos Velázquez, Francisco-Javier Cinco-Moroyoqui and Javier Hernández made key contributions antiproliferative studies, isolation process, and structural elucidation, respectively. All authors have made intellectual contributions and have given the final approval for the manuscript to be published.

Conflicts of Interest

The authors declare no conflict of interest.

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CAPÍTULO IV

Apoptosis induction in M12.C3F6 cells by triglycerides isolated from white shrimp muscle (*Litopenaeus vannamei*)

Artículo preparado para: Evidence-Based Complementary and Alternative Medicine

Apoptosis induction in M12.C3F6 Murine Cells by Bioactive Triglycerides Isolated from White Shrimp (*Litopenaeus vannamei*) Muscle

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Abstract

Cancer is the leading cause of death worldwide and the research efforts are turning to the search of chemopreventive/chemoprotective. Bioactive triglycerides were isolated from shrimp muscle by chloroformic extraction followed by solvent partition, RP-HPLC fractioning and open chromatographic column isolation. M12.C3F6 line cell was subjected to the effect of bioactive triglyceride, with an IC_{50} of 55.02 ± 7.66 εg/mL. The effect of bioactive triglyceride on cell apoptosis was analyzed by flow citometry where apoptosis induction was observed. Both early and late apoptosis had a significant increase from 10.45 ± 4.88 to 17.65 ± 5.02 and 9.1 ± 2.12 to 15.05 ± 3.04. The activation of caspases was detected in both executer and initiator groups. The activation of caspase 3 had significant increase compared to the negative control, and the signaling pathway activated was the extrinsic via caspase 8. This results prove that the bioactive triglyceride isolated from lipidic extract of white shrimp muscle induces the apoptosis of murine cancer cells via activation of caspases.

Keywords: Shrimp, chemopreventive triglycerides, apoptosis.

Introduction

Cancer, according to the World Health Organization, is the leading cause of death worldwide [1], and affects approximately one of every three individuals in Europe and in the United States of America, appearing as one of one hundred different kinds of this disease [2], with a mortality rate of approximately 20% [3]. The worldwide burden of cancer in the year 2012 was estimated as 14 million of new cases per year, and is expected to rise within the next two decades to 22 million new cases per year [4], for this reason research efforts are turning to the search for compounds capable of intervening in cancer cell spread.

More than 15,000 natural compounds and extracts have been obtained from marine organisms in the search of biological activities [5], and the contributions of the marine environment to therapeutic approaches for cancer are expected to increase in the future [6], among this marine organisms, our laboratory has been able to isolate compounds and fractions with antimutagenic and antiproliferative activities, including different species such as octopus [7], yellowtail fish, lisa fish and cazon fish [8] and white shrimp [9,10], one of the most popular seafood worldwide [11].

Antiproliferative activity in shrimp has been attributed to triglycerides esterified with eicosapentaenoic acid (EPA); this compounds are able to reduce the proliferation rate of M12.C3F6 murine cells [10]. Observations in several cancer cell lines, animal models and some epidemiological studies suggest that polyunsaturated fatty acids of marine species (PUFAs), specifically ϫ-3 fatty acids, are able to decrease cell proliferation and reduce the viability of cancer cells [12,13]. Purified, and specifically EPA, have been previously been reported as antiproliferative compounds [14], and specifically inducing cell apoptosis [15,16].

Apoptosis was first described by Kerr in 1972 [17], a cell process which is characterized by a stereotypical pattern of morphological events that includes cell

shrinkage, membrane blebbing, nuclear condensation and fragmentation, and finally the formation of sealed vesicles called apoptotic bodies [18]. Repression of apoptosis is one of the main underlying problems in the development of cancer, and disruption of the apoptotic program can promote tumor initiation, progression, and resistance to treatment [19]. Based on the above, the aim of the present study is to identify the induction of apoptosis by bioactive triglycerides isolated from shrimp muscle previously reported as antiproliferative.

Methodology

Testing Species

White shrimp (*Litopenaeus vannamei*) was obtained from a local market in Hermosillo, Sonora, Mexico, and transported in ice to the University of Sonora Seafood Laboratory. Shrimp muscle was separated, packed, and stored at ī 20 °C until analysis. Shrimp lipidic fraction was extracted following the methodology reported by Wilson-Sánchez [9]. Each part of shrimp sample was homogenized with five parts of $CHCl_3$ (w/w) in a blender at high speed for 1 min and the resulting mixture was poured into an Erlenmeyer flask and agitated for 40 min with the aid of a Wrist Action Burrel Shaker (Burrel Corporation, Pittsburg, PA, USA). The mixture was filtered through gauze and then through Whatman No. 1 filter paper (Whatman, Clifton, NJ, USA) under vacuum. Filtrate was evaporated to dryness under reduced pressure at 40 °C. The lipidic extract was then re-dissolved in methanol-hexane (1:1 v/v), agitated for 30 min and filtrated again through a Whatman No. 1 filter paper under vacuum.

The immiscible phases were separated in a separating funnel, hexane fraction was discarded and methanolic fraction was concentrated under reduced pressure at 40 °C, re-dissolved in chloroform, and dried under N_2 stream. All the process was performed in darkness, following the isolation process previously described by our group [10].

Isolation of Bioactive Component by Column Chromatography

The methanolic fraction was subjected to open column chromatography under gravity on silica gel (250 mm × 450 mm using silica, 60-120 mesh, Sigma, St. Louis, MO,

USA). Fraction M12 was poured onto the column and eluted using a mobile phase consisting of hexane: ethyl acetate (99:1). The eluents were monitored using Thin Layer Chromatography (TLC) testing plates coated with silica gel and contents were revealed using an iodide solution and observed under UV light. The fractions containing similar signals were combined.

Analysis of Lipidic Composition by RP-HPLC

Fractionation of lipidic fractions was carried out by semi-preparative HPLC using an Agilent Technologies HPLC station (Palo Alto, CA, USA) equipped with a Zorbax Eclipse XDB-C18 semi-preparative column (250 mm × 9.4 mm internal diameter; 5 µm particle size; Agilent Technologies). A guard column made of the same material was also used. Aliquots of 100 εL from each extract were injected into the column according to the modified procedure of [20]. Elution of components was performed using a flow rate of 2 mL/min and was continuously monitored by diode array detector (DAD) (Agilent Technologies) at 450 nm. Column temperature was maintained at 20 °C. Solvents used for elution were water (A), acetone (B), and hexane (C). Lipids were eluted from the column using a linear gradient from 70 % A, 30 % B to 100 % B in 5 min, and then a linear gradient from 100 % B to 70 % B, 30 % C up to minute 20 with a 3-min re-equilibration period at the initial conditions before application of the next sample. Fractions were collected using an Agilent Technologies fraction collector with a flow delay of 30 s, M12 was collected following the isolation process; the rest of the fractions were discarded.

Cell Lines

Cell line M12.C3.F6 (murine B-cell lymphoma) was kindly provided by Dr. Emil R. Unanue (Department of Pathology and Immunology, Washington University at St. Louis, MO, USA). Cell cultures were cultured in Dulbeccoôs modified Eagleôs medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 5% heat inactivated fetal calf serum and grown at 37 °C in an atmosphere of 5 % CO_2.

Antiproliferation Assay

The effect of shrimp lipidic fractions on the proliferation of the M12.C3F6 cell line was determined using the standard MTT assay [50]. Briefly, 10,000 cells (50 εL) were placed in each well of a flat96-well plate. After 12 h incubation at 37 °C in an

atmosphere of 5 % CO_2 to allow cell attachment, cell cultures were incubated with 50 εL of medium containing various concentrations of the lipidic fractions and incubated for 48 h. Shrimp lipidic fractions were first re-suspended in DMSO and diluted in supplemented DMEM media. Control cell cultures were incubated with DMSO (final concentrations of DMSO 0.06 %ï 0.5 % v/v). Control cell cultures did not show any evidence of cell damage. Prior to the last 4 h of the cell culture, 10 εL of MTT stock solution (5 mg/mL) were added to each well. Formazan crystals formed were dissolved with acidic isopropanol and the plates were read in an ELISA plate reader (Benchmark Microplate Reader; Bio-Rad, Hercules, CA, USA) using a test wavelength of 570 nm and a reference wavelength of 630 nm. Plates were normally read within 15 min after the addition of isopropanol.

Apoptosis Detection by Flow Citometry

Apoptosis of M12.C3F6 cell culture was detected by flow citometry following methodology described Vermes [21]. Cells were treated with bioactive compound and two positive controls, caffeic acid phenethyl ester (CAPE) and doxorubicin, with respective IC_{50}, cells where then double stained for FITC-annexin V (AnV) binding and for cellular DNA using of propidium iodide (PI). Briefly, cells were washed with PBA 1X, re-suspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl,). FITC-annexin V was added to a final concentration of 1 pg/ml annexin V, 0.1 mL of PI (10 pg/ml in binding buffer) was added resulting in a final concentration of 1 pg PI/ml cell suspension. The mixture was incubated for 10 min in the dark at room temperature and then measured by bivariate FCM. Apoptosis was measured at 12 and 20h.

Caspase activation

Caspase activation was measured by a fluorometric assay using caspase family fluorometric substrate kit II plus (Abcam, England). Briefly, cell apoptosis was induced with bioactive compounds and cisplatin, using IC_{50} of both compounds; 24 h later, cells were lysed and the cytosolic extract reacted with 7-amino-4-trifluoromethyl coumarin (AFC) conjugated substrates. Samples were read in a fluorometer (400 nm

excitation and 505 nm emission). Results were compared with un-induced control cells.

Results and Discussion

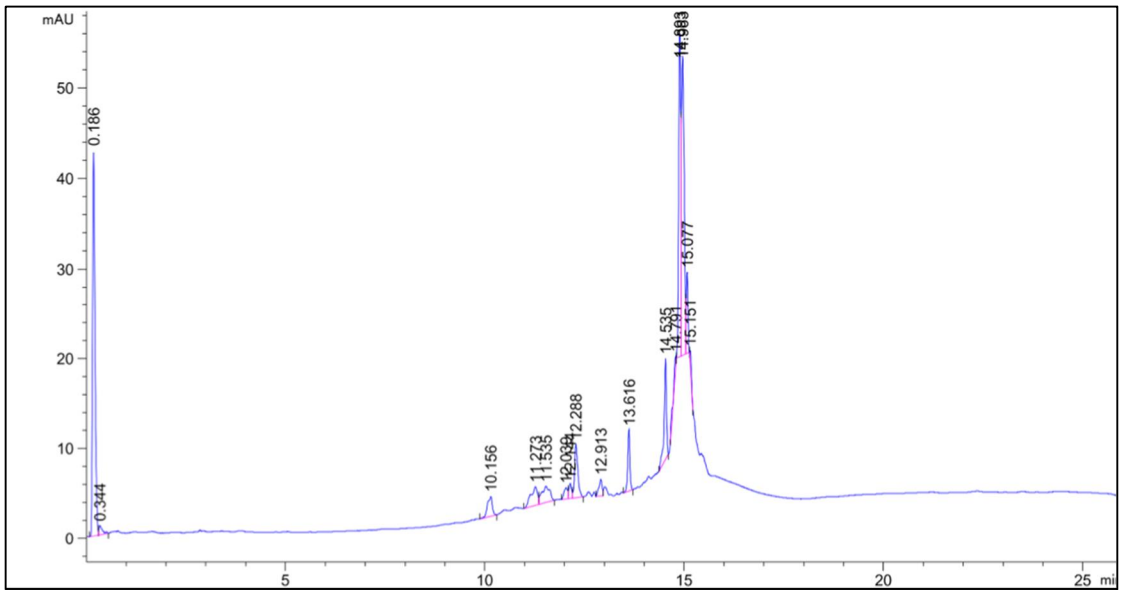
Crude extract, and methanolic fraction were obtained following our previous report [10]; where a bioactive triglyceride esterified with EPA and two saturated acids was obtained; even though antiproliferative bioactivity was previously reported in shrimp muscle [9], the bioactive triglyceride responsible for the highest antiproliferative activity was previously isolated at our laboratory.

Polyunsaturated fatty acids have been reported as antiproliverative compounds, specifically EPA exerts different activities according to the cell line tested. Cell cycle arrest at G_2/M has been reported in pancreatic cancer cells [22,23] and breast cancer [24] and also at G_0/G_1 in leukemia cell lines [25], but a triglyceride has not been reported as a apoptosis inductor.

Antiproliferative activity

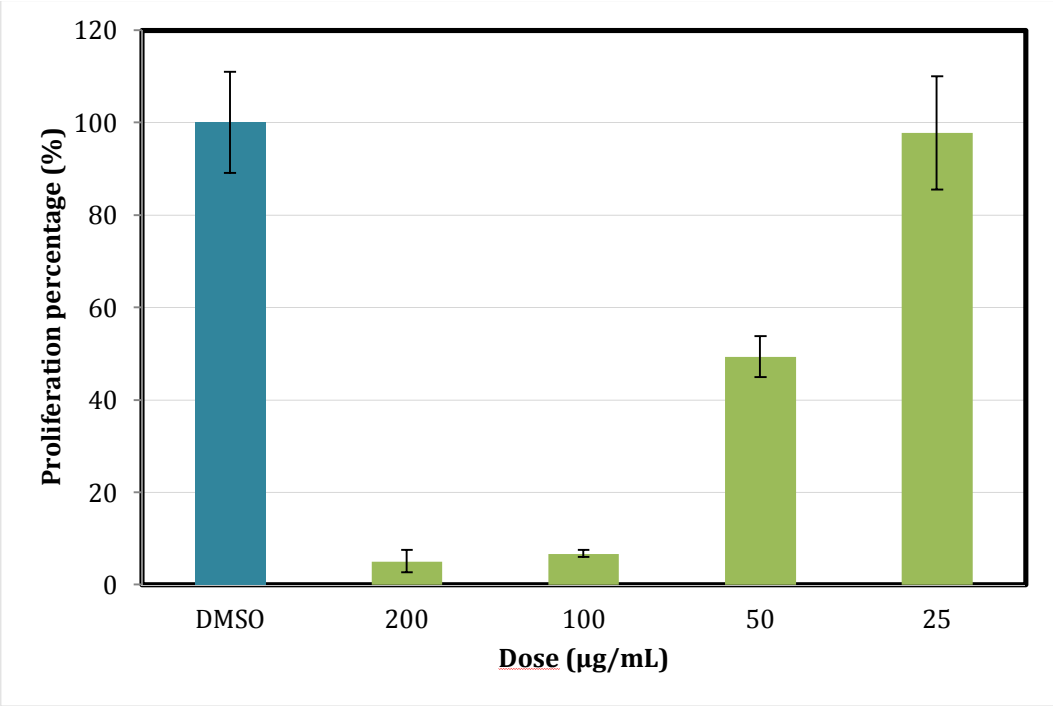
Antiproliferative activity of bioactive triglyceride was assessed following the MTT assay. One major compound was isolated with a retention time of 14.9 min following described methodology (Figure 1) and antiproliferative activity was confirmed (Figure 2).

Figure 1. RP-HPLC of bioactive triglyceride isolated from shrimp muscle



The isolated triglyceride showed an IC_{50} of 55.01 ± 7.66 µg/mL. Antiproliferative activity has been previously reported on polyunsaturated fatty acids [13,25,26].

Figure 2. Antiproliferative activity of triglyceride isolated from shrimp muscle on murine M12.C3F6 cells.



Apoptosis Detection by Flow Citometry

During the apoptotic process, phosphatidyl serine translocates from the internal to external leaflet of the plasmatic membrane, making it available for labeling by AnV, and once the membrane is broken, cell can also be labeled with PI, since it binds to DNA material inside the cell. Viable cells are negative for both stains (AnV and PI); in early apoptotic states, cells present a positive signaling for AnV, and, in late apoptosis, they present a positive signal for both AnV and PI [21]. Following this principle, apoptosis induction was studied by double staining of M12.C3F6 and further analyzed by flow cytometry (Figure 3).

Apoptosis was measured at 12 and 20 h after exposure, and both early and late apoptosis had a significant increase in this period of time from 10.45 to 17.65 and

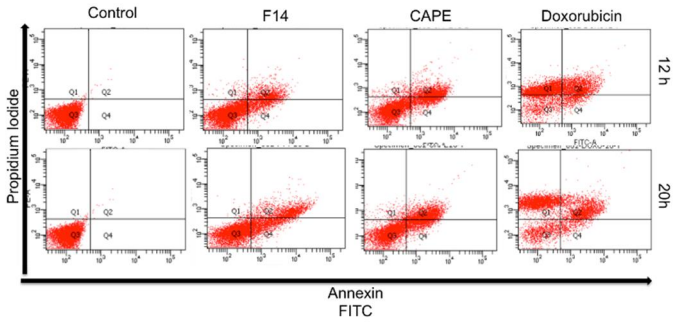
from 9.1 to 15.05, respectively. This results suggest that apoptosis is being induced by the bioactive triglyceride. This effect has been previously reported for polyunsaturated fatty acids found in marine species; docosahexaenoic acid (DHA) has been reported as apoptosis inductor in melanoma [26] and HT-29 [15] line cells; as for EPA, on HL-60 [25], lymphoma [16], HT-29 [15] and pancreatic [27] line cells.

Caspase activation

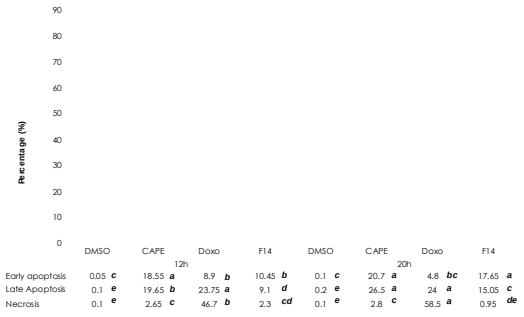
Since caspase activation is the main signaling cascade of cellular apoptosis, we measured activity of both, initiator (caspases 8, 10, 9 and 2) and executioner (caspases 3 and 7) caspases [28].

Caspases 3 and 7 were activated by bioactive triglyceride with significant difference (Figure 4). The activation of this pathway has been previously reported for the two main polyunsaturated fatty acids characteristically found in marine organisms, DHA [29] and EPA [29,30]. The activation of this signaling pathway can be due either intrinsic or extrinsic pathways; both groups of caspases were analyzed (Figure 5).

Figure 3. Induction of apoptosis by bioactive triglycerides isolated from shrimp muscle on M12.C3F6 murine cells. Cells were treated with 55 ε g/mL F14, 2 ε M CAPE and 10 ε M doxorubicin. **(A)** Quadrant location for the representative dot plots: lower left ï negative immunofluorescence (living cells); lower right ï annexin V positive (early apoptosis); upper left ï PI positive (necrosis), upper right ï annexin V and PI positive (late apoptosis). **(B)** Results are represented as the average of three different experiments. Different letters in the same row represent significant differences ($p<0.05$).

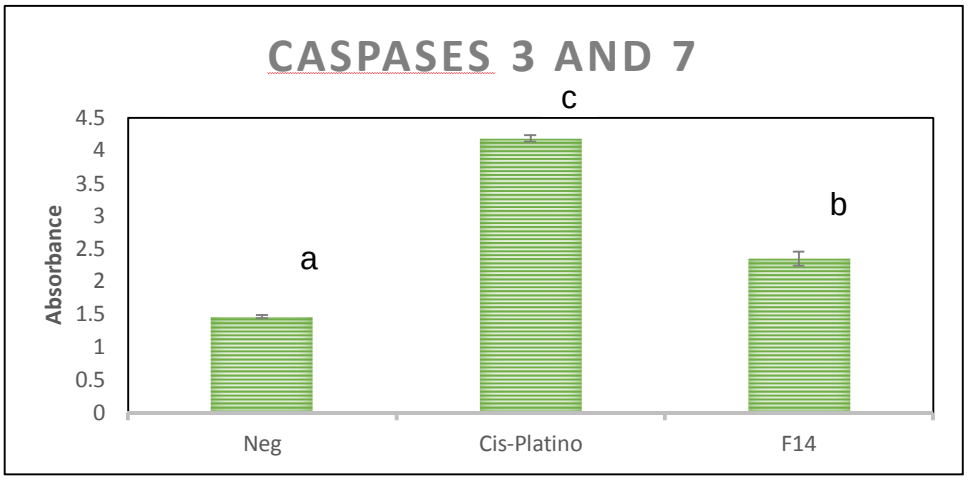


(A)



(B)

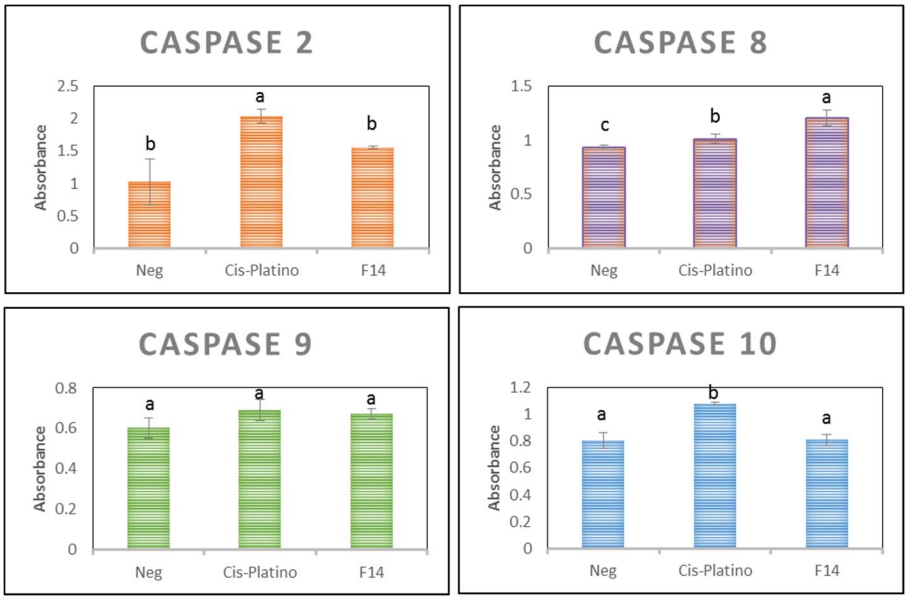
Figure 4. Activation of executioner caspases by the effect of bioactive triglyceride (F14)



Caspase 8 was activated by the bioactive triglyceride suggesting that the extrinsic pathway is the one that is taking place. These results agree with those of Arita and Giros [31,32] where the activation of the extrinsic pathways, specifically the activation of caspase 8, was reported when HL-60 and colorectal cancer cell lines were exposed to PUFAs.

Figure 5. Activation of initiator caspases by the effect of bioactive triglyceride (F14)

Intrinsic Pathway **Extrinsic Pathway**



Conclusion

The bioactive triglyceride isolated from white shrimp muscle exerts antiproliferative activity by activating the apoptosis mechanisms, specifically through the activation of the intrinsic pathway, with the activation of caspase 8; to our knowledge, this is the first time a bioactive triglyceride has been an apoptosis inductor.

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CONCLUSIONES

El extracto lipídico del músculo de camarón es una fuente de compuestos quimiopreventivos, incluyendo compuestos con actividad antioxidante, antimutagénica y antiproliferativa.

La actividad antiproliferativa que exhibe el extracto lipídico de camarón se debe a la presencia de triglicéridos sustituidos con ácido eicosapentaenóico y otros dos ácidos grasos poliinsaturados

La actividad antimutagénica se debe a la presencia de compuestos resultantes de la oxidación de los compuestos carotenoides, estos son apocarotenoides que presentan en su estructura anillos aromáticos.

El triglicrérido bioactivo es capaz de provocar la apoptosis de células de cáncer murino por medio de la activación de la vía extrinseca (Caspasa-8)

RECOMENDACIONES

Aun cuando estos compuestos bioactivos aislados en esta investigación muestran actividades biológicas prometedoras, se necesita más investigación para conocer su verdadero potencial quimioterapéutico.

Se recomienda buscar otras actividades biológicas en las diferentes fracciones del extracto lipídico de camarón, incluyendo las actividades antiinflamatoria y antiangiogénica; actividades biológicas importantes en la quimioprevención del cáncer

Se sugiere además, realizar ensayos para determinar el mecanismo de acción por el cual el compuesto antimutagénico está realizando su función, lo cual se puede establecer con la ayuda del ensayo cometa, y la intervención con la enzima citocromo P450.

Se aconseja continuar con los estudios de intervención de los compuestos antiproliferativos en el ciclo celular para estudiar si se está llevando a cabo un arresto celular dentro del mismo

Se recomienda realizar estudios *in vivo* con los compuestos antimutagénicos aislados de esta investigación, con el fin de establecer si estos ejercen su bioactividad en modelos animales.