

Antibacterial and antimycobacterial activity of white shrimp (*Litopenaeus vannamei*) exoskeleton and cephalothorax by-products extracts: fatty acids profile of the active hexanic shrimp cephalothorax extract

Actividad antibacteriana y antimicobacteriana de los subproductos exoesqueleto y cefalotórax del camarón blanco (*Litopenaeus vannamei*): perfil de ácidos grasos del extracto activo hexánico del cefalotórax del camarón blanco

López-Saiz CM¹, Coronado-Aceves EW², Tavera-Hernández R³, Espitia-Pinzón CI², Jiménez-Estrada M³, Morán-Corrales PG⁴, Hernández-Zazueta MS^{1*}

¹ Department of Research and Postgraduate Studies in Food, University of Sonora. Blvd. Luis Encinas and Rosales, CP 83000, Hermosillo, Sonora, México.

² Biomedical Research Institute. National Autonomous University of México. CP 04510. México City, México.

³ Institute of Chemistry. National Autonomous University of México. CP 04510. México City, México.

⁴ Department of Chemical Engineering and Metallurgy. University of Sonora. CP 83000. Hermosillo, Sonora, México.

ABSTRACT

The objective of this study was to evaluate the antibacterial and antimycobacterial potential of the by-products of white shrimp (*Litopenaeus vannamei*). The following extracts were obtained: exoskeleton hexanic, methanolic and aqueous extracts (ExHex, ExMe, ExAc); and cephalothorax hexanic, acetonic and methanolic extracts (CeHex, CeAce, CeMe). Antibacterial effect was determined by the broth microdilution method against Gram-positive bacteria: *Enterococcus faecalis* American Type Culture Collection (ATCC) 51299, *Staphylococcus aureus* ATCC 25293, and *Staphylococcus epidermidis*; Gram-negative bacteria: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC 10145, and *Salmonella typhimurium*; and *Mycobacterium bovis* bacillus Calmette-Guérin (*M. bovis* BCG) Danish strain. CeHex resulted active against all Gram-positive and Gram-negative bacteria (MIC₅₀ = 400 µg mL⁻¹) and against *M. bovis* BCG (MIC₁₀₀ = 250 µg mL⁻¹). Gas chromatography (GC) of CeHex identified oleic, linoleic, palmitic, stearic, behenic, palmitoleic and linolenic fatty acids. The strong antibacterial activity of CeHex and the identification of its main chemical constituents justify further studies on the clinical applications of this marine by-product.

Keywords: *Litopenaeus vannamei*, antimicrobial, *Mycobacterium*, by-products, fatty acids

RESUMEN

El objetivo de este estudio fue evaluar el potencial antibacteriano y antimicobacteriano de los sub-productos del camarón blanco (*Litopenaeus vannamei*). Fueron obtenidos los siguientes extractos: extracto hexánico, metanólico y acuoso del exoesqueleto (ExHex, ExMe, ExAc); y extracto hexánico, acetónico y metanólico del cefalotórax (CeHex, CeAce, CeMe). El efecto antibacteriano fue evaluado mediante el método de microdilución en caldo contra las bacterias

Gram-positivas: *Enterococcus faecalis* American Type Culture Collection (ATCC) 51299, *Staphylococcus aureus* ATCC 25293, y *Staphylococcus epidermidis*; bacterias Gram-negativas: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC 10145, y *Salmonella typhimurium*; y *Mycobacterium bovis* bacilo Calmette-Guérin (*M. bovis* BCG) cepa Danesa. CeHex resultó activo contra todas las bacterias Gram-positivas y Gram-negativas (MIC₅₀ = 400 µg mL⁻¹) y contra *M. bovis* BCG (MIC₁₀₀ = 250 µg mL⁻¹). Mediante cromatografía de gases (GC) de CeHex se identificaron los ácidos grasos: oleico, linoleico, palmítico, esteárico, behénico, palmitoleico y linolénico. La fuerte actividad antibacteriana de CeHex y la identificación de sus principales componentes químicos justifican estudios posteriores en las aplicaciones clínicas de este sub-producto marino.

Palabras clave: *Litopenaeus vannamei*, antimicrobiano, *Mycobacterium*, sub-productos, ácidos grasos

INTRODUCTION

Mexico's shrimp farming sector harvest a total of 177,000 tonnes in 2021 according to the National Commission of Aquaculture and Fisheries (CONAPESCA). Mexico has around 900 shrimp farms, 45 hatcheries and over 40 shrimp processing plants. Sinaloa is the main producer of farmed shrimp (40.3 %), followed by Sonora (39.7 %) and Nayarit (7.5 %) (TheFishSite, 2021).

Only 65% of the crustacean is used for human consumption, the rest corresponds to the exoskeleton and cephalothorax (Núñez-Gastélum *et al.*, 2011). The waste is almost completely discarded, except that only a little is processed as an excellent animal feed supplement (Nwana *et al.*, 2004).

Seafood by-products are valuable natural resources that show range of functionalities and hence potential materials for biomedical and nutraceutical industries (Se-

*Autor para correspondencia: Martín Samuel Hernández Sazueta
Correo electrónico: martinsamuel.hernandez@gmail.com

Recibido: 23 de diciembre de 2021

Aceptado: 7 de marzo de 2022

nevirathne and Kim, 2012; Hernández-Zazueta *et al.*, 2021a; Hernández-Zazueta *et al.*, 2021b).

Shrimp industry wastes have attracted attention due to the presence of valuable bioactive compounds as proteins, lipids, pigments (carotenoids), chitin/chitosan, oligosaccharides, vitamins, etc. (Sachindra *et al.*, 2006; Nirmal *et al.*, 2020). Bioactive evaluations reported include antimicrobial activity (*Stenotrophomonas maltophilia*, *Enterobacter cloacae* and *Bacillus subtilis*; *Lactobacillus helveticus*, *L. innocua*, *S. aureus*, *Citrobacter freundii*, *E. coli*, and *P. fluorescens*) (Vilar *et al.*, 2016; Djellouli *et al.*, 2020), antioxidant, ACE (Angiotensin I converting enzyme) inhibitory activity, and antiinflammatory (Nirmal *et al.*, 2020).

Natural products take the leading place in drug discovery of antimicrobial agents highlighting the fact that approximately 70% of antibiotics clinically used for treatment of infectious diseases are derived from nature (Brown *et al.*, 2014).

To the best of our knowledge, this is the first report of these *L. vannamei* by-products extracts activity against this set of clinical relevance bacteria (*E. faecalis*, *S. aureus*, *S. epidermidis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. typhimurium*; and *M. bovis* BCG).

MATERIALS AND METHODS

Shrimp material

A sample of white shrimp (*L. vannamei*) was obtained in the central-western region of the state of Sonora, in the town of Bahía de Kino (28° 49'22" N 111° 56'27" W). The raw material was separated into exoskeleton and cephalothorax, ground (Osterizer, Oster, USA) and stored in polyethylene bags at -18 °C until use.

Preparation of shrimp extracts

Twenty g of shrimp material were homogenized with 60 mL of solvent (proportion 1:3 weight/volume) (n-hexane, acetone, metanol or water, Químicos Fermont, Mexico) in a blender (Osterizer, Oster, USA) at high speed for 1 min and the resulting mixture was kept in an Erlenmeyer flask at room temperature for 24 h in darkness. Solids were filtered out (filter paper Whatman no. 1), the extracts concentrated by evaporation under reduced pressure at 30 °C in a rotary evaporator (HS-20055-N, Hahn vapor Hahnshin Scientific Co., Republic of Korea), and dried under N₂ stream (López-Saiz *et al.*, 2014; Osuna-Ruiz *et al.*, 2016). The following extracts were obtained: exoskeleton hexanic, methanolic and aqueous extracts (ExHex, ExMe, ExAc); cephalothorax hexanic extract, acetic and methanolic extracts (CeHex, CeAce, CeMe).

Antibacterial Activity

Bacterial strains

Bacterial strains used in this study: Gram-positive bacteria (*Enterococcus faecalis* American Type Culture Collection (ATCC) 51299, *Staphylococcus aureus* ATCC 25293, and *Staphylococcus epidermidis*) and Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae*, *Pseudo-*

monas aeruginosa ATCC 10145, and *Salmonella typhimurium*), obtained from the ceparium of the Department of Chemical Biological Sciences of the University of Sonora. Before testing, all bacterial strains were maintained frozen at -70 °C in 10 % glycerol broth.

Preparation of working solution

Each organic extract was dissolved in 100 % dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) (20 mg mL⁻¹) and maintained at room temperature for 1 h to assure their sterilization (Molina-Salinas *et al.*, 2006). These extracts were diluted with fresh Mueller Hinton broth (BD DIFCO, Sweden) to their final concentrations of 50, 100, 200 and 400 µg mL⁻¹.

Preparation of inoculum

Bacterial colonies grown on Mueller Hinton agar (MCD Lab, México) for 18 - 24 h (log phase of growth) were transferred to a sterile vial containing 15 mL of sterile 0.85 % saline solution. The bacterial suspension was disaggregated by agitation using a Genie II vortex, speed 3, for 1 minute, and left to stand for 10 min at room temperature. The supernatant was then adjusted to the optical density of OD_{630 nm} = ~0.095, a turbidity matching the 0.5 McFarland standard (1.5 x 10⁸ colony forming units CFU mL⁻¹).

Antibacterial assay

In vitro antibacterial studies were carried out by the broth microdilution method as described previously (Velazquez *et al.*, 2007; Navarro-Navarro *et al.*, 2013). Briefly, 15 µL (2.25 x 10⁶ CFU) of the inoculum (Velazquez *et al.*, 2007) were inoculated into each well of a flat 96-well microplate (Costar, Corning, USA), containing 200 µL of different concentrations of the organic extracts (50–400 µg mL⁻¹) in Mueller Hinton Broth (BD DIFCO, Sweden). Additionally, each antibacterial test included wells containing the culture media plus DMSO (2 %), in order to obtain a control of the solvent's antibacterial effect. Gentamicin (12 µg mL⁻¹) (AMSA, México) was used as positive control of bacterial growth inhibition. Bacterial cultures were incubated at 37 °C for 48 h. Plates were read at 630 nm in an enzyme-linked immunoassay (ELISA) microplate reader (Benchmark Microplate Reader, Bio-Rad, Hercules, USA) at 6, 12, 24, and 48 h. The optical density (OD_{630 nm}) was corrected by subtracting the OD_{630 nm} from wells with extracts alone in sterile broth. The minimal inhibitory concentration was defined as the lowest extracts concentrations that inhibited at least 50 % (MIC₅₀) or 90 % (MIC₉₀) of the bacterial growth after incubation at 37 °C for 24 h. MICs were determined using the following criteria (Baizman *et al.*, 2000; Velazquez *et al.*, 2007):

$$\text{MIC}_{50} : (\text{OD}_{630 \text{ nm}} \text{ untreated bacteria} - \text{OD}_{630 \text{ nm}} \text{ test concentration}) / (\text{OD}_{630 \text{ nm}} \text{ untreated bacteria}) \times 100 \geq 50 \%$$

$$\text{MIC}_{90} : (\text{OD}_{630 \text{ nm}} \text{ untreated bacteria} - \text{OD}_{630 \text{ nm}} \text{ test concentration}) / (\text{OD}_{630 \text{ nm}} \text{ untreated bacteria}) \times 100 \geq 90 \%$$

Antimycobacterial Activity of Shrimp Extracts

Mycobacterial strain

Mycobacterium bovis bacillus Calmette-Guérin (*M. bovis* BCG) Danish strain was obtained from the ceparium of

the BSL3 laboratory of the Biomedical Research Institute of the National Autonomous University of Mexico.

***M. bovis* BCG inoculum preparation**

M. bovis BCG strain was cultivated in 50 mL of Middlebrook 7H9 broth (Becton Dickinson, USA) supplemented with 0.2 % (v/v) glycerol, and 10 % (v/v) ADC (albumin, dextrose, catalase enrichment) (Becton Dickinson, USA) (MDB 7H9) to which 0.02 % (v/v) tyloxapol (Sigma-Aldrich, USA) was added. Bacteria was incubated at 37 °C until an OD_{600 nm} of 0.39 was reached (equivalent to 1 McFarland unit). Working bacteria solution was prepared by a 1:25 dilution in MDB 7H9 (Peñuelas-Urquidez *et al.*, 2013; Guzmán-Gutiérrez *et al.*, 2020).

Resazurin microtiter assay (REMA) to evaluate inhibitory activity against *M. bovis* BCG

This assay was adapted from Collins and Franzblau (1997). In 96-well polystyrene flat bottom plates, 200 µL of sterile distilled water were added to the perimetral wells, and 100 µL of MDB 7H9 broth were added to the remaining wells. Working solutions of extracts (2000 µg mL⁻¹) were distributed into the first well of each row, and 2-fold dilution series were made using the following four wells. 100 µL of inoculum was added to each test well. The final concentrations in-test ranged from 31.25 to 500 µg mL⁻¹. The controls of this experiment were: rifampicin (Sigma-Aldrich, USA) (concentrations of 16–9.7x10⁻⁴ µg mL⁻¹), MDB 7H9, MDB 7H9 with bacteria, extracts (without bacteria), 2.5 % DMSO, and 2.5 % DMSO with bacteria. The microplate was sealed with parafilm and incubated for 6 days at 37 °C. Further, 30 µL of 0.01 % resazurin sodium salt (weight/volume) (Sigma-Aldrich, USA) (Palomino *et al.*, 2002) were added to each well and plates were reincubated for 48 h. The minimum inhibitory concentration (MIC) was defined as the minimum concentration of crude extract that prevented the color shift from resazurin (blue) to resorufin (pink). Experiments were performed in triplicate.

Determination of the fatty acid profile

The fatty acid profile was determined as fatty acid methyl esters (FAMES), which were prepared adding to 10 mg of the CeHex, 200 µL of benzene and 200 µL of the derivatizing reagent Meth-Prep II (GraceTM AlltechTM). This reagent is a 0.2 N methanolic solution of (m-trifluoromethylphenyl) trimethylammonium hydroxide. The transesterification reaction was carried out at room temperature for 30 minutes to obtain the FAMES mixture. 1 µL of the FAMES mixture was injected to the gas chromatograph (Agilent 6890) equipped with a flame ionization detector (FID), and the AT-FAME column (30 m x 0.25 mm). The analytical conditions were: injection 1 mL, injector temperature 250 °C, detector temperature 250 °C. The temperature gradient in the column oven starts at 180 °C for 15 min, followed by 10 °C / min increments up to 230 °C. The FAME standards retention times were used to identify the chromatographic peaks of the samples. Fatty acid content was calculated, based on the normalized peak area of detected FAMES.

Statistical analysis

Antibacterial results were expressed as mean ± standard deviation of three independent experiments. Statistical analysis performed was one-way analysis of variance (Tukey) and the graphs of bacterial growth kinetics were made with GraphPad Prism © Version 5.01 software.

RESULTS AND DISCUSSION

Antibacterial activity

CeHex was the most potent extract, active against all Gram-positive and Gram-negative bacteria tested with a MIC₅₀ = 400 µg mL⁻¹; subsequently, CeAce was active against *E. faecalis*, *E. coli* and *K. pneumoniae* (MIC₅₀ = 100, 400 and 400 µg mL⁻¹, respectively). While CeMe resulted inactive. It is clear that the shrimp cephalothorax extracts were the most potent antibacterial samples tested. Non-polar or low polarity compounds may be the main responsible of the antibacterial activity described in CeHex. Despite antibacterials are collectively classified as large and polar compounds with relatively low lipophilicity (Mugumbate *et al.*, 2015), in this research increasing polarity of solvents diminished the antibacterial activity. Solvent polarity is usually the most influential factor on the yield of the extraction (Bayona *et al.*, 2018), has a great impact on selectivity, and influence directly on the solutes extracted, related to the chemical structure of the compounds (Lefebvre *et al.*, 2020). Recent research described that the shrimp oil extracted from cephalothorax (with a 1:1 hexane/isopropanol mixture) possesses an important amount of cholesterol (89.1 ± 0.6 mg/g) (Raju *et al.*, 2021), then it is possible that cholesterol is also present and abundant in the acetonic and methanolic extracts, affecting the antibacterial activity.

All exoskeleton extracts were inactive except for ExMe (MIC₅₀ = 50 µg/mL⁻¹), active against *E. faecalis*, which was the most susceptible bacteria to shrimp extracts, followed by *E. coli* and *K. pneumoniae* (Figure 1, Table 1).

Regarding *L. vannamei* waste, various studies have reported the inhibitory and bactericidal activity of chitosan prepared from shell waste against Gram-negative bacteria and Gram-positive bacteria (Vilar *et al.*, 2016); moreover, its antibacterial activity against *Xanthomonas sp.* isolated from leaves affected with citrus canker was proven (Mohanasrinivasan *et al.*, 2014).

A very interesting review about the utilization of seafood processing by-products was published describing peptides, oligosaccharides, fatty acids, enzymes, oils, and biopolymers isolated from fishes, crustacean shells, and shellfish, with many biological activities as antibacterial, antiviral, and anticancer (Senevirathne and Kim, 2012).

Moreover, marine sponges, produces interesting antibacterial compounds, in example: *Arenosclera brasiliensis* produces alkaloids active against resistant *S. aureus* and *P. aeruginosa* (Torres *et al.*, 2002); *Cribrochalina sp.* produces alkaloids (cribrostatin 3) active against *Neisseria gonorrhoeae* (Pettit *et al.*, 2000), just to mention a few (Laport *et al.*, 2009).

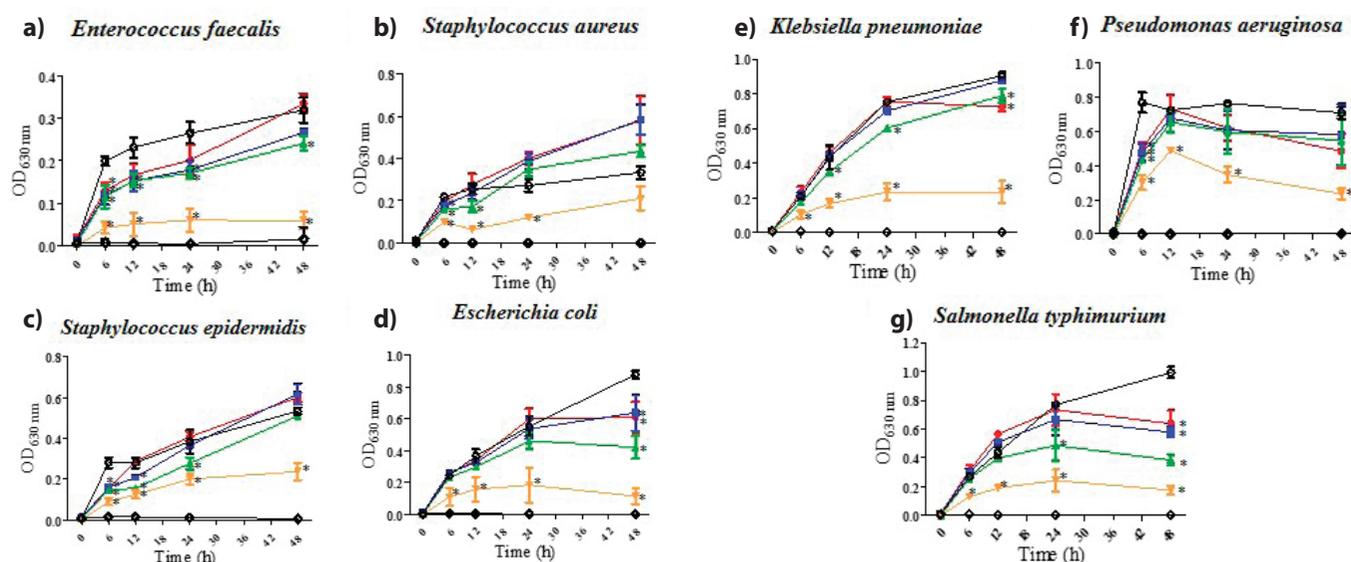


Figure 1. Antibacterial activity of shrimp cephalothorax hexanic extract, CeHex, evaluated at 50–400 µg mL⁻¹ (●, 50 µg mL⁻¹; ■, 100 µg mL⁻¹; ▲, 200 µg mL⁻¹; ▼, 400 µg mL⁻¹; ◊, gentamicin 12 µg mL⁻¹; ○, bacteria). All values represent mean of triplicate determinations ± SD. Significant differences (*p* < 0.05) from bacterial growth control are marked with an asterisk.

Figura 1. Actividad antibacteriana del extracto hexánico del cefalotórax de camarón, CeHex, evaluada a 50–400 µg mL⁻¹ (●, 50 µg mL⁻¹; ■, 100 µg mL⁻¹; ▲, 200 µg mL⁻¹; ▼, 400 µg mL⁻¹; ◊, gentamicina 12 µg mL⁻¹; ○, bacteria). Todos los valores representan el promedio de un triplicado ± Desviación Estándar. Diferencias significativas (*p* < 0.05) respecto al control de crecimiento bacteriano son marcados con un asterisco.

Table 1. Growth-inhibitory activity of shrimp exoskeleton, muscle, and head extracts against different Gram-positive and Gram-negative bacteria.

Tabla 1. Actividad inhibitoria del crecimiento de bacterias Gram-positivas y Gram-negativas por extractos de exoesqueleto y cefalotórax de camarón.

Strains	Crude shrimp extract ^a												
	ExHex		ExMe		ExAc		CeHex		CeAce		CeMe		
	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	
Gram-positive bacteria	<i>Enterococcus faecalis</i>	>400	>400	50	>400	>400	>400	400	>400	100	>400	>400	>400
	<i>Staphylococcus aureus</i>	>400	>400	>400	>400	>400	>400	400	>400	>400	>400	>400	>400
	<i>Staphylococcus epidermidis</i>	>400	>400	>400	>400	>400	>400	400	>400	>400	>400	>400	>400
Gram-negative bacteria	<i>Escherichia coli</i>	>400	>400	>400	>400	>400	>400	400	>400	400	>400	>400	>400
	<i>Klebsiella pneumoniae</i>	>400	>400	>400	>400	>400	>400	400	>400	400	>400	>400	>400
	<i>Pseudomonas aeruginosa</i>	>400	>400	>400	>400	>400	>400	400	>400	>400	>400	>400	>400
	<i>Salmonella typhimurium</i>	>400	>400	>400	>400	>400	>400	400	>400	>400	>400	>400	>400

^aConcentration in µg mL⁻¹

ExHex: Exoskeleton hexanic extract; ExMe: Exoskeleton methanolic extract; ExAc: Exoskeleton aqueous extract; CeHex: cephalothorax hexanic extract; CeAce: cephalothorax acetic extract; CeMe: cephalothorax methanolic extract.

Antimycobacterial activity

M. bovis BCG was used as an alternative to *Mycobacterium tuberculosis* H37Rv as it owns similar profiles of antibiotic susceptibility and offers a safer option for screening anti-tubercular compounds in a high-throughput format (Taneja and Tyagi, 2007; Altaf et al., 2010).

CeHex was the most active extract with a MIC₁₀₀ of 250 µg mL⁻¹, followed by CeAce with a MIC₁₀₀ of 500 µg mL⁻¹. The rest of the extracts (CeMe; ExHex, ExMe, and ExAc) were inactive against *M. bovis* BCG (Figure 2).

These results are in accordance to previous reports where non-polar extracts are recommended to find antimycobacterial compounds (Coronado-Aceves et al., 2016).

Previous studies have considered that an extract is active against mycobacteria if MIC is ≤250 µg mL⁻¹ (as exerted by CeHex); however, it is also relevant to consider toxicological studies (Coronado-Aceves et al., 2016; Jurno et al., 2019).

Chitosan obtained by deacetylation of chitin extracted from shrimp shell wastes has been used for the encapsulation of panchovillin, isolated from *Erythrina schliebenii*, and its antimycobacterial activity was demonstrated over *Mycobacterium indicus pranii* using *Galleria mellonella* larvae as an *in vivo* infection model (Rwegasila et al., 2016).

Another study investigated two edible marine algae, *Ulva lactuca* and *Ulva intestinalis*, finding that both extracts inhibit the mycobacterial biofilm development (Mukherjee et al., 2021).

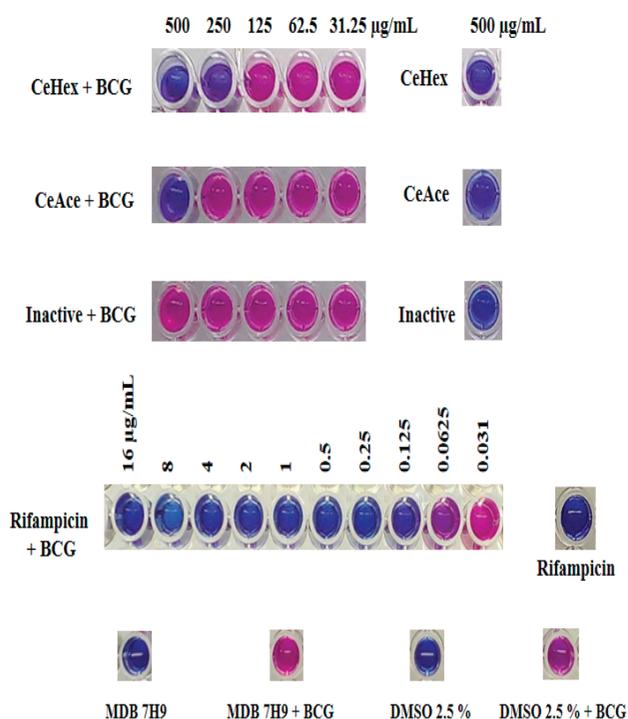


Figure 2. Antimycobacterial activity of shrimp extracts. CeHex: shrimp cephalothorax hexanic extract, $MIC_{100}=250 \mu\text{g mL}^{-1}$; CeAce: shrimp cephalothorax acetonic extract, $MIC_{100}=500 \mu\text{g mL}^{-1}$; 'Inactive' represents CeMe, ExHex, ExMe, and ExAc; BCG: *M. bovis* BCG.

Figure 2. Actividad antimicrobiana de extractos de camarón. CeHex: extracto hexánico del cefalotórax de camarón, $MIC_{100}=250 \mu\text{g mL}^{-1}$; CeAce: extracto acetónico del cefalotórax de camarón, $MIC_{100}=500 \mu\text{g mL}^{-1}$; 'Inactive' representa a CeMe; ExHex, ExMe, y ExAc; BCG: *M. bovis* BCG.

Finally, remarkable reviews have been published describing more than 250 antimycobacterial metabolites from marine natural products (Daleto *et al.*, 2016; Wang *et al.*, 2018).

Determination of the fatty acid profile

Some investigations have been carried out to elucidate the content of primary and secondary metabolites present in shrimp, as well as its biological activity of extracts of different polarity and/or fractions of the muscle, exoskeleton, head, and tail (Núñez-Gastélum *et al.*, 2011; López-Saiz *et al.*, 2014).

Shrimp muscle is reported to have a high content of high-quality protein and a low proportion of fatty acids (López-Saiz *et al.*, 2016; AlFaris *et al.*, 2021) and consequently, it is the part of the shrimp with the highest commercial value. The exoskeleton, head, and tail are often discarded or transformed into feed for aquaculture or supplemented as feed for animals (Nwanna *et al.*, 2004). Various bioactive compounds have been described from all parts of the shrimp (Mandeville *et al.*, 1992; Heu *et al.*, 2003; Sachindra *et al.*, 2006; López-Saiz *et al.*, 2014; Bharathi *et al.*, 2019), which is why the waste generated from this crustacean has attracted attention in recent years. It has been possible to separate chitin, proteins,

and lipids from shrimp waste (Núñez-Gastélum *et al.*, 2011) and it has been reported that pigments such as astaxanthins and fatty acids are found within the lipid fraction (Armenta *et al.*, 2002; Kandra *et al.*, 2012; López-Cervantes *et al.*, 2010).

The presence of polyunsaturated fatty acids has been described in shrimp oil obtained from cephalothorax, mainly EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) (Núñez-Gastélum *et al.*, 2011; Takeungwongtrakul *et al.*, 2012; Gulzar and Benjakul, 2018). These fatty acids belong to the group of ω -3 and therefore, they are widely valued for their nutraceutical and medicinal applications.

Oleic acid has been detected within the most abundant fatty acids extracted from shrimp cephalothorax (Takeungwongtrakul *et al.*, 2012).

In order to know a more complete profile of fatty acids present in CeHex, derivatization was carried out to form the methyl esters of the fatty acids and they were analyzed by gas chromatography (GC) (Figure 3). Fatty acid methyl esters were identified with the use of standards.

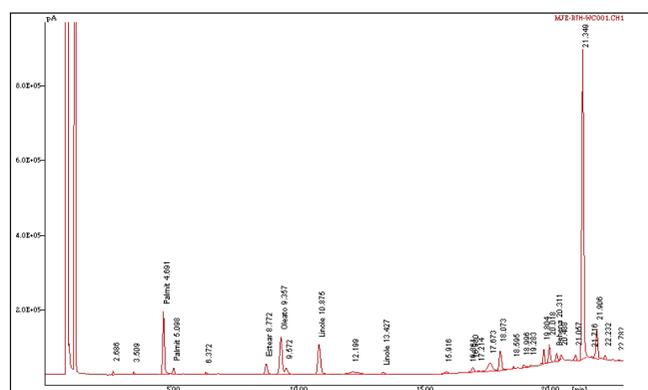


Figure 3. Chromatogram of fatty acid methyl esters of CeHex.

Figure 3. Cromatograma de ésteres metílicos de ácidos grasos de CeHex.

Table 2 shows the retention times of the detected peaks, the area percentage, and the methyl ester to which it corresponds according to its retention time. The fatty acid methyl esters that were identified in the highest proportion are oleate, linoleate, and palmitate (Figure 4). Other fatty acid methyl esters that were identified in lower proportions are stearate, palmitoleate, linolenate, and behenate (Figure 4). In another investigation where shrimp head oil was analyzed by gas chromatography, 14 fatty acid methyl esters were identified, of which it is confirmed that the main fatty acids are oleic, in a similar proportion to linoleic, followed by palmitoleic.

Table 2. Identification of fatty acid methyl esters.

Tabla 2. Identificación de ésteres metílicos de ácidos grasos.

# peak	Methyl ester	Fatty acid	Retention time (min)	% Area
3	Methyl palmitate	C16:0	4.691	25.764
4	Methyl palmitoleate	C16:1n7	5.098	2.866
6	Methyl stearate	C18:0	8.772	8.338
7	Methyl oleate	C18:1n9	9.357	29.294
9	Methyl linoleate	C18:2n6	10.875	27.896
11	Methyl linolenate	C18:3n3	13.427	1.806
23	Methyl behenate	C22:0	20.311	4.036

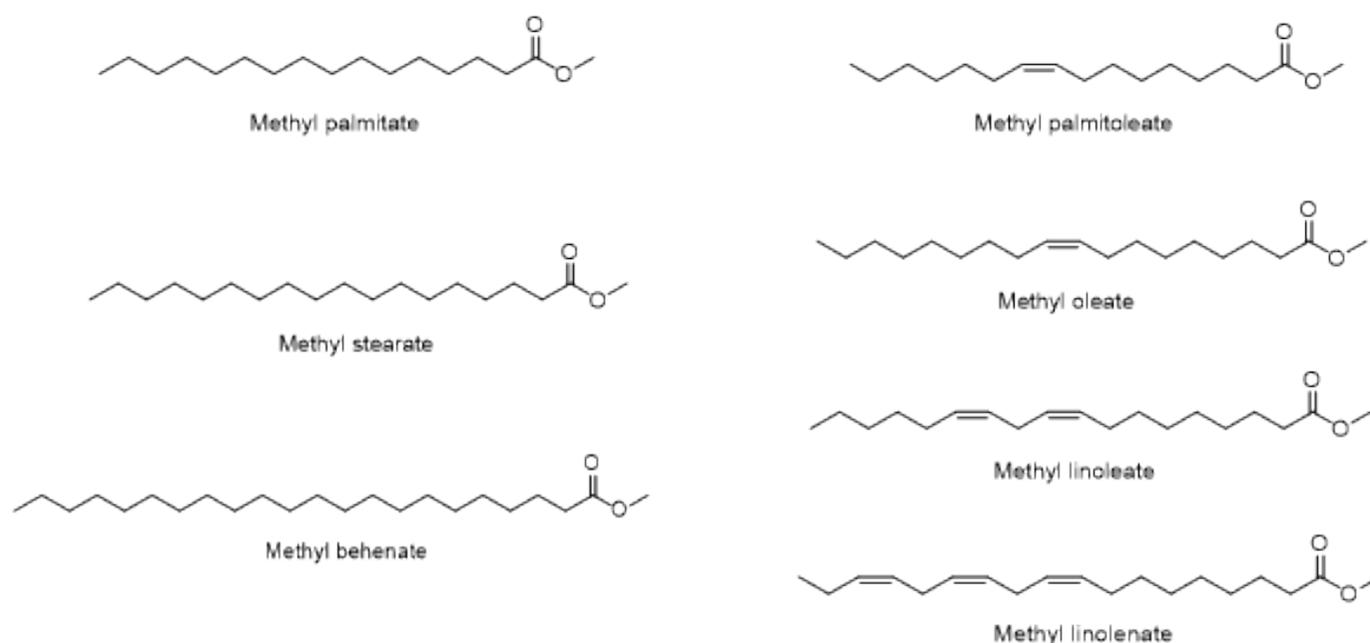


Figure 4. Structures of fatty acid methyl esters identified by GC.

Figura 4. Estructuras de los ésteres metílicos de ácidos grasos identificados mediante GC.

Similarly, the methyl esters of fatty acids C16: 1n7, C18: 0, and C18: 3n3 were identified in lower proportions (Núñez-Gastélum *et al.*, 2011).

DHA, EPA, among other fatty acids could not be identified by gas chromatography due to the lack of standards. Additionally, other studies of shrimp by-products show that the highest proportion of fatty acids are unsaturated (Heu *et al.*, 2003; Núñez-Gastélum *et al.*, 2011; Takeungwongtrakul *et al.*, 2012; Gulzar and Benjakul, 2018).

Regarding the antibacterial activity of the FFA found in CeHex, their main target is the cell membrane, producing disruption of the electron transport chain (ETC) and oxidative phosphorylation, interfering with cellular energy production, inhibition of fatty acid biosynthesis enzyme activity, impair active nutrient uptake, and induces autolysis and leakage of cell metabolites by pore formation (Desbois and Smith, 2010; Yoon *et al.*, 2018).

Oleic acid treatment in *S. aureus* increases membrane permeability and fluidity, leading to cell death (Chamberlain *et al.*, 1991); while, a linolenic acid treatment induces the release of intracellular content. Moreover, linoleic acid induces the disruption of the ETC in *S. aureus* (Greenway and Dyke, 1979). Also, oleic acid or linoleic acid, produce lysis of *Streptococcus faecalis* (Carson and Daneo-Moore, 1980).

Finally, regarding to the anti-*M. bovis* BCG activity of CeHex, MIC values of linolenic acid (α - and γ - form) and conjugated linoleic acid (CLA) against the viability of *M. tuberculosis* were determined as $75 \mu\text{g mL}^{-1}$ and $100 \mu\text{g mL}^{-1}$, respectively (Choi, 2016). Palmitic, linoleic and oleic acid have MICs of 25-50, 50-100, and $100 \mu\text{g mL}^{-1}$ against *M. tuberculosis* H37Rv (Sandoval-Montemayor *et al.*, 2012). Increased levels of myristic, palmitic, oleic, and linoleic fatty acids in sera of guinea pigs induced tuberculocidal effect toward *M.*

bovis BCG (Kochan and Berendt, 1974); long-chain fatty acids (oleic, linoleic, myristic, lauric, and palmitic) were reported to be mycobactericidal; while unsaturated fatty acids showed strong bactericidal activity in low concentrations (Kondo and Kanai, 1972; Kanetsuna, 1985).

CONCLUSIONS

Shrimp cephalothorax was the most promising by-product tested with antibacterial and antimycobacterial potential. Hexanic (non-polar) extract of shrimp cephalothorax resulted the most active against Gram-positive, Gram-negative bacteria, and *M. bovis* BCG. GC analysis of CeHex demonstrated the presence of three main, and four minoritarian, fatty acids. The strong antibacterial activity of CeHex and the identification of its main chemical constituents justify further studies on its biomedical and nutraceutical applications of this marine by-product. To the best of our knowledge, this is the first report of the antibacterial properties and chemical characterization of CeHex.

ACKNOWLEDGEMENTS

Author Martin Hernandez-Zazueta thanks Consejo Nacional de Ciencia y Tecnología (CONACYT-Mexico) for his Ph.D. scholarship [grant numbers: 329334] and the funding provided for this project [grant number: A1-S-9762]. Coronado-Aceves E.W. thanks "Dirección General de Asuntos del Personal Académico (UNAM)" for the post-doctoral fellowship. Tavera-Hernández R. is thankful to Consejo Nacional de Ciencia y Tecnología (Scholarship 662794) and Programa de Maestría y Doctorado en Ciencias Químicas, UNAM. The authors are thankful to Lucía del Carmen Márquez, Lucero Mayra Ríos Ruíz, Eréndira García, Elizabeth Huerta Salazar and Rubén Gaviño Ramírez for technical assistance.

REFERENCES

- AlFaris, N.A., Alshammari, G.M., AlTamimi, J.Z., AlMousa, L.A., Alagal, R.I., AlKehayez, N.M., Aljabryn, D.H., Alsayadi, M.M. and Yahya, M.A. 2021. Evaluating the effects of different processing methods on the nutritional composition of shrimp and the antioxidant activity of shrimp powder. *Saudi Journal of Biological Sciences*. 29: 640-649.
- Ataf, M., Miller, C. H., Bellows, D. S. and O'Toole, R. 2010. Evaluation of the *Mycobacterium smegmatis* and BCG models for the discovery of *Mycobacterium tuberculosis* inhibitors. *Tuberculosis*. 90: 333-337.
- Armenta, R.E., Guerrero-Legarreta, I. and Huerta, S. 2002. Extracción de caroproteínas a partir de residuos de camarón fermentados. *Academia Mexicana de Investigación y Docencia en Ingeniería Química*. 1: 49-55.
- Baizman, E. R., Branstrom, A. A., Longley, C. B., Allanson, N., Sofia, M. J., Gange, D., and Goldman, R. C. 2000. Antibacterial activity of synthetic analogues based on the disaccharide structure of moenomycin, an inhibitor of bacterial transglycosylase. *Microbiology*. 146: 3129-3140.
- Bayona, L. M., Videnova, M., and Choi, Y. H. 2018. Increasing metabolic diversity in marine sponges extracts by controlling extraction parameters. *Marine drugs*. 16: 393.
- Bharathi, R., Vigneshpriya, D. and Krishnaveni, N. 2019. Comparison of proximate and fatty acid composition of shell of marine edible shrimps, *Heterocarpus gibbosus* (Bate, 1888) and *Aristeus alcocki* (Ramadan, 1938). *International Journal of Zoology and Applied Biosciences*. 4: 75-79.
- Brown, D. G., Lister, T. and May-Dracka, T. L. 2014. New natural products as new leads for antibacterial drug discovery. *Bioorganic & medicinal chemistry letters*. 24: 413-418.
- Carson, D.D. and Daneo-Moore, L. Effects of fatty acids on lysis of *Streptococcus faecalis*. 1980. *Journal of Bacteriology*. 141: 1122-1126.
- Chamberlain, N. R., Mehrtens, B. G., Xiong, Z. H. U. O., Kapral, F. A., Boardman, J. L. and Rearick, J. I. 1991. Correlation of carotenoid production, decreased membrane fluidity, and resistance to oleic acid killing in *Staphylococcus aureus* 18Z. *Infection and Immunity*. 59: 4332-4337.
- Choi, W. H. 2016. Evaluation of anti-tubercular activity of linolenic acid and conjugated-linoleic acid as effective inhibitors against *Mycobacterium tuberculosis*. *Asian Pacific Journal of Tropical Medicine*. 9: 125-129.
- Collins, L. A., and Franzblau, S. G. 1997. Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. *Antimicrobial Agents and Chemotherapy*. 41: 1004-1009.
- Coronado-Aceves, E.W., Sánchez-Escalante, J.J., López-Cervantes, J., Robles-Zepeda, R. E., Velázquez, C., Sánchez-Machado, D. I., and Garibay-Escobar, A. 2016. Antimycobacterial activity of medicinal plants used by the Mayo people of Sonora, Mexico. *Journal of Ethnopharmacology*. 190: 106-115.
- Daletos, G., Ancheeva, E., Chaidir, C., Kalscheuer, R., and Proksch, P. 2016. Antimycobacterial metabolites from marine invertebrates. *Archiv der Pharmazie*. 349: 763-773.
- Desbois, A. P. and Smith, V. J. 2010. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. *Applied Microbiology and Biotechnology*. 85: 1629-1642.
- Djellouli, M., López-Caballero, M. E., Arancibia, M. Y., Karam, N. and Martínez-Alvarez, O. 2020. Antioxidant and antimicrobial enhancement by reaction of protein hydrolysates derived from shrimp by-products with glucosamine. *Waste and Biomass Valorization*. 11: 2491-2505.
- Greenway, D. L. A. and Dyke, K. G. H. 1979. Mechanism of the inhibitory action of linoleic acid on the growth of *Staphylococcus aureus*. *Microbiology*. 115: 233-245.
- Gulzar, S. and Benjakul, S. 2018. Ultrasound waves increase the yield and carotenoid content of lipid extracted from Cephala thorax of pacific white shrimp (*Litopenaeus vannamei*). *European Journal of Lipid Science and Technology*. 120: 1700495.
- Guzmán-Gutiérrez, S. L., Silva-Miranda, M., Kregel, F., Huerta-Salazar, E., León-Santiago, M., Díaz-Cantón, J. K., Espitia-Pinzón, C. and Reyes-Chilpa, R. 2020. Antimycobacterial Activity of Alkaloids and Extracts from *Tabernaemontana alba* and *T. arborea*. *Planta Medica*. 88: 53-61.
- Hernández-Zazueta, M. S., García-Romo, J. S., Noguera-Artiaga, L., Luzardo-Ocampo, I., Carbonell-Barrachina, Á. A., Taboada-Antelo, Campos-Vega, R., Rosas-Burgos, E.C., Burboa-Zazueta, M. G., Ezquerra-Brauer, J. M., Martínez-Soto, J. M., Santacruz-Ortega, H. C. and Burgos-Hernández, A. 2021a. *Octopus vulgaris* ink extracts exhibit antioxidant, antimutagenic, cytoprotective, antiproliferative, and proapoptotic effects in selected human cancer cell lines. *Journal of Food Science*. 86: 587-601.
- Hernández-Zazueta, M. S., Luzardo-Ocampo, I., García-Romo, J. S., Noguera-Artiaga, L., Carbonell-Barrachina, Á. A., Taboada-Antelo, Campos-Vega, R., Rosas-Burgos, E. C., Burboa-Zazueta, M. G., Ezquerra-Brauer, J. M. and Burgos-Hernández, A. 2021b. Bioactive compounds from *Octopus vulgaris* ink extracts exerted anti-proliferative and anti-inflammatory effects *in vitro*. *Food and Chemical Toxicology*. 151: 112119.
- Heu, M.S., Kim, J.S. and Shahidi, F. 2003. Components and nutritional quality of shrimp processing by-products. *Food Chemistry*. 82: 235-242.
- Jurno, A. C., Netto, L. O. C., Duarte, R. S., and Machado, R. R. P. 2019. The search for plant activity against tuberculosis using breakpoints: A review. *Tuberculosis*. 117: 65-78.
- Kandra, P., Challa, M.M. and Kalangi P.J.H. 2012. Efficient use of shrimp waste: present and future trends. *Applied Microbiology and Biotechnology*. 93: 17-29.
- Kanetsuna, F. 1985. Bactericidal effect of fatty acids on mycobacteria, with particular reference to the suggested mechanism of intracellular killing. *Microbiology and Immunology*. 29: 127-141.
- Kochan, I., and Berendt, M. 1974. Fatty acid-induced tuberculocidal activity in sera of guinea pigs treated with bacillus Calmette-Guerin and lipopolysaccharide. *Journal of Infectious Diseases*. 129: 696-704.
- Kondo, E. and Kanai, K. 1972. The lethal effect of long-chain fatty acids on mycobacteria. *Japanese Journal of Medical Science and Biology*. 25: 1-13.
- Laport, M. S., Santos, O. C. S., and Muricy, G. 2009. Marine sponges: potential sources of new antimicrobial drugs. *Current pharmaceutical biotechnology*. 10: 86-105.
- Lefebvre, T., Destandau, E., and Lesellier, E. 2021. Selective extraction of bioactive compounds from plants using recent extraction techniques: A review. *Journal of Chromatography A*. 1635: 461770.
- López-Cervantes, J., Adan-Bante, N.P. and Sánchez-Machado, D.I. 2010. Separation and biochemical characterization of the products from fermented shrimp wastes. In: Le Behan, E. (Ed.), *Sea By-Products as Real Material: New Ways of Application*. Transworld Research Network. 2010: 1-16.

- López-Saiz, C.M., Hernández, J., Cinco-Moroyoqui, F.J., Velázquez, C., Ocaño-Higuera, V.M., Plascencia-Jatomea, M., Robles-Sánchez, M., Machi-Lara, L. and Burgos-Hernández, A. 2016. Antimutagenic compounds of white shrimp (*Litopenaeus vannamei*): isolation and structural elucidation. Evidence-Based Complementary and Alternative Medicine. 2016: 1-7.
- López-Saiz, C.M., Velázquez, C., Hernández, J., Cinco-Moroyoqui, F.J., Plascencia-Jatomea, M., Robles-Sánchez, M., Machi-Lara, L. and Burgos-Hernández, A. 2014. Isolation and structural elucidation of antiproliferative compounds of lipidic fractions from white shrimp muscle (*Litopenaeus vannamei*). International Journal of Molecular Sciences. 15: 23555–23570.
- Mandeville, S., Yaylayan, V. and Simpson, B.K.. 1992. Proximate analysis, isolation and identification of amino acids and sugars from raw and cooked commercial shrimp waste. Food Biotechnology. 6: 51-64.
- Mugumbate, G., and Overington, J. P. 2015. The relationship between target-class and the physicochemical properties of antibacterial drugs. Bioorganic & medicinal chemistry. 23: 5218-5224.
- Mukherjee, G., Mukhopadhyay, B. and Sil, A. K. 2021. Edible marine algae: a new source for anti-mycobacterial agents. Folia microbiologica. 66: 99-105.
- Mohanasrinivasan, V., Mishra, M., Paliwal, J. S., Singh, S. K., Selvarajan, E., Suganthi, V., and Devi, C. S. 2014. Studies on heavy metal removal efficiency and antibacterial activity of chitosan prepared from shrimp shell waste. Biotech. 4: 167-175.
- Molina-Salinas, G. M., Ramos-Guerra, M. C., Vargas-Villarreal, J., Mata-Cárdenas, B. D., Becerril-Montes, P., and Said-Fernández, S. 2006. Bactericidal activity of organic extracts from *Flourensia cernua* DC against strains of *Mycobacterium tuberculosis*. Archives of Medical Research. 37: 45-49.
- Navarro-Navarro, M., Ruiz-Bustos, P., Valencia, D., Robles-Zepeda, R., Ruiz-Bustos, E., Virués, C., Hernández, J., Domínguez, Z. and Velazquez, C. 2013. Antibacterial activity of Sonoran propolis and some of its constituents against clinically significant *Vibrio* species. Foodborne Pathogens and Disease. 10: 150-158.
- Nirmal, N. P., Santivarangkna, C., Rajput, M. S. and Benjakul, S. 2020. Trends in shrimp processing waste utilization: An industrial prospective. Trends in Food Science & Technology. 103: 20-35.
- Núñez-Gastélum, J. A., Sánchez-Machado, D. I., López-Cervantes, J., Paseiro-Losada, P., Sendón, R., Sanches-Silva, A.T., Costa, H.S., Aurrekoetxea, G.P., Angulo, I. and Soto-Valdez, H. 2011. Evaluación físico-química de aceite pigmentado obtenido de la cabeza de camarón. Grasas y Aceites. 62: 321-327.
- Nwanna, L. C., Balogun, A. M., Ajenifuja, Y. F. and Enujiugha, V. N. 2004. Replacement of fish meal with chemically preserved shrimp head in the diets of African catfish, *Clarias gariepinus*. Journal of Food Agriculture and Environment. 2: 79-83.
- Osuna-Ruiz, I., López-Saiz, C. M., Burgos-Hernández, A., Velázquez, C., Nieves-Soto, M., and Hurtado-Oliva, M. A. 2016. Antioxidant, antimutagenic and antiproliferative activities in selected seaweed species from Sinaloa, Mexico. Pharmaceutical Biology. 54: 2196-2210.
- Palomino, J. C., Martin, A., Camacho, M., Guerra, H., Swings, J., and Portaels, F. 2002. Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis*. Antimicrobial Agents and Chemotherapy. 46: 2720-2722.
- Peñuelas-Urquides, K., Villarreal-Treviño, L., Silva-Ramírez, B., Rivadeneyra-Espinoza, L., Said-Fernández, S., and León, M. B. D. 2013. Measuring of *Mycobacterium tuberculosis* growth: a correlation of the optical measurements with colony forming units. Brazilian Journal of Microbiology. 44: 287-290.
- Pettit, G. R., Knight, J. C., Collins, J. C., Herald, D. L., Pettit, R. K., Boyd, M. R. and Young, V. G. 2000. Antineoplastic agents 430. Isolation and structure of cribostratins 3, 4, and 5 from the Republic of Maldives *Cribochalina* species. Journal of Natural Products. 63: 793-798.
- Raju, N., Gulzar, S., Buamard, N., Ma, L., Ying, X., Zhang, B., and Benjakul, S. 2021. Comparative Study of Astaxanthin, Cholesterol, Fatty Acid Profiles, and Quality Indices Between Shrimp Oil Extracted From Hepatopancreas and Cephalothorax. Frontiers in Nutrition. 8.
- Rwegasila, E., Mubofu, E.B., Nyandoro, S. S., Erasto, P. and Munissi, J. J. 2016. Preparation, characterization and *in vivo* antimycobacterial studies of panchovillin-chitosan nanocomposites. International Journal of Molecular Sciences. 17: 1559.
- Sachindra, N.M., Bhaskar, N. and Mahendrakar, N.S. 2006. Recovery of carotenoids from shrimp waste in organic solvents. Waste Management. 26: 1092-1098.
- Sandoval-Montemayor, N. E., García, A., Elizondo-Treviño, E., Garza-González, E., Alvarez, L. and del Rayo Camacho-Corona, M. 2012. Chemical composition of hexane extract of *Citrus aurantifolia* and anti-*Mycobacterium tuberculosis* activity of some of its constituents. Molecules, 17(9), 11173-11184.
- Senevirathne, M., and Kim, S. K. 2012. Utilization of seafood processing by-products: medicinal applications. Advances in Food and Nutrition Research. 65: 495-512.
- Takeungwongtrakul, S., Benjakul, S. and H-kittikun, A. 2012. Lipids from cephalothorax and hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*): Compositions and deterioration as affected by iced storage. Food Chemistry. 134: 2066-2074.
- Taneja, N. K. and Tyagi, J. S. 2007. Resazurin reduction assays for screening of anti-tubercular compounds against dormant and actively growing *Mycobacterium tuberculosis*, *Mycobacterium bovis* BCG and *Mycobacterium smegmatis*. Journal of Antimicrobial Chemotherapy. 60: 288-293.
- TheFishSite: Mexican shrimp sector set for 177,000 tonne year. [Consulted in December 21, 2021] 2021. Available in: <https://thefishsite.com/articles/mexican-shrimp-sector-set-for-177-000-tonne-year>
- Torres, Y. R., Berlinck, R. G., Nascimento, G. G., Fortier, S. C., Pessoa, C. and de Moraes, M. O. 2002. Antibacterial activity against resistant bacteria and cytotoxicity of four alkaloid toxins isolated from the marine sponge *Arenosclera brasiliensis*. Toxicon. 40: 885-891.
- Velazquez, C., Navarro, M., Acosta, A., Angulo, A., Dominguez, Z., Robles, R., Lugo E, Goycoolea FM, Velazquez EF, Astiazaran H, and Hernandez, J. 2007. Antibacterial and free-radical scavenging activities of Sonoran propolis. Journal of Applied Microbiology. 103: 1747-1756.
- Vilar-Junior, J. C., Ribeaux, D. R., Alves da Silva, C. A., Campos-Takaki, D. and Maria, G. 2016. Physicochemical and antibacterial properties of chitosan extracted from waste shrimp shells. International Journal of Microbiology, 2016: 7.
- Wang, L., Wang, J., Liu, J. and Liu, Y. 2018. Antitubercular marine natural products. Current Medicinal Chemistry. 25: 2304-2328.