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Escuela de Ciencias Biológicas e Ingeniería

Essential oils as bioactive substances for the control of White Spot Syndrome Virus (WSSV) in *P. vannamei* shrimp farming

Trabajo de integración curricular presentado como requisito para la obtención del título de Bióloga

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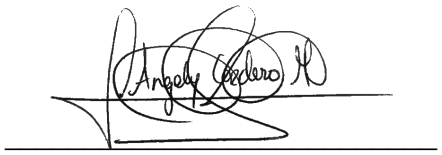

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DEDICATION

To my mom, Gladys Macías. All my achievements, I owe to her.

ABSTRACT

White spot syndrome virus (WSSV) affects wild and farmed shrimp. WSSV is highly contagious and can produce mortalities of up to 100% in a few days, causing significant economic losses globally. A common way to treat diseases that affect shrimp farming is antibiotics, but their indiscriminate use induces microbial resistance and affects the organisms' microbiota. In this sense, many of the medicinal plant products are safe and do not pollute the environment. Some medicinal plants are known to have potent antiviral properties. In this work, the anti-WSSV activity of 10 essential oils (EOs) as antiviral agents was evaluated in *Penaeus vannamei*. Initially the primary hemocyte culture in L-15 medium was adapted to test the active doses of EOs *in vitro*. Next, the toxicity of EOs was determined *in vitro* and *in vivo*, in primary culture (by reducing MTT) and in the early stages of *P. vannamei* (by direct exposure). Finally, the active doses of EOs were validated *in vivo*, incorporating them into food for 10 days. Then the treated shrimp were challenged via injection with WSSV at a dose that causes 50% mortality. The primary culture was viable for 7 days; in the *in vitro* screening, the best results against WSSV were obtained with the EOs of *Syzygium aromaticum* (EO3), *Zingiber officinale* (EO7), and *Cymbopogon citratus* (EO10). Regarding toxicity, these three oils affected the viability of hemocytes by 25% at the two highest concentrations tested (100, 10 μg/ml). These same concentrations affected about 15% of the Zoea when exposed to EO3 and EO10, while the Mysis was only affected about 15 % by EO10 at the highest concentration assessed. Juvenile shrimp showed significant improvements ($p<0.05$) in cumulative survival rates after being treated with EOs, for EO7 and EO10 was 40% and for EO3 was 30%. These results illustrate the anti-WSSV effect of EOs and that the inclusion in shrimp's diet can exert a protective effect against WSSV by improving the survival rates of farmed shrimp.

Keywords: White spot syndrome virus, *Litopenaeus vannamei*, essential oils, shrimp farming.

RESUMEN

El virus del síndrome de la mancha blanca (WSSV) afecta a camarones silvestres y de cultivos. El WSSV es altamente contagioso y puede producir mortalidades de hasta el 100% en pocos días ocasionando importantes pérdidas económicas a nivel global. Una forma habitual para tratar enfermedades que afectan al cultivo del camarón es el uso de antibióticos, pero su uso indiscriminado induce la resistencia microbiana y afecta la microbiota propia de los organismos. En este sentido, los productos de plantas medicinales son seguros y no contaminan el ambiente. Se sabe que muchas plantas medicinales tienen poderosas propiedades antivirales. Debido a esto, en este trabajo se evaluó la actividad anti-WSSV de 10 aceites esenciales (EO) como agentes antivirales en *Penaeus vanammei*. Inicialmente se adaptó el cultivo primario de hemocitos en medio L-15 para determinar las dosis activas de los EOs *in vitro.* Seguidamente se determinó *in vitro* e *in vivo* la toxicidad de los EOs, en cultivo primario (mediante la reducción del MTT) y estadios tempranos de *P. vanammei* (por exposición directa). Finalmente, se validó *in vivo* las dosis activas de los EOs, incorporando en el alimento y suministrando por 10 días. Luego los camarones tratados fueron desafiados vía inyección con WSSV a una dosis que provoca el 50% de mortalidad. El cultivo primario fue viable por 7 días, en el cribado *in vitro* los mejores resultados se obtuvieron con los EOs de *Syzygium aromaticum* (EO3), *Zingiber officinale* (EO7) y *Cymbopogon citratus* (EO10) frente al WSSV. En cuanto a la toxicidad estos 3 aceites afectaron en un 25% la viabilidad de los hemocitos a las dos concentraciones más altas testeadas (100, 10 μg/ml). Estas mismas concentraciones afectaron alrededor del 15% de las Zoea cuando fueron expuestas al EO3 y EO10, mientras que las Mysis sólo se vieron afectadas en un 15% por el EO10 a la mayor concentración evaluada. Los camarones juveniles mostraron mejoras significativas ($p<0.05$) en las tasas de supervivencia acumulada después de ser tratados con los EOs, para el EO7 y EO10 la mejora fue del 40% y para el EO3 del 30%. Estos resultados ilustran el efecto anti-WSSV de los EOs y que la inclusión en la dieta alimenticia del camarón puede ejercer un efecto protector frente al WSSV mejorando las tasas de supervivencia del camarón de cultivo.

Palabras clave: Virus del síndrome de la mancha blanca, *Litopenaeus vannamei*, aceites esenciales, cultivo de camarón.

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1. Introduction

The Pacific white shrimp *Litopenaeus vannamei* is the most commercially important shrimp species worldwide. Currently, *L. vannamei* is produced in at least 50 countries, although the industry is concentrated in two regions, Asia and America. Ecuador is the principal producer in South America, 2020 exporting 676 TM, approximately representing 3,600 million dollars (CNA, 2021). Shrimp farming constitutes a significant national income, generating around 261,000 direct and indirect jobs in rural regions where there are few job alternatives for citizens; therefore, this industry directly affects poverty indicators in the country.

The shrimp farming industry is affected by various viral, bacterial, and fungal diseases. Viruses are the most critical disease-causing agents. Several viral pathogens, including white spot syndrome virus (WSSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), infectious myonecrosis virus (IMNV), Taura syndrome virus (TSV), yellow head virus (YHV), white tail disease (WTD), covert mortality nodavirus (CMNV), Laem–Singh virus (LSNV) and shrimp hemocyte iridescent virus (SHIV), affect *P. vanammei* farms (Arulmoorthy et al., 2020; Lightner et al., 1999).

WSSV is the most devastating and widespread viral problem reported for shrimp; the disease has spread rapidly in shrimp farming regions of the world. WSSV is highly virulent; mortality can be rapid (3-10 days) and affect up to 100% of individuals. The economic cost of the disease to the shrimp aquaculture industry worldwide, since it first appeared and spread, is estimated at up to $ 15 billion, increasing by $ 1 billion annually and accounting for approximately 10 % of world shrimp production (Oakey et al., 2019). In late 1999, WSSV spread in Ecuador, drastically decreasing shrimp export by nearly 70%, causing a great financial impact due to catastrophic economic losses (Bir et al., 2017).

In 2019, the Republic of China established an agreement with the Ecuadorian government stating that shrimp whose PCR tests give positive results for the presence of the WSSV will not be accepted. This measure represents a challenge for the shrimp producing industry since it increases production costs and China is the leading buyer of Ecuadorian shrimp (CNA, 2019).

Viral diseases, such as the WSSV are not curable. Although it has been more than two decades since its appearance, no antiviral products have been developed. In aquaculture, a good alternative in the prevention and treatment of diseases is the use of natural products, such as those derived from medicinal plants because they are safe sources and inexpensive and do not generate resistance to pathogens compared to antibiotics, thus avoiding the tremendous environmental impact that antibiotics can cause (Escamilla-Montes et al., 2019). Moreover, studies have demonstrated the use of essential oils (EOs) for shrimp nutrition as a strategy to strengthen animals' immune system and reduce the potential for infection by WSSV. EOs have shown antimicrobial, antioxidant, and anti-inflammatory effects (Júnior et al., 2018).

In the last few years, herbal products have been used as an alternative practice against WSSV control (Velmurugan et al., 2012). According to Anh et al. (2009), *Phyllanthus amarus* extract was evaluated for virucidal activity against WSSV, showing excellent results. In addition, several researchers have used a mixture of different herbal medicinal plants to increase the immunity of shrimp against the WSSV (Citarasu et al., 2006; Yogeeswaran., 2012). Thus, the main objective of this research is to evaluate the potential use of 10 essential oils obtained from plants as anti-WSSV agents.

1.1. Problem statement

In Ecuador, *P. vannamei* shrimp farming represents the main aquaculture activity, and shrimp are currently the first non-oil export product. The shrimp industry is considered one of the engines of the country's economy; between January and June of the current year, it generated 27.5% of total non-oil exports (BCE, 2021). Nevertheless, shrimp aquaculture is affected by diseases caused by viruses and bacteria, as well as low prices, sanitary barriers, and high production costs. WSSV affects shrimp exports, especially to the Popular Republic of China, the leading importer of Ecuadorian shrimp. China recently imposed sanitary barriers, given its refusal not to receive shrimp that are positive for WSSV. Therefore, an efficient and consumer-friendly alternative is needed to to prevent and control WSSV in shrimp farming, and thus reduce the massive financial losses (billions of dollars) in the penaeid shrimp aquaculture industry.

1.2. Hypothesis

- Essential oils have anti-WSSV properties and can be used safely to control white spots in shrimp farming**.**

1.3. Objectives

General objective:

▪ To evaluate the potential of essential oils as antiviral compounds against white spot syndrome virus (WSSV) in *P. vannamei* shrimp farms.

Specific Objectives:

▪ Obtain ten essential oils from commonly used plants.

▪ Develop a protocol for shrimp cell culture that lasts at least 7 days.

▪ Conduct *in vitro* screening to determine the antiviral effect of EOs in shrimp cell lines.

▪ Determine the toxicity of EOs in *in vitro* and *in vivo* shrimp models.

▪ Validate *in vivo* the active doses of essential oils against WSSV.

2. Background

Litopenaeus vannamei

The Pacific white shrimp (*Litopenaeus vannamei*, formerly *Penaeus vannamei*) is the most commonly cultured species in Pacific-rim Asia and Latin America (Boone, 1931; Briggs et al., 2004). It is colloquially known as white-legged shrimp or Mexican white shrimp because of its greyish-white color. *Litopenaeus vannamei* is native to the Pacific coast of Mexico, Central and South America, where water temperatures generally remain above 20ºC for the whole year. This species lives in tropical marine habitats. They usually live in muddy sediments down to a depth of 75 m. They can also grow in fresh water, but growth is slower below 10 ppt salinity. (Rosas *et al.*, 2001). Female shrimp grow faster than males. The weight of the mature female *L. vannamei* is about 30-45 g, and they can spawn 100,000 – 250,000 eggs. The world production of Pacific white shrimp reached over 4 million tons in 2019, increasing sharply between 2013 and 2019 (FAO, 2019).

White Spot Syndrome Virus (WSSV)

White spot syndrome virus is a double-stranded DNA virus (80-120nm x 250-380 nm) that is responsible for the white spot disease (WSD) in farm-raised shrimp globally (Bir *et al.*, 2017; Durand et al., 1997). It possesses a sizeable ovaloid lipid envelope with a unique tail-like appendage (Walker and Mohan, 2009). Taxonomically, WSSV belongs to the family *Nimaviridae* and is the sole member of the genus *Whispovirus* (Bir et al., 2017; Verbruggen et al., 2016).

At least 45 structural proteins make the three morphological distinct layers of virions (Tsai et al., 2004; Li et al., 2007). The nucleocapsid, a bacilliform structure (~70 nm x ~300 nm), lies at the core of the virions and contains the dsDNA and nine proteins. Furthermore, includes a basic DNA-binding protein (VP15) and a giant protein (VP664) form the stacked ring subunits which protrude through the overlaying tegument (Leu et al., 2005; Witteveldt et al., 2005). The tegument layer comprises at least four structural proteins, including the major capsid protein VP26 and three minor proteins denominated VP36A, VP39A, and VP95 (Tsai et al., 2006; Xie et al., 2006). The envelope is a tri-laminar lipid membrane, 6-7 nm thick, composed of 28 viral proteins including VP19 and VP28 that extend from the outer surface of the membrane and are responsible for the induction of the protective response in shrimps (Tsai et al., 2006; Xie et al., 2006). Furthermore,

VP28 and VP26 are abundant, representing approximately 60% of the viral envelope (Sánchez, 2010; Walker and Mohan, 2009).

History of the outbreak and world distribution

White Spot Disease (WSD) was first found in June 1992 in a cultured kuruma shrimp (*Penaeus japonicus* BATE, 1888) in the Fujian Province of China (Zhan et al., 1998; Jiang 2001). One year later, the disease spread to several prefectures of Japan and Korea (Nakano et al., 1994; Park et al., 1998). Over the next few years, WSSV infected farmed shrimp raised in Thailand, Vietnam, Indonesia, Malaysia, and India, causing hundreds of million dollars in economic losses for the shrimp industry every year. By 1995, the disease arrived in America, first reported at a local farm in Texas. Then, it spread rapidly to Nicaragua, Honduras, and Guatemala by mid-January 1999. In Ecuador, WSSV was first identified in May 1999 (Alday de Graindorge 2000). The National shrimp production of *P. vannamei* was about 100 000 tonnes and fell 50% by 2000. As a result, shrimp exports dropped 70%, generating a billions of dollars in losses.

Treatments against WSSV

Shrimp farms used at least one antibiotic to treat disease or promote growth. Oxytetracycline, florfenicol, sarafloxacin, and enrofloxacin are the antibiotics maximum often applied. However, the overuse of antibiotics in aquaculture production has become one of the biggest challenges to develop effective and sustainable therapies since the amount transferred to the environment varies from 1% (chloramphenicol) to 90% (oxytetracycline) (Bermúdez-Almada et al., 2012). Moreover, Hekton et al. (1995) outline that approximately 70-90% of the antibiotic used in cultured organism therapy ends up in the environment and sediments.

As an alternative to the indiscriminate use of antibiotics for shrimp culture, Biofloc Technology (BFT) can be used. This technology consists of *in situ* production of beneficial microorganisms in fish and shrimp acuaculture. In this system, shrimp and fish are grown with zero or minimum water exchange, avoiding discharging dirty water into the environment. A continuous water flow in the entire water column is required to induce the macroaggregate (biofloc) formation. Thus, the nutrients in the water will create a heterogeneous and stable microbial community that plays vital roles in 1) maintenance of water quality, 2) nutrition, by reducing feed conversion ratio (FCR) and decreasing feed costs, and 3) competition with pathogens for substrate and nutrients, due

to the production of inhibitory compounds and interferens in the bacterial *quorum*-sensing communication (Emerenciano, Martínez-Córdova, Martínez-Porchas and Miranda-Baeza, 2017; Pilotto et al., 2018).

In 2018, Pilotto et al. (2018) found that the bacteriome of shrimp reared in BFT was more diversified and more prosperous when compared to that from animals reared in clear seawater, where the predominant bacterial community were Vibrionaceae. Additionally, bio floc can act as immunostimulants, enhancing the shrimp's innate immune system, even altering the expression of genes related to the shrimp immune response, which might be attributed to the ability of the BFT to induce changes in shrimp microbiota.

Glucans are known as "biological response modifiers" and are found in the cell wall of algae, fungi, and yeast. Glucans interact with multiple receptors in microorganisms, invertebrates, and mammals and initiate several immune responses. The use of yeast glucans of marine and terrestrial origin has been reported in the literature, suggesting that yeast are excellent sources of beta glucans, which in turn confer protection to penaeid shrimp against pathogenic microorganisms. However, not all beta-glucans have immunostimulant properties; they vary by source and even within the same source. According to a study by Ochoa Álvarez et al. (2021) beta-glucans, which are obtained from some marine yeasts that live in *Penaeus vannamei* culture ponds, have a stronger protective effect on white shrimp in the presence of WSSV virus particles compared to that of *Saccharomyces cerevisiae* terrestrial yeast.

Research on antiviral plant extracts has been ongoing since 1950, and several plant compounds have been found to have nonspecific immune-stimulating properties in shrimp. Thus, plant products have been proven to be an affordable and efficient way to strengthen the shrimp immune system and reduce the potential of being infected by WSSV. In that sense, plant essential oils for animal nutrition have been shown to have antimicrobial, antioxidant, immunomodulation, and anti-inflammatory effects (Gianennas et al., 2013). For example, thymol and terpinene can stimulate fish immunity (Vaseeharan and Thaya 2013), EOs of *Thymus vulgaris* and *Thymus zygis* have been shown to exhibit antimicrobial, antifungal, antioxidant, and antiviral activity (Botelho et al., 2007; Rota et al., 2008; Nazzaro et al., 2013; Hernández-Hernández et al., 2014). However, EOs may lose their biological activity by volatilization or degradation triggered by high temperatures,

oxidation, and UV light (Soliman et al., 2013). Further research on EOs is warranted to optimize their use in shrimp aquaculture.

3. Methodology

3.1 Essential oils extraction

To obtain essential oils, the steam distillation method was applied following Božović, et al. (2017), using an essential oil extractor (Figmay, Argentina). Plant material was exposed to steam to release the essential oil through evaporation. When the vapor and essential oil vapors condensed, they were separated and collected in amber glass bottles. EOs were extracted from 10 plant sources: *Origanum vulgare* (EO1), *Azadirachta indica* (EO2), *Syzygium aromaticum* (EO3), *Cinnamomum verum* (EO4), *Melaleuca alternifolia* (EO5), *Thymus vulgaris* (EO6), *Zingiber officinale* (EO7), *Allium sativum* (EO8), *Citrus sinensis* (EO9) and *Cymbopogon citratus* (EO10). For the antivirulence and toxicity assays, the EOs were emulsified in Tween-80 (Figure 1) (Barik et al., 2019; Pavoni et al., 2020), and (1x) phosphate-buffered saline (PBS) was used to dilute EOs to various concentrations.

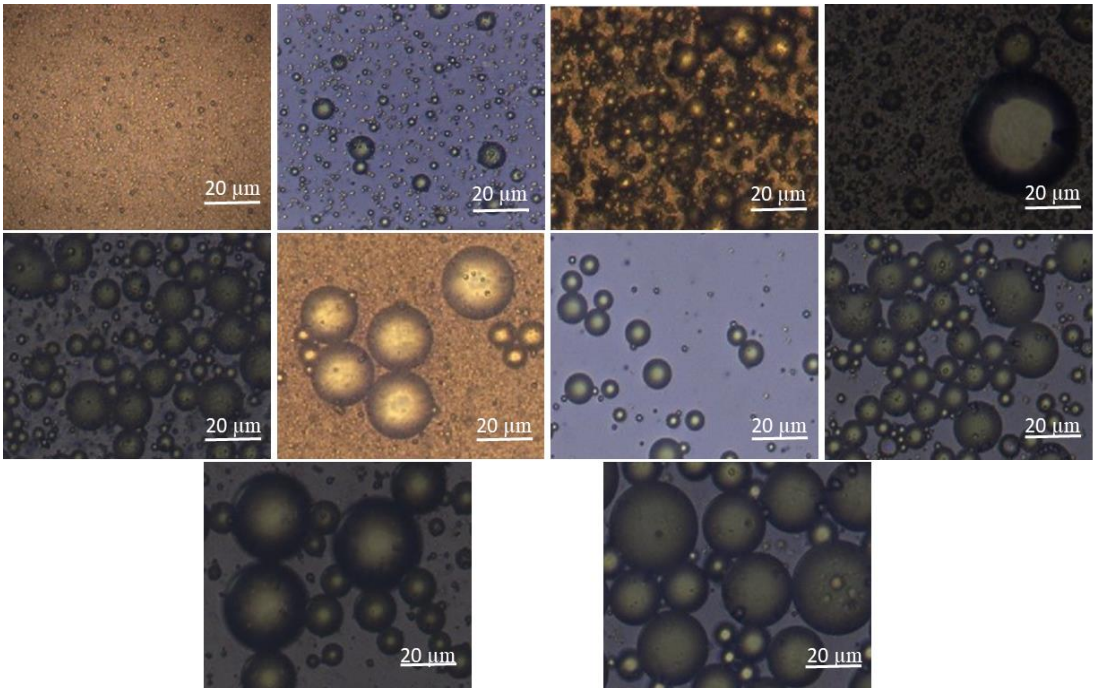

Fig 1. Essential oil emulsions. Images were captured using an inverted fluorescence microscope (EVOS® FL Cell Imaging System).

3.2 Preparation of WSSV inoculum

A bioassay was performed with healthy shrimp from Palmar Experimental Station located in Santa Elena-Ecuador weighing 6.79±1.02 g. Ground viral source tissue was obtained following Dominguez's et al. (2019) methodology. Moribund shrimp were collected every 4 hours until day 7 post-infection, macerated, and stored at -80°C. A sample (10 shrimp) was used for PCR and histopathology analyses to confirm the presence of WSSV and determine the typical clinical signs of the virus. For histopathological analysis, the shrimp were fixed with Davidson's AFA solution and the tissues were processed according to the procedures described by Bell and Lightner. (1983). On the other hand, PCR analyses were performed following the methodology described by Pradeep et al. (2008).

The methodology described by Li et al. (2014) was followed with slight modifications to obtain the viral inoculum. A 50 g of infected shrimp muscle was mash in 100 ml PBS on ice and centrifuged at 6000 x g for 20 min at 4°C. The supernatant was centrifuged at 10000 x g for another 20 min at 4°C using an ultracentrifuge (Sorvall Discovery 90SE), the supernatant was collected in a Falcon tube and filtered through 0.45 μm and 0.22 μm membranes. WSSV inoculum was aliquoted, and subsequently, their virulence was assessed in a challenge test.

3.3 Development of primary hemocyte culture

A permanent shrimp cell line is essential to investigate and develop new therapies for emerging prawn viruses. Numerous efforts have been made to establish a cell line to evaluate viral agents that affect penaeid culture since the initial attempt in 1978 (Fontaine,1978). However, to date it has not been successfully achieved. Despite this, the *in vitro* cell culture technique represents a powerful tool to study the pathogenesis and viral infection mechanism of WSSV. In what way primary hemocyte culture was developed is described in detail below.

Shrimp weighing 6.79±1.02 g were used for hemolymph extraction. Following a rigorous sterilization process, they were submerged in a 4% hypochlorite solution prepared in distilled water, then rinsed several times in cold, sterile seawater and their surface disinfected by wiping with 70% ethanol. To avoid clotting, hemolymph was drawn aseptically using 1 ml syringes filled previously with 200 μl of anticoagulant (10% sodium citrate). Hemolymph was pooled, then aliquoted in Eppendorf tubes (1.5 ml), and centrifuged (800 x g at 24°C for 5 min). The supernatant was

discarded, and the pellet was resuspended in L-15 medium. Following several researchers (Li et al., 2014; Sivakumar et al., 2019), the medium was supplemented with 1% glucose, 15% fetal bovine serum, penicillin (1000 IU/ml), streptomycin (1000 μg/ml), gentamicin (250 μg/ml), and amphotericin B (250 μg/ml) (pH: 7.2), and homogenized.

Subsequently, an aliquot of the sample was fixed in 3.7% (V/V ratio) formaldehyde. Hemocytes were counted using a Neubauer hemocytometer, and hemocyte concentration was adjusted to $5x10^5$ cells/ml for the assay. Hemocytes were transferred to cell culture flasks (Corning®CellBIND®) containing 5 ml of L-15 medium ($5x10^5$ cells/ml), the flasks were incubated at 27°C in 5.0% CO_2. It was essential to change the culture medium in the first 24 hours, and every 2 days after that. The culture was examined every day with an inverted microscope (EVOS® FL Cell Imaging System).

3.4 Screening anti-WSSV of EOs

To evaluate the *in vitro* activity of the EOs, primary culture of the hemocytes obtained by the process described above was performed, with a concentration of 500,000 cells/ml. The hemocytes were placed in 96-well cell culture plates (Nunc™ MicroWell™) with a final volume of 200 μl of L-15 medium per well. Primary cell culture was incubated for 24 h at 27 °C and 5.0% CO_2, the supernatant was removed and then 180 μl per plate were added to new plates. Thereafter, 10 μl of the 10 EOs at four different concentrations (1.25, 2.5, 5, 10 μg/ml) and WSSV inoculum (10 μl) were added. Hemocytes without EOs and WSSV inoculum (200 μl of fresh culture medium) were used as controls. Microplates were incubated at 27°C for 72 h. To establish the effects of EOs against WSSV, the viability of hemocytes was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or an MTT reduction assay. After 72 hours of incubation, the supernatant was removed from all wells and 200 μl of Minimum Essential Medium (MEM) were added with MTT (5 mg/ml MTT in MEM), and incubated at 27°C for 4h. The supernatant was then discarded and 5ml of dimethyl sulfoxide (DMSO) were used to dissolve the formazan crystals. Lastly, the microplate was read at 620 nm using a microplate reader (Varioskan™ LUX, Thermo Scientific™).

3.5 *In vitro* and *in vivo* toxicity of EOs

In order to establish the active anti-WSSV and non-lethal doses in *L. vannamei*, the *in vitro*

toxicity of EOs was initially evaluated by MTT reduction assay, following the protocol described by Domínguez et al. (2018). Primary cell cultures of hemocytes were set up at a concentration of 500,000 cells/ml in 96-well plates with Hank's balanced salt solution, supplemented with Cl_2Mg and Cl_2Ca. The primary cell cultures were incubated for 75 min at 25°C, the supernatant was removed, and 100 µl of Hank's balanced salt solution were immediately added to the plates. Hank's balanced salt solution were used as a vehicle to dilute EOs to various concentrations (1.25, 2.5, 5, 10, 100 µg/ml). From these solutions, 100 µl were added to each well. Six replicates for each concentration were evaluated and a control of hemocytes without EOs was included. After 120 min of exposure, 10 µl of MTT (5 mg/ml MTT in Hanks) were added to all wells. Then, the microplate was incubated for 120 min at 25°C. The supernatant was discarded, 200 µl of DMSO were used to dissolve the formazan crystals, and the microplate was read at 620 nm. The number of viable cells counted were transformed into percentages of cell viability, using the cell viability of hemocytes that did not receive EOs as the 100% benchmark. The following formula was applied:

$$\text{Cell viability OD} = (\text{OD exposed cells}/\text{OD control cells}) \times 100\%$$

In vivo toxicity was evaluated in two early stages of *L. vannamei*: Zoea (Z2) and Mysis (M2). Several concentrations of the oils were used (2.5, 5, 10 µg/ml), and three replicates of each concentration were evaluated. The EOs were applied proportionately to each experimental unit's total volume of water (40 larvae). A control with vehicle solution was included. The larvae were observed for 96 hours. The data were converted to survival percentages, taking into account the 100% survival of the larvae that did not receive EOs.

3.6 *In vivo* evaluation of the anti-WSSV activity of essential oils

3.6.1 Virus inactivation by direct exposure to essential oils

In vivo validation of active doses of essential oils against WSSV, *L. vannamei* shrimp (n=100, initial weight 6.79±1.02 g) were distributed homogeneously into aquariums with an operational capacity of 40 L of seawater and provided with constant aeration. The bioassay was performed for 7 days, and three essential oils emulsified at three different concentrations were evaluated (1.25, 2.5, 10 µg/ml). The viral inoculum was exposed to the oil emulsions for 15 min. Immediately thereafter, shrimp were challenged with WSSV by injecting 100 µl of EO+WSSV

(V/V ratio) in the fifth segment of the shrimp. As positive control shrimp were injected with 100 µl of viral inoculum. Vehicle control shrimp were injected with 100 µl of PBS without virions and EOs. Shrimp were fed a commercial 35% protein feed (2.8% of shrimp body weight) once a day. The temperature fluctuated between 23-25°C. Uneaten food and waste material were removed by siphoning every 2 days before feeding, and exchanging 50% of the water in the tank. Throughout the bioassay, mortality was recorded every 2 h post-infection.

3.6.2 Protective effect of essential oils against-WSSV

L. vannamei shrimp (n=300, initial weight 14.95±2.11 g) were distributed in equal numbers into 20 aquariums and treated with EO active doses for 10 days. EOs were incorporated to the shrimp's food at three concentrations: 1.25, 2.5, and 10 µg/ml (1.5% of shrimps body weight) twice a day. After 10 days of treatment, the viral inoculum was exposed to the oil emulsions for 15 min. Immediately shrimp were challenged with WSSV as 100 µl of EO+WSSV (V/V ratio) were injected into the fifth segment of the shrimp. As positive control shrimp were injected with 100 µl viral inoculum. Vehicle control shrimp were injected with 100 µl of PBS without virions and EOs. The temperature fluctuated between 23-25°C. Uneaten food and waste material were removed by siphoning every day before feeding, and 50% of the water was exchanged every day. Mortality was registered every 2 h during 8 days post-infection, and a survival analysis was performed with the data.

3.6.3 Data Analysis

All experiments were done in 6 replicates. The results were expressed as an average (±standard deviation) of the replicates. Statistical analyses were performed to determine significant differences ($P \leq 0.05$) using one-way ANOVA, after verification of the normality and variance homogeneity assumptions. Dunnett analysis was applied (control and treated groups) when significant differences were detected. The data expressed in percentages were transformed (using arcsine), and the assumptions were fulfilled before performing the statistical analysis. Mortality rate was estimated using the Kaplan-Meier analysis; a non-parametric maximum likelihood estimate (MLE) of the survival function. The estimation of significant differences in the survival rate among groups was done using Kaplan-Meier comparison tests. The P-value was calculated using the log-rank test. All analyses were performed using the SPSS statistical software (version 21).

4. Results

4.1 Ground viral source tissue

A challenge test was performed to obtain WSSV infected muscle (Fig. 2). After 24 hours, the survival rate of the shrimp challenged with WSSV begins to decrease until it reaches almost 40% at 108 hours. Mortality increased, showing a maximum peak at 100 hours post-infection and cumulative mortality of 56.67%. Shrimps were confirmed to be infected by WSSV through histopathologic analysis (Fig. 3) and nested polymerase chain reaction (PCR) (Fig. 4).

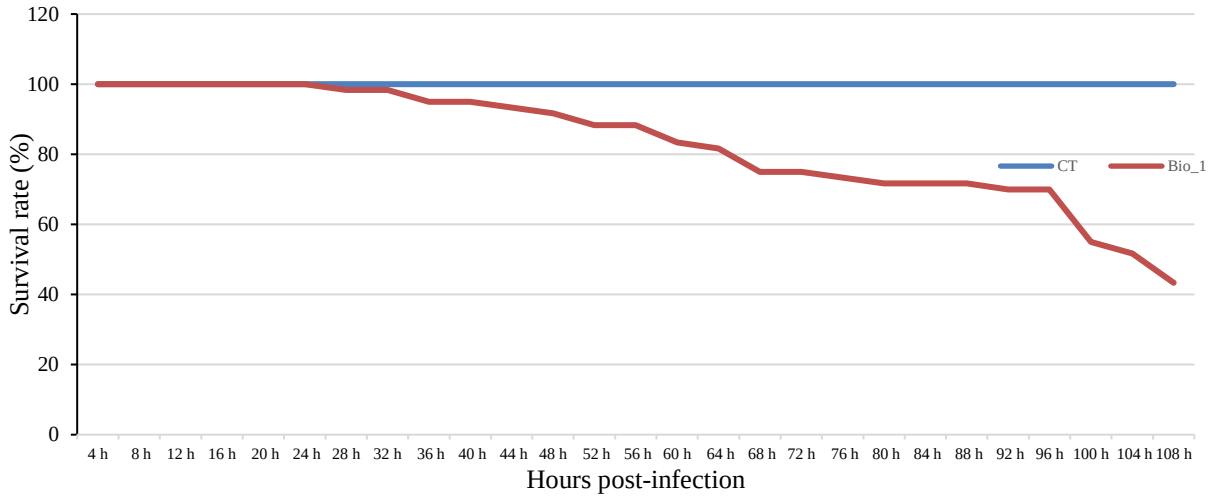

Fig. 2. WSSV infection challenge. Blue line represents the control group (n=90 shrimps). The orange line represents the mean survival rate of 9 aquariums (n= 90 shrimps) 108 hours post-infection.

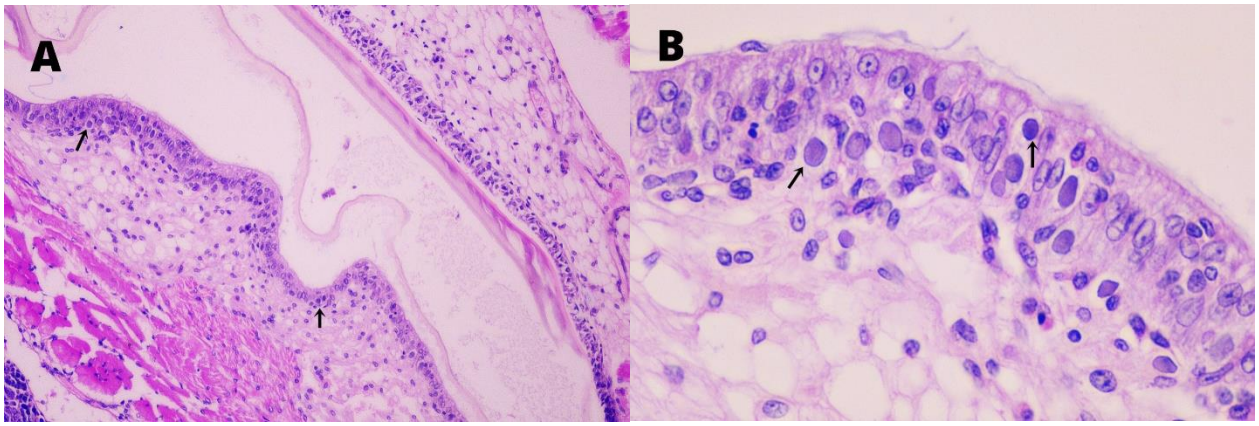

Fig. 3. Stomach epithelial tissue of shrimp infected with WSSV. A) Arrows indicate stomach epithelial cells severely affected by WSSV, 10x lens B) A close-up of WSSV-affected cells for better illustration, 40x lens.

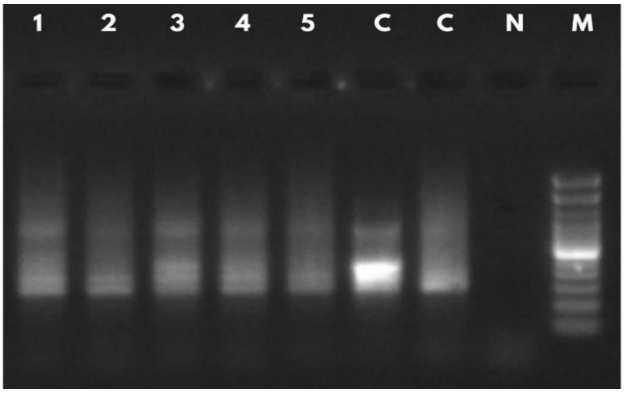

Fig. 4. Nested PCR in a shrimp sample 72 hours post-infection, (1) pleopod tissue, (2)(3)(4)(5) muscle tissue, (C)(C) WSSV positive control, (N) Negative, (M) Marker.

4.2 Viral inoculum

To evaluate and validate the virulence of the viral inoculum, 2 bioassays were performed with healthy *P. vanammei* shrimps. In both bioassays, the first signs of disease were detected between 24 and 36 hours post-infection. During the 6 days that the trials lasted, the results of the first challenge test showed a cumulative mortality rate of 100%, while in the second, it was 92%, showing that the viral inoculum is lethal (Fig. 5). Mortalities peaks were recorded between 52- and 84-hours post-infection; these findings agree with those obtained by Escobedo-Bonilla et al. (2006).

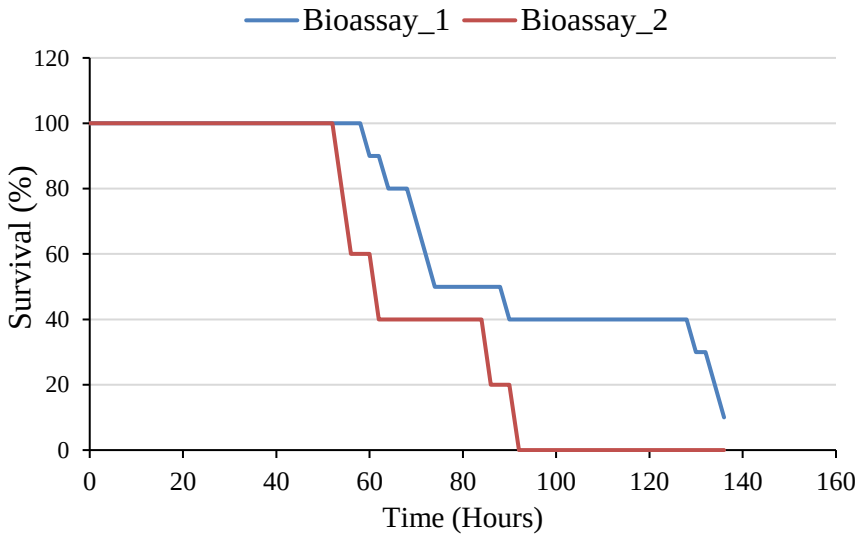

Fig. 5. Virulence test. The Red line represents the mean survival rate of 9 aquariums (n= 90 shrimps) 92 hours post-infection. Blue line represents the mean survival rate of 9 aquariums (n= 90 shrimps) 136 hours post-infection.

4.3 Primary hemocyte culture

In the primary hemocyte culture, a high concentration of hemocytes showed a rapid multiplication and proliferation of cells, swiftly attaining a confluent monolayer that depleted the medium's nutrients in just a few days. To obtain a shrimp cell line that lasts at least 7 days and avoid a gradual decline in the number of viable cells, different concentrations of hemocytes were tested (25,000, 50,000, 100,000, 250,000, 500,000 cells/ml) and was found better results using 50,000 cells/ml.

L-15 medium used for shrimp cell culture exhibited the capability to support the migration and survival of the hemocytes cells satisfactorily. The first migrating cells had a round appearance and adhered to the surface 24 hours after seeding (Fig 6). A confluent cell monolayer could be achieved in 3-4 days. The hemocytes cells migrated actively within the first 5 days, and then some of the cells began to detach.

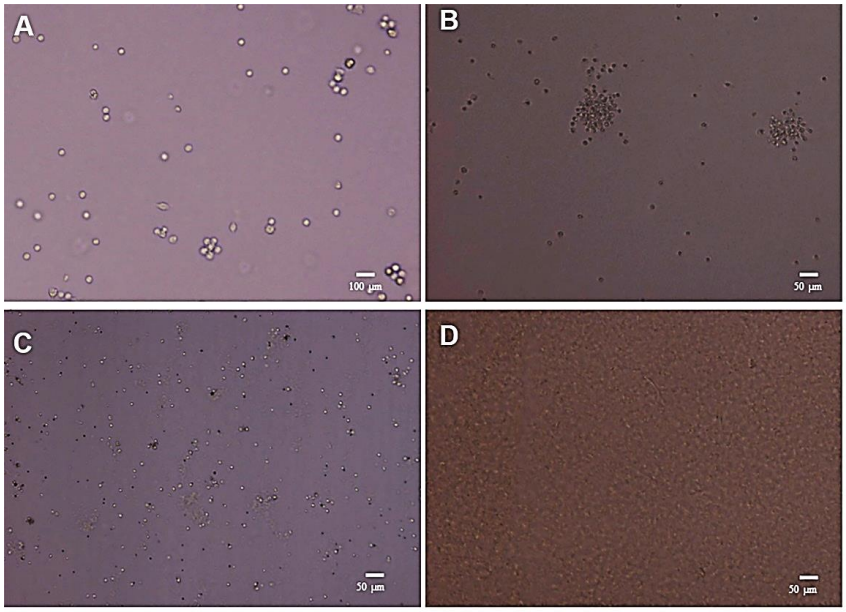

Fig. 6. Primary hemocyte culture in L-15 medium. A) Initial culture, cells in suspension or semi suspension. B) 24 h after seeding, adhered cells. C) 42 h after seeding, initiation of monolayer formation. D) Confluent monolayer throughout the cell culture flask, 5 days after seeding. Images were captured using an inverted fluorescence microscope (EVOS® FL Cell Imaging System).

4.4 Screening anti-WSSV

To identify the antiviral properties of essential oils against WSSV, the effects of 10 EOs on hemocytes were evaluated at different concentrations (1.25, 2.5, 5, 10 µg/ml). The essential oil that

resulted in the highest survival rates at the highest concentration evaluated was EO10 (Fig 7A). At 5 and 2.5 µg/ml, a cumulative survival rate of 60 and 80%, respectively, were obtained with EO3 (Fig 7A, 7B). At the lowest concentration evaluated, best results were obtained with EO7 (Fig 7C). For the *in vivo* tests, the three oils that showed a cumulative survival rate ≥ 80% were used**.**

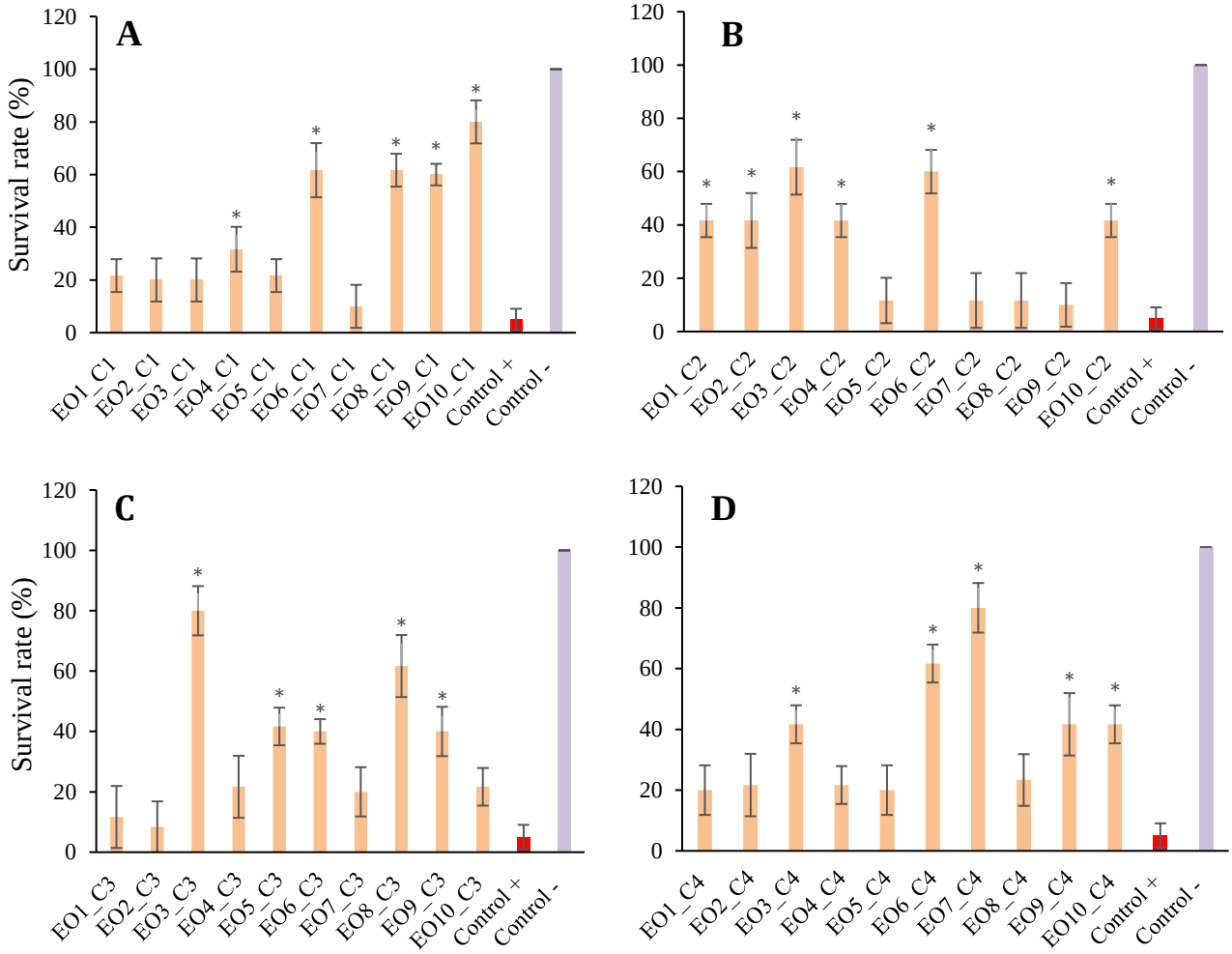

Fig. 7. Screening anti-WSSV effect of 10 essentia oils: EO1=*Origanum vulgare*, EO2=*Azadirachta indica*, EO3=*Syzygium aromaticum*, *EO4=Cinnamomum verum*, *EO5=Melaleuca alternifolia*, EO6=*Thymus vulgaris*, EO7=*Zingiber officinale*, EO8=*Allium sativum*, EO9=*Citrus sinensis* and EO10=*Cymbopogon citratus. C4*, *C3*, *C2*, *C1*correspond to concentrations of 1.25, 2.5, 5, 10 µg/ml, respectively. *Indicates a significant difference ($p < 0.05$) in comparison to the control as determined by an Anova and post-hoc Dunnett test for multiple comparisons.

4.5. Toxicity of EOs, *in vitro* and *in vivo* shrimp models.

4.5.1. *In vitro* toxicity of EOs

To examine whether essential oil compounds have toxic properties on hemocyte cells, different concentrations of EO3, EO7, and EO10 were applied. The three essential oils evaluated significantly affected the viability of hemocytes (Fig 8). Concentrations of 100 and 10 µg/ml resulted in 75% to 85% of cell viability. EO10 was the most toxic towards hemocytes because it affected them at three different concentrations, causing a drop of 15% of hemocytes survivorship, even at a 5 µg/ml concentration. On the other hand, concentrations of 5, 2.5, and 1.25 µg/ml of EO3 and EO7 did not show toxic effects because the viability of the hemocytes was not affected by the oils compared to the control.

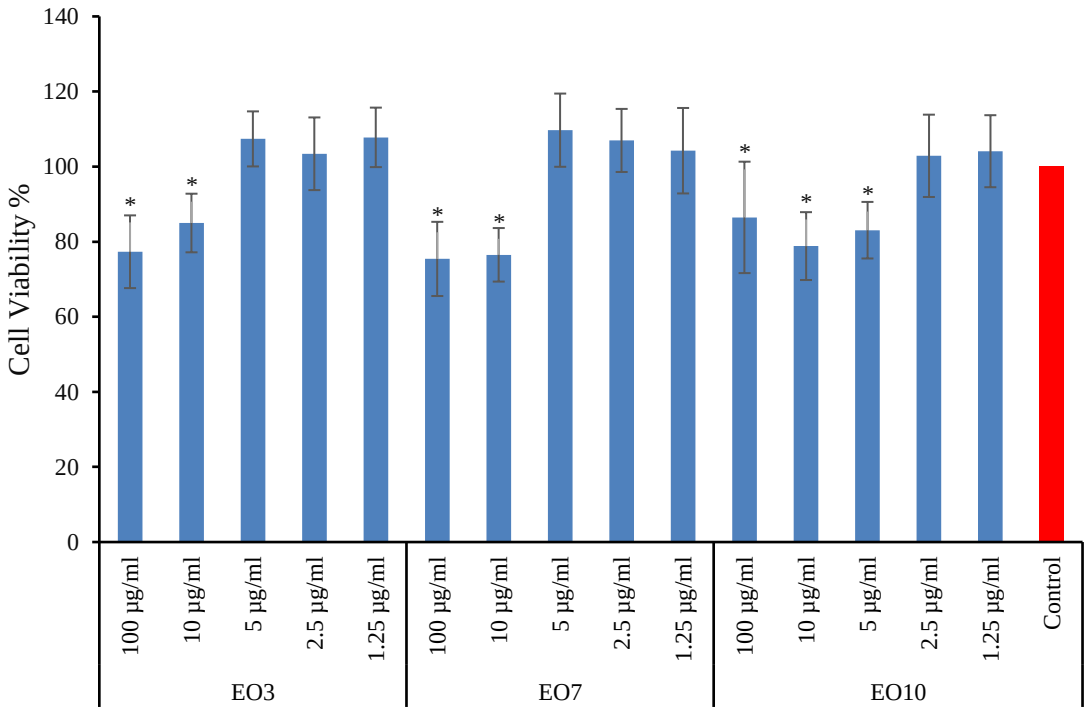

Fig. 8. Percentage of cell viability among three essential oils and control**.** Three essential oils from *Syzygium aromaticum, Zingiber officinale* and *Cymbopogon citratus* were applied at five concentrations (1.25, 2.5, 5, 10, 100 µg/ml) to the primary hemocyte culture. *Indicates a significant difference ($p < 0.05$) in comparison to the control as determined by an Anova and post-hoc Dunnett test for multiple comparisons.

4.5.2. *In vivo* toxicity of EOs

Of the three essential oils evaluated, EO7 did not show toxic effects on Z2 larvae of *P.*

vanammei at any of the concentrations tested. Regarding EO3 and EO10, both significantly affected the survival of the zoea, such that the three concentrations assessed with EO10 (2.5, 5, 10 µg/ml) affected more than 15% of the population than the control (Fig 9). Regarding the M2 larvae exposed to the three essential oils, the results showed that EO10 affected their survival at the highest concentration evaluated (10 µg/ml). In contrast, EO3 and EO7 did not show cytotoxic effects since the accumulated survival was different from the control (Fig. 10).

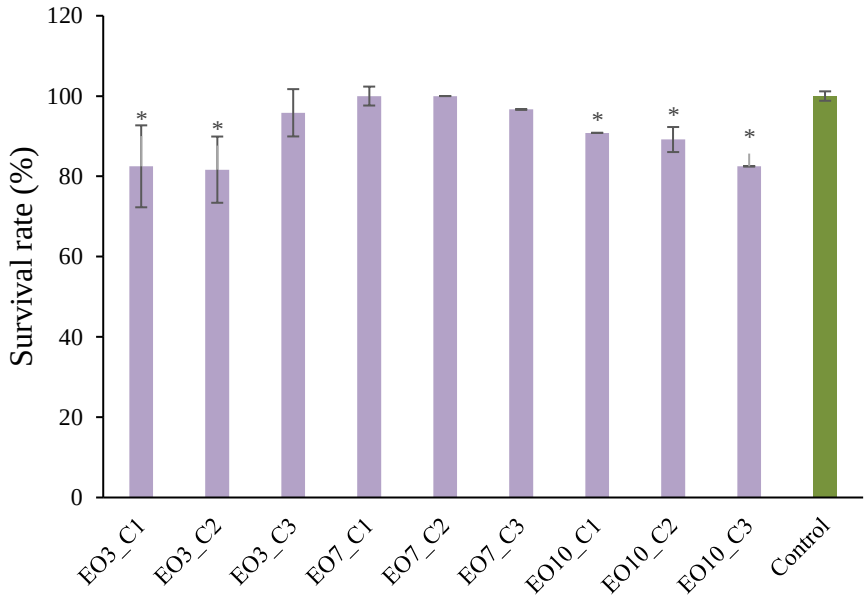

Fig. 9. In vivo toxicity in Zoea stage. Three essential oils *Syzygium aromaticum, Zingiber officinale,* and *Cymbopogon citratus* were applied in three concentrations (2.5, 5, 10 µg/ml). *Indicates a significant difference ($p < 0.05$) in comparison to the control as determined by an Anova and post-hoc Dunnett test for multiple comparisons..

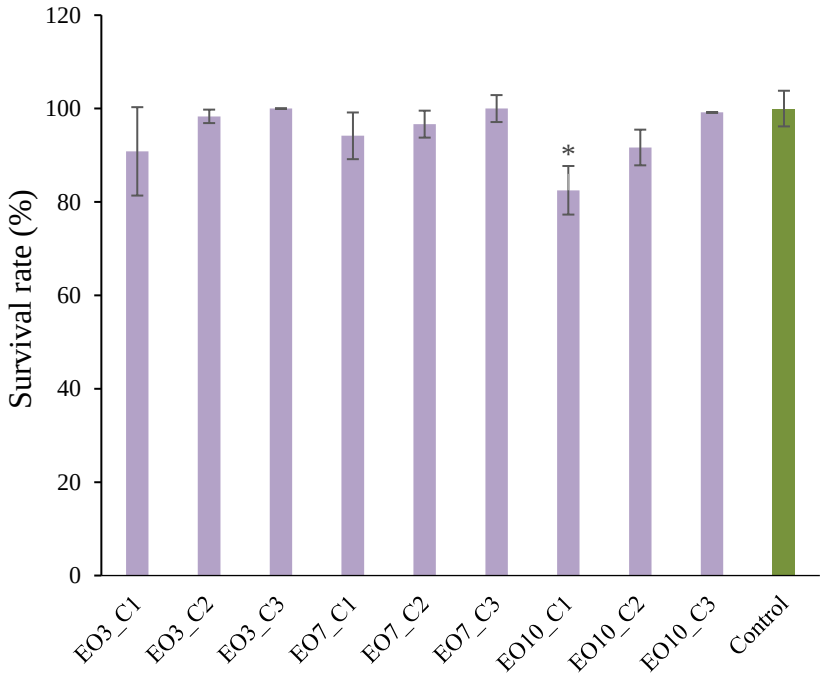

Fig. 10. In vivo toxicity in Mysis stage. Three essential oils *Syzygium aromaticum, Zingiber officinale* and *Cymbopogon citratus* were applied in three concentrations (2.5, 5, 10 µg/ml). *Indicates a significant difference ($p<0.05$) in comparison to the control as determined by an Anova and post-hoc Dunnett test for multiple comparisons**..**

4.6 Challenge test

After 10 days of applying the EOs at the active doses previously tested *in vitro*, the shrimp were challenged with viral inoculum, using a concentration that causes $50\% \pm$ mortality in a healthy population of *P. vannamei*. The cumulative mortality rate in the three oils was significantly reduced compared to the control group (Fig 11). A survival analysis was performed with the data obtained in the Challenge test (Fig 12). The analysis showed significant differences in the mortality hours of the shrimp treated with oils compared to the control. At 162 hours post-infection, the shrimp treated with EO10 showed a cumulative survival of 89%, while in control, survival of 47% was recorded. The analysis corroborated that EO10 was the most effective; however, it is essential to emphasize that the dose was higher. These results together demonstrate the antiviral effect of essential oils and the protection exerted towards shrimp.

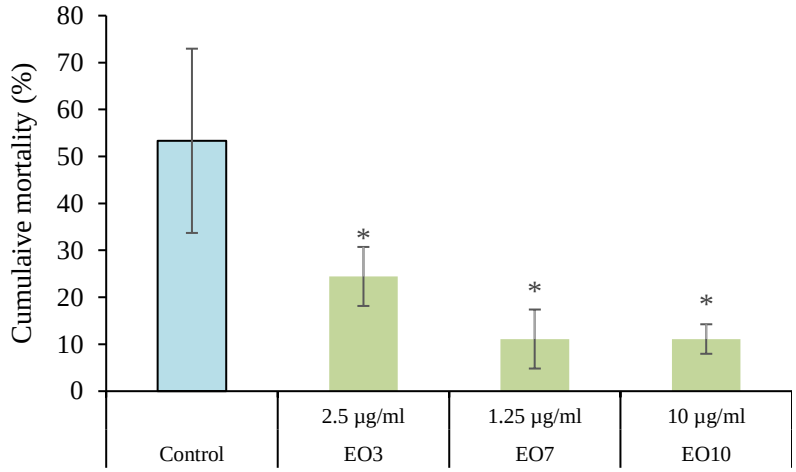

Fig. 11. Percentage of mortality among three different treatments and control. *Syzygium aromaticum, Zingiber officinale* and *Cymbopogon citratus* were applied in four concentrations (1.25, 2.5, 10 µg/ml) to food and supplied to shrimps infected with WSSV. *Indicates a significant difference ($p < 0.05$) in comparison to the control as determined by an Anova and post-hoc Dunnett test for multiple comparisons.

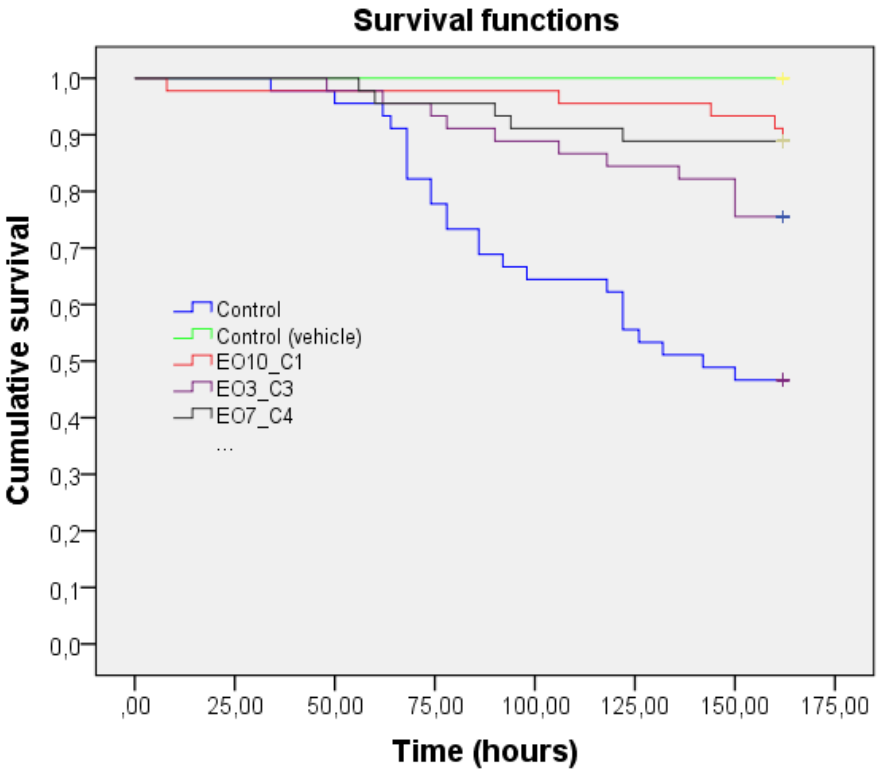

Fig. 12. *In vivo* survival analysis in juvenile shrimp challenged with WSSV. Three essential oils *Syzygium aromaticum, Zingiber officinale* and *Cymbopogon citratus* were applied at different concentrations C4, C3, C1 =1.25 2.5, 10 µg/ml.

5. Discussion

In the present study, it was possible to identify three essential oils with anti-WSSV properties: *Syzygium aromaticum*, *Zingiber officinale,* and *Cymbopogon citratus*. These three oils, when added to the feed and supplied to the shrimp, managed to improve survival significantly when challenged by WSSV. In general, the results obtained offer a consumer-friendly alternative to control WSSV in the farming of *Pennaeus vanammei.*

Compared with other organs and tissues of penaeid shrimp, hemocytes adhere and proliferate more rapidly; moreover, hemocyte cultures represent a non-invasive method, unlike the extraction of vital organs for shrimp, due to this particularity, in this study was carried out a primary culture from hemocytes. Our results agree with Jose et al. (2010) managing to maintain the viability of the hemocytes for 7 days. Although other researchers affirm to developed a hemocyte culture in L-15 medium and reported the maintenance of the cells for 20 and 30 days (Ellender et al., 1992; Jiang et al., 2006), in our study, this was not possible.

Cell culture-based toxicological assays are indispensable tools to evaluate functional additives used in the shrimp industry. Notwithstanding multiple advantages of cell-based *in vitro* assays, such as fewer financial resources and reduced number of animals used for toxicity assessment, this type of assays has limitations. They do not accurately reflect the intricate processes that occur in an organism (Lanzerstorfer et al., 2021). For this reason, in this study, toxicological analyzes were performed in both *in vitro* and *in vivo* models. Results were similar in one and the other, but when shrimp were challenged with WSSV, the EO10 applied at 10 μg/ml turned out to be the best treatment against-WSSV. This corroborates that cell culture systems can only provide a first understanding of *in vivo* conditions since the physiology of an organism is highly complex. Similar results regarding the toxicity of essential oils have been reported by Domínguez-Borbor et al. (2020).

To evaluate anti-WSSV products as strategies to control the disease, different inoculation routes as immersion, *per os,* and intramuscular inoculation in *L. vannamei* is used. Several authors have mentioned that *per os* via feeding and immersion inoculation simulates an infection closely linked to what happens in nature, but it has been described that the reproducibility of these methods

is not efficient, resulting in contradictory findings (Laramore, 2007; Gitterle et al., 2006; Pérez et al., 2005). This is because the *per os* inoculation is based on the supply of infected material in which there is a probability of ingesting or not ingesting or that the ingested viral load is not adequate to cause the disease. Regarding intramuscular inoculation, even though the natural shrimp barrier is evaded, it has been the most efficient and reproducible method in the experimental infection of WSSV in penaeid shrimp (Escamilla-Montes et al., 2019; Van Thuong et al., 2016). In this study, we initially used the *per os* method to obtain WSSV viral particles, but to validate the effectiveness of the oils we used intramuscular inoculation.

A quantitative analysis of the chemical composition of the extracted essential oils was not performed in this study. Nonetheless, the extraction of the EOs was carried out separately, so a mixture of oils was not produced. In previous studies, the chemical composition of oils that showed anti-WSSV activity was investigated. The major constituent of *Syzygium aromaticum* essential oil was euglenol (80%); *Zingiber officinale* was mainly composed of citral (neral 9.1% and geranial 10.5%), α-zingiberene (17.4%), camphene (7.8%), (E,E)-α-farnesene (6.8%) and β-sesquiphellandrene (6.7%). Meanwhile for *Cymbopogon citratus*, citral α (40.8%) and citral β (32%) were the most abundant constituents (Shah et al., 2011).

Essential oils have been reported several active biological properties including antiviral activity in human health (Nadjib, 2020; Brand et al., 2016). Specifically, EOs exert antiviral activity against Herpes simplex virus, serotypes 1 (HSV-1) and 2 (HSV-2) and Caprine alphaherpesvirus-1 (Almeida et al., 2018; Benencia et al., 2000; Camero et al., 2019), where the EOs action modes is known. Regarding the mechanism of action of EOs on WSSV, this has not been investigated since it is the first study using the EO3, EO7 and EO10 in the penaeid shrimp culture. Nevertheless, Reichling (2021) stated the potential mechanisms of antiviral effects of essential oils on enveloped viruses include: direct antiviral effect when the viruses are outside the host's cells, disruption of the envelope, inhibition of entry into the host's cells, inhibition of the virus replication and/or other viral activities inside the cell, antioxidant effect that reduces viral titers and anti-inflammatory effect in virus-infected tissues.

EOs evaporate easily at normal temperatures as they contain volatile substances. They are relatively easy to acquire, friendly to the environment as they break down quickly in soil and water.

(Valková et al., 2022). In the present study, the oil of *Cymbopogon citratus* was the most effective, significantly improving the survival of shrimp previously treated with the oil and then challenged with WSSV. These findings denote the great potential that essential oils show as an effective tool for controlling WSSV in shrimp farming.

6. Conclusion

The initial screenings allowed to identify of three essential oils (*Syzygium aromaticum*, *Zingiber officinale*, and *Cymbopogon citratus*) with anti-WSSV activity and their active concentrations, the highest concentration evaluated showed toxic effects for both hemocytes and early stages of *P. vanammei*. In particular, the best results were obtained with the *Cymbopogon citratus* oil at the highest concentration, which shows that for juvenile shrimp, this concentration is not toxic and instead provides a protective effect against the virus. In general, these results illustrate the antiviral properties of EOs against WSSV, and they can be included in the shrimp diet for the control of WSSV.

7. Recommendations

In this work, ten essential oils were evaluated, of which a large percentage of anti-WSSV was found, so new sources of EOs should be evaluated to have other substances that can be used to treat emerging pathogens that affect the aquaculture industry. EOs should not be supplied in the diet of the early larval stages of *P. vanammei* as they can be toxic; oils should be incorporated into the diet of juvenile shrimp. EOs could also be combined to improve the anti-WSSV response; this approach was not addressed in the study. Finally, the bioactive compounds of EOs should be identified through mass spectrometry to enhance the anti-WSSV effect.

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