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RESEARCH ARTICLE

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Effect of microencapsulated phenolic compound extracts of *Maclura tinctoria* (L.) Steud on growth performance and humoral immunity markers of white leg shrimp (*Litopenaeus vannamei*, Boone, 1931) juveniles

Jose S. Diaz[1], María C. Flores-Miranda[2], Norma Almaraz-Abarca[3], Arturo Fierro-Coronado[4], Antonio Luna-Gonzalez[4], Manuel Garcia-Ulloa[4] and Hector A. Gonzalez-Ocampo[4]

[1] Universidad Autónoma de Sinaloa-Escuela de Biología. Blvd. de las Américas y Universitarios. s/n, Col. Universitarios. 80010 Culiacan, Sinaloa, Mexico. [2] Universidad de Guadalajara. Estudios para el Desarrollo Sustentable de Zonas Costeras, Dept., CUCSUR. Av. Gomez Farias No. 82, San Patricio Melaque, Jalisco, Mexico. [3] Instituto Politécnico Nacional, Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional-Unidad Durango, Sigma 119, 20 de Noviembre II, 34220 Durango, Durango, Mexico. [4] Instituto Politécnico Nacional, Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional-Unidad Sinaloa, Blv. Juan de Dios Batiz Paredes 250, Col. San Joachin. 81101 Guasave, Sinaloa, Mexico.

Abstract

Aim of study: : The effect of microencapsulated phenolic compound extracts of *Maclura tinctoria* (MTBE) on growth performance and humoral immunity markers of the white leg shrimp *Litopenaeus vannamei* juveniles (0.5 ± 0.2 g initial weight) was studied.

Area of study: M. tinctoria was collected from Hampolol, Campeche, and Arroyo del Agua, Culiacan, Sinaloa, Mexico.

Material and methods: Three MTBE inclusions (0.5, 1, and 2.5 g MTBE/kg, Purina®) were compared with a control commercial feed (Purina®) during 30 days. Nine phenolic acids, nine flavonols, four dihydro-flavonoids, four flavones, and seven unidentified phenolic compounds were determined fin the MTBE using a Perkin Elmer® HPLC chromatograph and diode array-detection.

Main results: The mean concentrations of total phenolic compounds, total flavonoid compounds, and condensed tannins were 198.05 ± 5.59 mg gallic acid equivalent (GAE) g-1 dw, 78.57 ± 1.80 quercetin equivalent g^{-1}, and 28.32 ± 0.33 mg epicatechin equivalent g^{-1}, respectively. The ferric reducing antioxidant power and the total antioxidant capacity, respectively, averaged 28.32 mg GAE mL^{-1} and 10.9 mg ascorbic acid equivalent mL^{-1}. Survival, weight gain, and specific growth rate of *L. vannamei* were similar among the experimental diets. The dietary inclusion of MTBE at 0.5 g/kg of food showed significant higher ($p < 0.05$) plasma hemocyte lysate protein (1.35 ± 0.055 µg mL^{-1}), prophenoloxidase (0.47 ± 0.15, Abs. 492 nm), and superoxide anion (O_2.-) activity (0.21 ± 0.07, Abs. 630 nm).

Research highlights: The supplementation of MTBE at 0.5 g/kg of food could be considered as a potential alternative additive for *L. vannamei* diet in the juvenile production, since it improved the response of the humoral immunity markers at post larval life stages, when cultivated shrimp are more susceptible to be infected by pathogens.

Additional key words: nutraceutical; ethnobotany; prophenoloxidase; phenoloxidase; superoxide anion; immunostimulant supplements

Abbreviations used: FRAP (ferric reducing antioxidant power assay); GAE (gallic acid equivalent); HLS (hemocytes lysate supernatant); MTBE (microencapsulated phenolic compound extracts of *Maclura tinctoria*); PO (phenoloxidase); proPO (prophenoloxidase); SGR (specific growth rate); SO (superoxide anion); TAC (total antioxidant capacity); TPC (total phenolic compounds); WSSV (White Spot Syndrome Virus)

Authors' contributions: JAS performed the experiment, analysed the data and wrote the draft paper. CFM analyzed the data and wrote the paper. NAM designed and wrote the paper. AFC analyzed the data. ALG analyzed the data, wrote the paper and contributed reagents and materials. MGU analyzed data, and made critical revisions of the manuscript. HAG conceived and designed the experiment, analyzed the data and critical revision of the manuscript. All authors read and approved the final manuscript.

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Correspondence should be addressed to Hector A. Gonzalez-Ocampo: hgocampo@yahoo.com, or Manuel Garcia-Ulloa: turbotuag@hotmail.com (shared corresponding authors)

Introduction

Shrimp aquaculture is one of the main economic activities in tropical and subtropical areas, which supply this product at a world scale reaching more than four million tons. The success of white shrimp (*Litopenaeus vannamei* Boone 1931) production depends on reducing the impact of diseases and epizootics (Zhang *et al.*, 2016; Dewangan *et al.*, 2017), mainly during its larval and juvenile stages. Among the most important pathogens that cause diseases in larval and juvenile stages of *P. vannamei* are White Spot Syndrome Virus (WSSV) and Taura Syndrome Virus (TSV), and *Vibrio* species (Cheng *et al.*, 2011; Rajkumar *et al.*, 2017). Intensification of culture applications has led to diseases causing up to 80% economic losses in hatcheries (mainly within 60–80 days post-stocking) (Kibenge, 2019)); *e.g.* total losses caused just by WSSV have amounted to 3 billion US dollars per year (Yang *et al.*, 2016). For decades, the use of antibiotics has been a common practice for the prevention of shrimp diseases (Bermúdez-Almada & Espinosa-Plascencia, 2012); however, it is well documented that the application of these chemicals is a management practice that involves dangers and risks to the environment and human health (Thornber *et al.*, 2020). Therefore, the search for alternative compounds that are environmentally-friendly, harmless to human health, and do not compromise shrimp production are a priority (Romero *et al.*, 2012).

The use of alternative substances and microorganisms with antimicrobial and immunostimulant activities to reduce diseases impact (Peraza-Gómez *et al.*, 2014; Trejo-Flores *et al.*, 2016) and diminish the application of antibiotics (Cabello, 2006) in the shrimp industry has been increasing. Allelopathic powders and plant extracts are immunomodulator and immunostimulant additives commonly used in safeguarding animal health (Tiwari *et al.*, 2018). Among these products, the phenolic compounds-based extracts have been described as potential immunostimulants, antipathogenic, and antistress agents in aquaculture (Chakraborty & Hancz, 2011).

The *Maclura tinctoria* tree is a deciduous, semideciduous, and even perennial arboreal native species of dry and wet tropical regions from Mexico to Argentina (Berg, 2001). Although antibacterial, antioxidant, and immunostimulant benefits of *M. tinctoria* extracts have been proved in laboratory assays (Lamounier *et al.*, 2012; Matson-Robles *et al.*, 2015), their chemical properties are commonly reduced by temperature, light, and environmental conditions (Santos-Buelga *et al.*, 2019). The microencapsulation technique represents a viable alternative (Ozkan *et al.*, 2019) to preserve the allopathic properties of *M. tinctoria'* extracts. There are few studies on the use of some phenolic compounds in shrimp culture (Sudheer *et al.*, 2011; Chandran *et al.*, 2016; Tomazelli-Júnior *et al.*, 2018), but so far, supplementation of microencapsulated phenolic extracts of *M. tinctoria* in diets for *L. vannamei* juvenile has not been described yet. In the present work, we studied the effect of microencapsulated phenolic compound extracts of *M. tinctoria* on growth performance (final weight gain, specific growth rate, and survival) and humoral immunity markers (protein concentration in plasma and hemocytes, phenoloxidase activity, and superoxide anion concentration) of white shrimp *L. vannamei* juveniles.

Material and methods

Plant material

Three samples of *M. tinctoria* fruits were obtained from 20 reproductive trees (60 samples) from two tropical dry forest areas of Mexico (19°56´32” N, 90°22´32” W, and 24° 52´42.39” N, 107°17´47.53” W). Dried samples were pulverized with a domestic blender (Sunbeam®, USA), then, 5.5 g powder per sample were extracted with 30 mL of 50% ethanol (Baker®) and kept isolated from light in amber jars until laboratory analysis. For microcapsules, the core materials were *M. tinctoria* fruit extracts (MTBE) and fish oil (Pescadería Mora®, Mex), wall materials consisted of food grade gum arabic E 414 (Alfred L. Wolf®, Mex) and maltodextrin DE® 10 (Chemistry LEFE®, Mex), according to (González-Ocampo *et al.*, 2016).

Extraction, identification, quantification, and microencapsulation of *M. tinctoria* phenolic compounds

To obtain the MTBE microcapsules, the bark powder was mixed with ethanol, stirred, and kept in the dark for 24 h. Thereafter, a solution was prepared with 183 g of bark powder and 1 L of absolute ethanol (50%) FAGALAB®. The solution was stirred for 24 h, filtered through an analytical sieve with a mesh (90 μm) (WS Tyler®, USA) to remove solids, and subsequently kept in amber jars (Almaraz-Abarca *et al.*, 2013).

The microencapsulation technique was developed in the Aquaculture Laboratory of the CIIDIR-IPN Sinaloa Research Center, and is being processed for a patent (MX/a/2016/007504) at the *Instituto Mexicano de la Propiedad Intelectual*, IMPI (González-Ocampo *et al.*, 2016).

Phenolic content

The phenolic composition of MTBE was determined through high performance liquid chromatography, HPLC (Perkin-Elmer®, Series 200 HPLC system, Shelton, CT, USA) using a Perkin Elmer® Brownlee Analytical C18 column (4.6 × 250 mm, 5 µm) and diode array-detection (DAD) system (Perkin Elmer Series 200, USA), with a modified gradient method (Campos & Markham, 2007). Maximal absorbance was achieved employing a gradient method with mobile phase, which consisted of a mixture of water, 0.05% orthophosphoric acid (2.5 pH, Baker®, USA) (A), and acetonitrile (J. T. Baker®, USA) (B) as follows: 100(A):0(B) 0-12 min; 87(A):13(B) 12-20 min; 67(A):33(B) 20-32 min, and 57(A):43(B) 32-42 min, at a flow rate of 1 mL min-1. The identified phenolic chromatograms were registered at 260 nm and structural information of compounds was compared with the reference of compounds and its retention time (RT) from Apin Chemicals Limited® (Abingdon, Oxon, UK) standard: caffeic acid (RT: 53.13 min; λ_{max}), *p*-coumaric acid (RT: 37.2 min; λ_{max}: 294sh, 308), quercetin (RT: 45.95 min; λ_{max}: 260, 268sh, 299sh, 370), rutin (quercetin-3-*O*-[rhamnosyl(1-6) glucoside]; RT: 33.74 min; λ_{max}: 255, 264sh, 294sh, 355), apigenin (RT: 59.60 min, 267, 290sh, 335), quercitrin (quercetin-3-*O*-rhamnoside, RT: 38.54, λ_{max}: 255, 264sh, 295sh, 348), morin (RT: 45.4, λ_{max}: 254, 264sh, 298sh, 354), hesperidin (RT: 39.34, λ_{max}: 284, 335sh), and naringenin (RT: 52.25, λ_{max}: 289, 335sh). Spectral information was obtained from the phenolic compounds' spectral data described by Mabry *et al.* (1970) and Campos & Markham (2007).

Antioxidant capacity

The total antioxidant capacity (TAC) was calculated by the ferric reducing antioxidant power (FRAP) method (Oyaizu, 1986) and the molybdate VI reduction to molybdate V assay, modified by Prieto *et al.* (1999). The FRAP assay is based on the reduction power of the plant extracts. The ferric ion (Fe^{3+}) is reduced to ferrous ion (Fe^{2+}) forming the blue complex (Fe2+ TPTZ-1), which increases the absorption at 593 nm; samples and standard (ascorbic acid) were read after 30 min. Results are expressed as the extract concentration required to reach an absorbance value of 0.5 EC_{50} (effective concentration at 50%) in equivalent milligrams of ascorbic acid per milliliter of extract (mg EAA mL^{-1}) and equivalent milligrams of ascorbic acid per gram of microcapsules (mg EAA g^{-1}). The calibration curve of ascorbic acid (Eq. 1) was prepared with four concentrations of ascorbic acid (10-40 µL combined with a volume of methanol to reach 1 mL as final volume). The absorbance values of the samples were substituted in the equation and the results were adjusted to the dilution factor used and expressed in equivalent milligrams of gallic acid per gram of microcapsules (mg GAE g^{-1}).

$$\mathrm{Abs}_{700} = 0.0711\,[\text{Ascorbic acid}] - 0.1057, \quad \mathrm{r} = 0.9963 \quad [1]$$

TAC was determined by the method of reducing molybdate VI to molybdate V, following the procedure described Prieto *et al.* (1999). Each sample of 100 µL was mixed with 1000 µL of molybdate solution and incubated in a thermoblock (Thermolyne® Dry Bath DB28125) for 90 min at 95 °C. Once all samples had been cooled to room temperature (in the dark), the absorbance at 695 nm was recorded. A calibration curve (A695 = 0.0729-0.0747 [gallic acid], correlation coefficient r = 0.9947) (Eq. 2) was prepared with different concentrations of gallic acid (0.5, 1.0, 2.5, 5.0, 7.5. and 10 mg mL^{-1}).

$$Abs_{695} = 0.0729\,[\text{gallic acid}] - 0.0747, \quad r = 0.9947 \quad [2]$$

The absorbance values of the sample were substituted in the equation, and the results were adjusted to the used dilution factor and expressed in mg GAE mL^{-1}.

Bioassay

Juveniles of *L. vannamei* (0.5 ± 0.2 g) were obtained from the "Cuate Mechado" shrimp farm (Guasave, Sinaloa, Mexico). Experimental units consisted of 120-L plastic tanks with 80 L of seawater (34-35 mg L^{-1}) supplied with constant aeration. Stocking density was adjusted at 10 shrimp per tank. MTBE treatments were tested as a feeding additive with three replicates and coded as follows: CTRL, control group fed with commercial feed (Purina®, 35% protein); T1, 0.5 g MTBE/kg of feed; T2, 1 g MTBE/kg of feed; and T3, 2.5 g MTBE/kg of feed. Shrimps were fed twice a day (9:00 and 17:00 h) at a feeding ration of 7% of total biomass per tank, for 30 days. The tanks were cleaned (residues of food and wastes) by siphoning the bottom every three days. Clean seawater was added to replace water loss by evaporation and siphoning up to 120 L from each thank every five days.

Growth and survival of *L. vannamei*

The final weight gain, specific growth rate, and survival of *Litopenaeus vannamei* juveniles were evaluated at the end of the study. Final weight gain (%) was obtained following the procedure of Amaya *et al.* (2007). The specific growth rate (SGR) (% d-1) was calculated in percentage using the Ziaei-Nejad *et al.* (2006) equation (Eq. 3):

$$\mathrm{SGR}\,(\%\ \mathrm{d}^{-1}) = 100\left(\frac{ln\mathrm{W_t} - ln\mathrm{W_0}}{t}\right) \quad (3)$$

where *t* is the bioassay period (d), $\ln W_0$ is the natural logarithm of the initial shrimp´s weight (g), and $\ln W_t$ is the natural logarithm of the final weight (g). Survival (%) was calculated according to Ziaei-Nejad *et al.* (2006).

Hemolymph collection

At the end of the bioassay, the hemolymph was extracted from the ventral area of the shrimp's cephalothorax by using insulin syringes (27G × 13 mm) containing SIC-EDTA Na_2 (450 mM NaCl, 10 mM KCl, 10 mM Hepes, and 10 mM EDTA, Na_2) pH 7.3 (Hernández-López *et al.*, 1996) as anticoagulant, at a proportion of 2:1 (anticoagulant:hemolymph).

Hemolymph analysis

Hemocytes from plasma and lysate supernatant (HLS) were obtained following the method of Vargas-Albores *et al.* (1993). Protein concentration in HLS was determined using the Bradford (1976) method using bovine serum albumin (BSA, Sigma®) as standard. The Bradford method involves the binding of Coomassie Brilliant Blue G-250 to protein and the absorbance is determined at 595 nm.

Phenoloxidase, prophenoloxidase, and superoxide anion

Humoral response of *L. vannamei* juvenile was determined with the activity of phenoloxidase (PO) and prophenoloxidase (proPO) enzymes and the superoxide anion concentration. PO for small samples was measured spectrophotometrically (490 nm min^{-1} g $protein^{-1}$) according to Hernández-López *et al.* (1996), by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) and the amount of inactive proPO in samples containing also PO activity was calculated as total available proPO minus PO activity before trypsin treatment. The superoxide anion (O_2^-) was quantified (630 nm) using the methodology of Song & Hsieh (1994) based on SIC-EDTA buffer to wash hemocyte pellets; the cytoplasmic formazan was read at 630 nm in a Thermo Spectronic Genesys 2 spectrophotometer.

Physicochemical seawater parameters

Salinity, pH, dissolved oxygen, and temperature were determined in each tank before cleaning, siphoning, and water replacement. Nitrites, nitrates, and ammonium (Strickland & Parsons, 1972) were measured at the beginning and end of the bioassay and throughout the study remained within the optimum levels for shrimp culture (Brock & Main, 1994).

Statistical analysis

ANOVA was applied to determine the differences of protein in plasma and HLS, PO and proPO activities, SGR, and superoxide anion concentration (mean ± SD) among treatments. Results of SGR were $\log_{10}$ transformed to normalize its distribution. If significant differences were found ($p < 0.05$), a *post-hoc* Tukey honestly significant difference (HSD) with multiple comparisons of Scheffé, Bonferroni and Holm was used to identify the differences.

Results

Phenolic compounds profile of *M. tinctoria*

The chemical and rehydration properties of the microcapsules are detailed on Table 1. The physical properties of the microencapsulated phenolic compounds fulfill

Table 1. Chemical and rehydration properties of the microcapsules containing the phenolic compounds extracts of *M. tinctoria.*

Chemical properties		Rehydration properties	
TPC	198.05±5.59	Humidity (%)	4.64±0.25
TF	78.57±1.80	Water activity (aw)	0.15±0.003
CT	29.51±2.6	Moistening time (s)	2340±103.8
FRAP EC_{50}	1.09±0.44	Dissolution time (s)	3960±60
TAC	28.32±0.33	Hygroscopicity (%)	14.19±1.50

TPC = total phenolic compounds (mg GAE g^{-1}). TF = total flavonoids (mg EQ g^{-1}). CT = condensed tannins (mg ECA g^{-1}). FRAP EC_{50} = antioxidant capacity by ferric reducing antioxidant power (mg EAG mL^{-1}). TAC = total antioxidant capacity by molybdate reduction (mg GAE mL^{-1}). GAE = equivalents of gallic acid. EQ = equivalents of quercetine. ECA = equivalents of epicatechin the requirements for their storage and preservation, revealing their viability as an antioxidant supplement for shrimp feeds.

Thirty-three phenolic compounds were identified in the *M. tinctoria* extracts, of which, nine were phenolic acids, nine flavonols (two methylquercetin-3-glycosides, two kaemferol-3-O-glycosides, and four quercetin-3-O-glycosides), four dihydro-flavonoids, and four flavones (one apigenin glycoside, two luteolin glycosides, and one chrysoeriol glycoside). Seven were unidentified phenolic compounds (Fig. 1).

Antioxidant capacity

The antioxidant capacity of the microcapsules, containing the phenolic compound extracts of *M. tinctoria*, averaged 28.32± 0.33 mg GAE mL^{-1} and 10.9 ± 0.44 mg

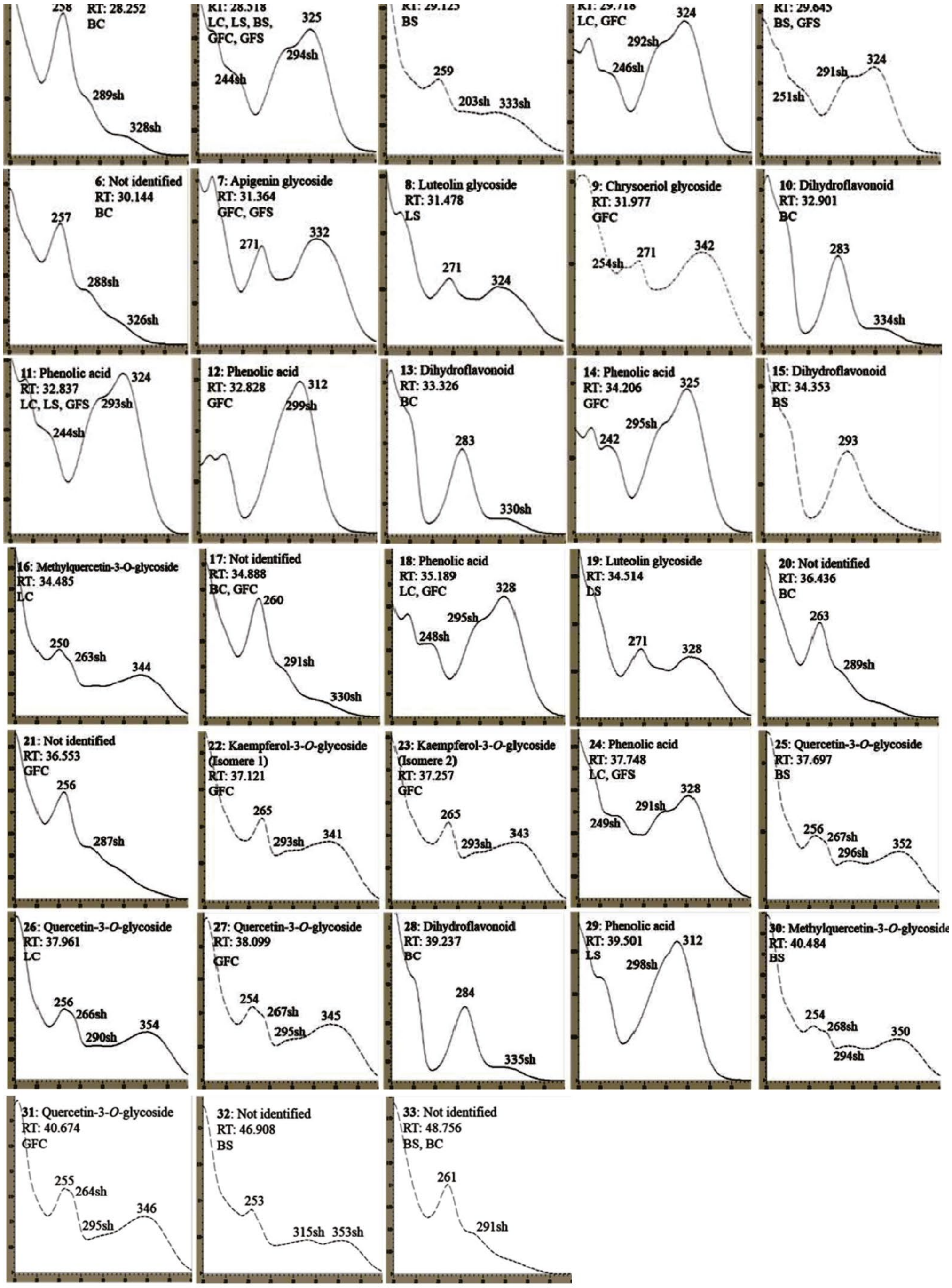

Figure 1. UV spectra, λ_{max}, and retention time (RT, min) of phenolic compounds detected in *Maclura tinctoria* from Sinaloa and Campeche, Mexico. BC: bark of samples from Campeche. BS: bark of samples from Sinaloa. LC: leaves from Campeche. LS: leaves from Sinaloa. GFC: fruits from Campeche. GFS: fruits from Sinaloa.

ascorbic acid equivalent mL^{-1} for TAC and FRAP, respectively (Table 1).

Effect of phenolic extracts microcapsules of *M. tinctoria* on growth performance and survival of *L. vannamei*

No significant differences ($p > 0.05$) were found in shrimps' final weight among treatments (Fig. 2a). SGR was not significantly different ($p > 0.05$) among groups; CTRL and T1 treatments showed the highest SGR with 5.94 ± 0.6% and 5.96 ± 0.7%, respectively (Fig. 2b). Survival was 96.5% in CTRL and T1. In T2 and T3, survival was 100% (Fig. 2c).

Plasma, HLS and total protein

The T1 group displayed a significantly higher ($p < 0.05$) HLS protein content (1.35 ± 0.55 µg mL^{-1}) than T2 and T3 groups (Fig. 3a), however, none of the groups showed statistically significant differences when compared to the control group. Total and plasma protein showed no significant differences ($p > 0.05$) (Figs. 3b and 3c).

Concentrations of superoxide anion and PO (proPO) activity

Superoxide anion concentration showed no significant difference between T1 and T2 groups. T1 group had a higher superoxide anion concentration (0.21, Abs. 630 nm) than T3 groups, but none of the groups was different from the control group (Fig. 4) ($p < 0.05$).

The activity of PO (activated proPO) in HLS (Fig. 5A) and total PO (Fig. 5B) was significantly higher ($p < 0.05$) in T1 as compared to treatments T2 and T3.

Discussion

Numerous studies have established the economic impact due to diseases and mortality caused by diverse pathogens during larval and postlarval stages in L. vannamei aquaculture (Doyle, 2016; de Torres Bandeira *et al.*, 2019), and have focused on the use of diverse plant extracts for shrimp immunostimulation (Chakraborty & Hancz, 2011).

In this study, the phenolic compounds detected in the fruits (glycosides, flavanones, flavonoids, and xanthones) are similar to previous reports using wood, bark and

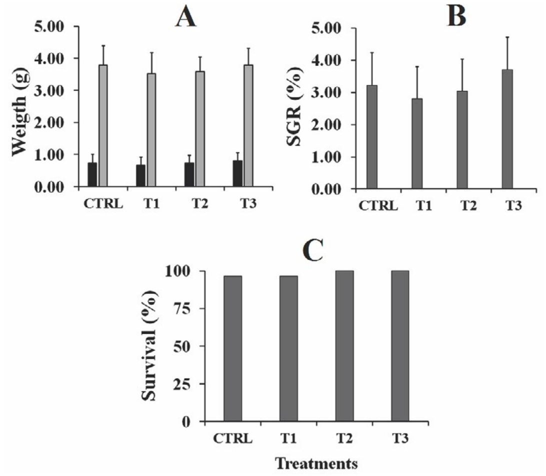

Figure 2. Initial weight [black bars] and final weight [grey bars] (A), specific growth rate, SGR (B), and survival (C) of *Litopenaeus vannamei* juveniles fed with different dietary inclusions of microencapsulated phenolic extracts of *Maclura tinctoria.* Treatments: CTRL, control group fed with commercial feed (Purina®, 35% protein); T1, 0.5 g of MTBE/kg of feed; T2, 1 g of MTBE/kg of feed; and T3, 2.5 g of MTBE/kg of feed. Bars are means ± SD.

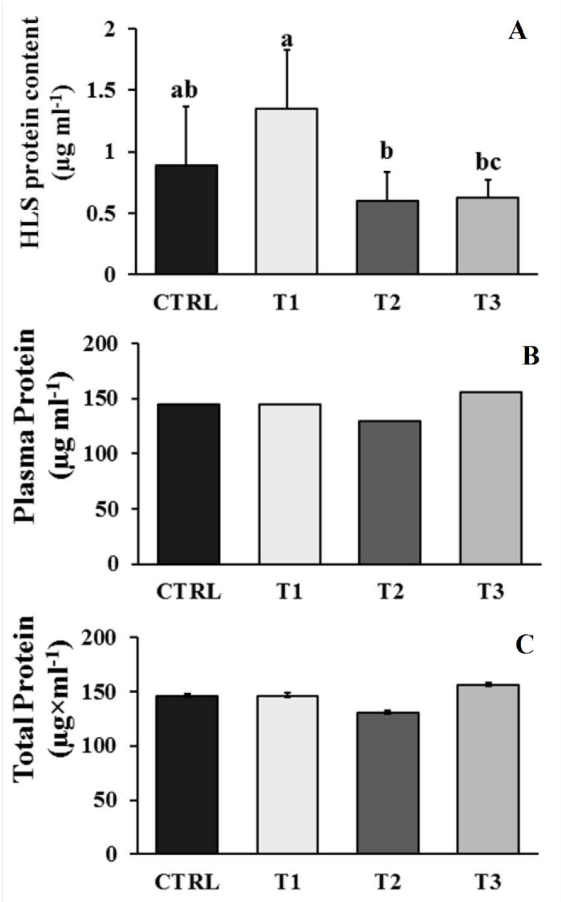

Figure 3. HLS protein content (A), HLS plasma protein (B) and total protein (C) of *Litopenaeus vannamei* juveniles fed with different dietary inclusions of microencapsulated phenolic extracts of *Maclura tinctoria* (MTBE). CTRL, control diet; T1, 0.5 g of MTBE/kg of feed; T2, 1 g of MTBE/kg of feed; T3, 2.5 g of MTBE/kg. Bars are means ± SD. Different letters indicate significant differences ($p < 0.05$).

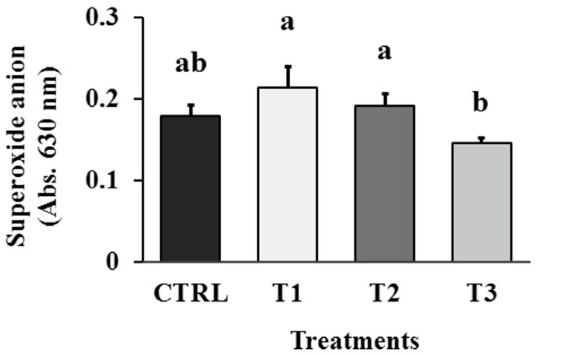

Figure 4. Superoxide anion concentrations in *Litopenaues vannamei* juveniles fed with different dietary inclusions of microencapsulated phenolic extracts of *Maclura tinctoria* (MTBE). CTRL, control diet; T1, 0.5 g of MTBE/kg of feed; T2, 1 g of MTBE/kg of feed; T3, 2.5 g of MTBE/kg of feed. Bars are means ± SD. Different letters indicate significant differences ($p < 0.05$).

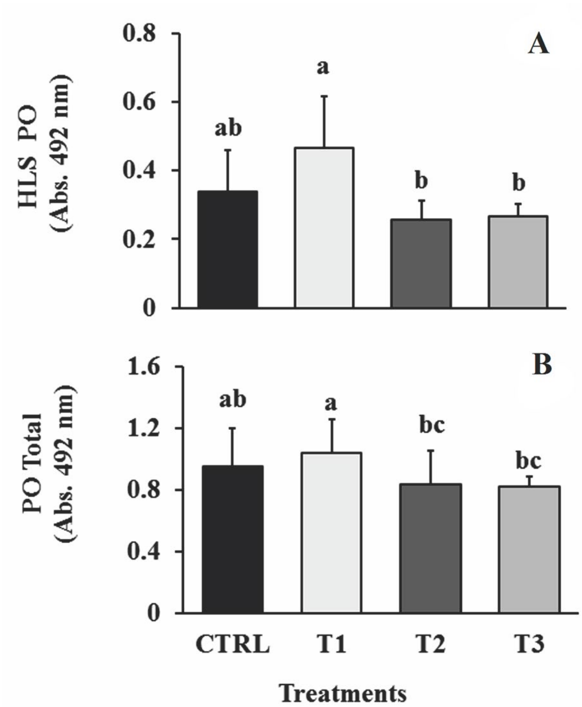

Figure 5. Phenoloxidase (PO) activity in hemocytes lysate supernatant (HLS) (A) and total PO (B) of *Litopenaues vannamei* juveniles fed with different dietary inclusions of microencapsulated phenolic extracts of *Maclura tinctoria* (MTBE). CTRL, control diet; T1, 0.5 g of MTBE/kg of feed; T2, 1 g of MTBE/kg of feed; T3, 2.5 g of MTBE/kg of feed. Bars are means ± SD. Different letters indicate significant differences ($p < 0.05$).

leaves from Maclura genus (Lamounier *et al.*, 2012; Matson-Robles, *et al.*, 2015), which independently of the plant tissue used, the phenolic compounds showed antioxidant, antibacterial, and immunostimulant properties. The MTBE supplied to shrimp feeds have not affected the *L. vannamei* juvenile growth and survival, as resulted in the present study. Similar observations were reported by Subramanian *et al.* (2013). These authors reported a higher SGR in white shrimp fed with a dietary inclusion of the Korean black raspberry, Bokbunja Rubus coreanus extract during four weeks. As *M. tinctoria,* the Korean black raspberry contains total phenolic compounds (TPC) and total flavonoids (Yang & Choi, 2017), which are directly related to the antioxidant activity and improve the growth in earlier stages of aquaculture species (Hussain *et al.*, 2020).

Survival showed no significant differences ($p > 0.05$) between treatments and the control group. This survival pattern of *L. vannamei* had been reported earlier when adding other phenolic extracts from species such as Rhodiola rosea (Wang *et al.*, 2017b), Yucca schidigera, and *Quillaja saponaria* (Hernández-Acosta *et al.*, 2016) to the shrimp's diet. The humoral system could be able to respond to the treatments since shrimps infected with pathogens and treated with phenolic extracts have shown higher survival rates (Júnior, *et al.*, 2018).

The plasma protein includes protein from enzymes, hormones and metabolites. Increment of proteins in the plasma has been described to be caused by a diverse factors such as the stress or the presence of pathogens in the plasma (Gholamhosseini *et al.*, 2020). In the present study, no significant higher plasma protein was determined among CTRL and treatments. This might be because the MTBE supplied in the feed was not detected as a threat by the immune system of the *L. vannamei* juveniles. Nevertheless, the protein content in the HLS was significantly higher at 0.5 mg kg^{-1} but not in the plasma and total protein content. High protein in HLS has been reported to be a response of the humoral system that increases the production of enzymatic proteins (Valentim-Neto *et al.*, 2015; Wang *et al.*, 2017a); commonly, crustaceans with a good immune system present a high protein content in hemocytes (Lamela *et al.*, 2008). In the present study, supplementation with MTBE in the treated *L. vannamei* juveniles could have activated their humoral response. Live pathogen challenge bioassay, together with immune gene expression need to be considered to demonstrated the effectiveness of microencapsulated MTBE in shrimp juvenile culture.

Although the superoxide anion (SO) showed no significant differences (p>0.05) among treatments, it decreased at higher MTBE concentrations, being significantly lower (p<0.05) in the T3 than T1 and T2. It is suggested that the highest MTBE supplementation (2.5 mg of MTBE per kilogram of feed) could reduce the SO anion production. The antioxidant system of *L. vannamei* has been previously stimulated with alcoholic plant extracts (Esquer-Miranda *et al.*, 2016; Júnior *et al.*, 2018), and, in some cases, polyphenols have ameliorated the phagocytosis of the retinal pigment of epithelial cells (Liu *et al.*, 2018). Supplementation with MTBE could stimulate the antioxidant system of *L. vannamei* juvenile as seen in T1, and increase its PO activity, which agrees with the reports by Vanichkul *et al.* (2010), Hsieh *et al.* (2013), and Wang *et al.* (2017b). Extracts from the species *Uncaria tormentosa* showed lower Trolox equivalent (3.610 ± 0.054 mmol g^{-1}) (Júnior *et al.*, 2018) than any of the extracts tested in the present study. Their lower TAC but higher TPC (554.60 ± 0.015 mg GAE g^{-1}) was reported accompanied by a significant higher survival rate of *L. vannamei* at 2 and 4% of *U. tormentosa* to eliminate the clinical signs of WSSV. Similar high survival rates were reported by Sudheer *et al.* (2011), who added *Rhizophora mucronata*, and *Sonneratia* spp. to the feed of *Penaeous monodon* challenged with WSSV, and *Ceriops* tagal extracts to the shrimp diet. The microencapsulated phenolic compound extracts doses used in the present study could be high in relation to the shrimp's weight. It is necessary to calculate the MTBE dose based on the concentration of phenolic extracts to properly determine this critical effect and correlate it with shrimp survival and growth rate.

In *L. vannamei*, the proPO system plays a key role in the humoral response against pathogen infections and the production of proPO is increased some days after infection (Wu *et al.*, 2017). The significantly higher proPO activity ($p < 0.05$) found in HLS could be attributed to the stimulation by the microencapsulated phenolic compounds supplied; although survival showed no significant differences ($p > 0.05$), the shrimp juvenile in T2 and T3 showed 100% survival. Phenolic extracts increase the gene expression related to the immune system and the production of antioxidant enzymes (Subramanian *et al.*, 2013). The 33 encapsulated phenolic compounds determined in the present study may have stimulated the humoral system and antioxidant capacity, increasing survival and growth in shrimps. These phenolic compounds have been proven to possess the ability to trap superoxide anions and hydroxyl radicals (Choi *et al.*, 2017; Hamed *et al.*, 2017).

In conclusion, MTBE supplementation did not show negative effects on shrimp survival and growth. There were no significant differences in any of the tested humoral immunity markers of the white leg shrimp (*L. vannamei*) (HLS protein, superoxide anion, proPO, and phenoloxidase). In this sense, further research is needed considering lower MTBE concentrations, feeding periods, and the effects on other humoral agents, like total hemocytes count, immune gene expressions, and/or antioxidant enzymatic processes in *L. vannamei,* including catalase and superoxide dismutase. To the best of our knowledge, this is the first report on the effect of microencapsulated phenolic compound extracts of *M. tinctoria* on phenoloxidase activity, protein content, survival, growth specific rate, and anion superoxide concentration in *L. vannamei* juveniles.

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