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Phytochemical composition and immunobiological activity of Hawthorn *Crataegus mexicana* nanoencapsulated in Longfin yellowtail *Seriola rivoliana* leukocytes

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ABSTRACT

The aims of this study were to determine the presence of phenolic compounds in Hawthorn *Crataegus mexicana*, species native to Mexico, nanoencapsulated (*CmNano*) with maltodextrin at 100 and 170 °C (*CmNano100* and *CmNano170*) and its antioxidant and immunological effects in Longfin yellowtail *Seriola rivoliana* leukocytes. The phytochemical study revealed an important level of total phenolic (TPC), flavonoid (TFC) and tannin (CTC) contents in *CmNano100*, which correlated with a strong antioxidant capacity. *CmNano100* or 170 were safe or not cytotoxic for head-kidney (HKL) and peripheral blood (PBL) leukocytes. The *in vitro* study demonstrated that *CmNano* increased the percentage of phagocytic cells, stimulated the production of reactive oxygen species, and modulated antioxidant ability by increasing superoxide dismutase activity in leukocytes with respect to the control group. In addition, *CmNano100* also increased the transcription of proinflammatory cytokine IL-1β and down-regulated MyD88 and TNF-α mRNA transcription. These results suggest that maltodextrin nanoencapsulates protected and maintained the antioxidant properties of *C. mexicana*. In addition, they enhanced antioxidant and immunological parameters in Longfin yellowtail *S. rivoliana* leukocytes. Therefore, this study provides novel insights of *CmNano* for its potential application as functional food in aquaculture.

1. Introduction

Currently, prophylactic administration of natural immunostimulants, such as herbal plants or fruits is considered as promising alternative in aquaculture because of their broad-spectrum multi-beneficial functions, cost effectiveness, and environmental friendliness. In addition, consumption of plant-based foods rich in phytochemical compounds is associated with good health status and disease prevention [1]. Several plants are potential sources of phenolic compounds, which are one of the major bioactive compounds that can serve as natural antioxidants [2]. In Mexico, a great variety of plants and fruits exist with a wide genetic diversity useful for agriculture, industry, and medicine. Unfortunately, many of them have not been explored or are exploited inappropriately. The Hawthorn *Crataegus mexicana*, called "tejocote" or "manzanita" which means "little apple", has a great deal of medicinal applications, but only a few of its

biological activities have been analyzed [3,4]. Tejocote (*C. mexicana*) is native to Mexico and considered a typical fruit since pre-hispanic times although different varieties can be found in other parts of the world [5]. In China, species of *Crataegus* are used as medicinal herbs and have showed strong antioxidant properties [6]. For example, over 150 compounds (especially phenolic compounds) have been identified in *C. pinnatifida* [7]. Concerning *C. mexicana*, some flavonoids have been identified, such as quercetin and its derivatives: glycone type and carotenoid β-carotene [8].

Despite all health benefits of medicinal plants, limitations have been reported for commercial-scale application because they are very susceptible to environmental stresses, such as oxygen, enzymes, light, high temperature, and acidic or alkaline pH, which have resulted in degradation and losses of their antioxidant capacity [9]. In recent years, the interest in microencapsulation of susceptible compounds has increased. Maltodextrin-spray-drying is regarded as an economical and

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effective process in the food industry and the most popular and common materials for encapsulating active compounds [10,11]. For example, Comunian et al. [12] found that microencapsulation of chlorophyllide using maltodextrin was adequate for protection from environmental conditions during storage. In another study, maltodextrin was selected over many other edible materials for its high-water solubility, low viscosity, mild flavor, and efficient use in the encapsulation of saffron extracts *Crocus sativus* [13].

Based on these considerations, the aim of this study was to investigate the content of phenolic compounds of *C. mexicana* nanoencapsulated (*CmNano*) at 100 and 170 °C and their antioxidant and immunostimulant effects in leukocytes of Longfin yellowtail (*Seriola rivoliana*), one of the most important fish with potential for aquaculture in Mexico with great conversion indices and growth rates. *S. rivoliana* has been commercially cultured for the last few years in southern U.S.A. and Hawaii and currently in Mexico by Kampachi Farms with a Hawaii-based mariculture. However, a few studies have been performed on the immune system and nutrition of this important species. The knowledge of *C. mexicana* chemical properties is one of the first steps to its application as antioxidant and immunostimulant in aquaculture.

2. Materials and methods

2.1. Maltodextrin

The carrier agent used in this study was maltodextrin Inamalt 110 ° (11–11.4 DE) and purchased from Industrializadora de Maiz Company, Guadalajara, Jalisco Mexico.

2.2. Crataegus mexicana

The tejocote (*Crataegus mexicana*) fruits were obtained from Felipe Angeles Supermarket, Guadalajara, Jalisco Mexico, selecting those without physical or mechanical damage and disease conditions. The fruits were washed with water and disinfected with a sodium hypochlorite solution (2 ppm) for 15 min. Then, the fruits were drained to remove water excess. Each fruit was cooked in a steam jacketed kettle (6E-KKT, Blodget, USA) in distilled water at 100 °C for 50 min, and then made into pulp. The pulp was diluted with distilled water (3:1) and filtered with an 11-μM muslin cloth. Thereafter, the pulp was stored at −10 °C until use. Aqueous solutions of maltodextrin (26%, w/w) were used for pulp encapsulation after spray-drying (SD-Basic, LabPlant, UK) at two different temperatures (100 and 170 °C). The drying air flow rate was set to 70 m^3/h. The nanoencapsulates (*CmNano*) obtained were packed in plastic bags, hermetically sealed, and stored in a desiccator at room temperature until analyses.

2.3. Determination of active phenolic compound contents

Briefly, two g of each sample (*CmNano100* or *CmNano170*) were extracted with seven mL of methanol-acetic acid solution (80:20, v/v) in an ultrasonic bath (812, Polyscience, USA) for 30 min. Then, the extract was centrifuged at 2200 rpm (Z326, Hermle, DE) for 10 min. The supernatant was decanted into a Falcon tube and the residual pellet was extracted once again. The supernatants were mixed, and this final extract was protected against the light. This extract was used to quantify polyphenols, flavonoids, tannins, and the antioxidant capacity of *C. mexicana* when nanoencapsulated.

2.3.1. Total polyphenol content (TPC)

The TPC was determined according to the method described by Singleton et al. [14] with some modifications. The Folin-Ciocalteu reagent (125 μL), distilled water (1.25 mL) and the sample (250 μL) were mixed and the reaction was stopped after exactly 5 min. Then, 20% of aqueous Na_2CO_3 (375 μL) and distilled water (500 μL) were added, and the solution was homogenized and placed in darkness at room temperature for 2 h. Absorbance was measured at 750 nm (in sextuplicate) using an Ultraviolet–visible spectrophotometer (Thermo Scientific, Waltham, MA, USA). A standard curve was created using gallic acid as the standard substance with a concentration range of 0–150 μg/ml. A blank sample consisting of methanol (80%) and the reagents were used as reference. The results were expressed in mg of gallic acid equivalent per g of sample in dry weight basis (mg GAE g^{-1} DW).

2.3.2. Total flavonoid content (TFC)

The aluminum chloride colorimetric method was modified for the determination of total flavonoids [15]. A volume of 250 μL of the extract was added to 75 μL of 5% $NaNO_2$ solution and 1 mL of distilled water. The mix was allowed to remain for 5 min before adding 75 μL of $AlCl_3$ (10%) and incubated for 6 min. Then, 500 μL of NaOH (1 M) and 600 μL of distilled water were added. After 15 min of incubation, the absorbance was measured at 510 nm (in triplicate). A standard curve was created using quercetin as the standard substance with a concentration range of 0–8000 μg/ml. A blank sample consisted of dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA), and the reaction mix was used as reference. The total flavonoid content (TFC) was expressed in mg of quercetin equivalents (QE) per gram of the extract in dry weight basis (mg QE g^{-1} DW).

2.3.3. Determination of condensed tannin content (CTC)

The CTC analysis was performed according to the method described previously [16]. Absorption was measured using a UV–vis spectrophotometer (Thermo Scientific, Waltham, MA, USA) at 500 nm using methanol as the blank. The CTC was expressed as milligrams of catechin equivalent per gram of dry weight (μmol CAE g^{-1} DW) using the calibration curve of (+)-catechin.

2.4. Antioxidant capacity analysis

2.4.1. Free radical scavenging activity

To determine the antioxidant activity of *C. mexicana*, the method based on the reduction of methanolic solution of colored free radical DPPH was used. The changes in color from deep-violet to light-yellow were measured at 515 nm in an UV–vis light spectrophotometer (Thermo Scientific, Waltham, MA, USA). Radical scavenging activity [17] was expressed as percentage using the EC50 (efficient concentration): the amount of extract (mg of DW) needed to obtain 50% activity per one mL of the initial solution.

2.4.2. Determination of ferric-reducing antioxidant power

The FRAP assay was measured according to Benzie and Strain, [18]. The working solution was prepared by mixing $FeCl_3$ solution (20 mM, 2.5 mL), TPTZ solution (10 mM 2,4,6-tripiridil-S-triazine in 40 mM HCl, 2.5 mL) and the acetate buffer (300 mM, 25 mL). Samples with serial concentrations (75 μL) were added to the working solution (1425 μL), and the mixture was incubated at 37 °C for 30 min. Absorbance was measured at 593 nm. The negative control was DMSO. Trolox solutions were used to perform the calibration curve. The results were expressed as mmol equivalents of Trolox/L of sample.

2.5. In vitro study and experimental design

Head kidney and peripheral blood leukocytes were obtained based on a previously described methodology [19]. Briefly, head-kidney was removed and passed through 100 μm cell strainers in sRPMI [RPMI-1640 culture medium (Gibco, Waltham, MA, USA) with 0.35% sodium chloride, 100 IU mL^{-1} penicillin (Flow), 100 mg mL^{-1} streptomycin (Flow), 10 IU mL^{-1} heparin (Sigma, St. Louis MO USA), and 5% fetal bovine serum (Gibco, Waltham, MA, USA)]. To obtain peripheral blood leukocytes, blood samples were immediately withdrawn from the caudal vein with a heparinized syringe under sterile conditions and

obtained according to Reyes-Becerril et al. [20]. Finally, leukocytes cells were observed and counted with a TC20 Coulter Particle Counter (BioRad, Hercules, CA, USA) and adjusted to 1.2 x 10^6 cells mL^{-1} of sRPMI using a viability of more than 95% for *in vitro* experiments.

One milliliter of head-kidney (HKL) and peripheral blood leukocytes (PBL) of Longfin yellowtail (*Seriola rivoliana*) containing 1.2 x 10^6 cells mL^{-1} were dispensed into 24-well flat-bottomed microtitre plates (Nunc, Thermo Scientific, Waltham, MA, USA) or 90 μL in 96-well plates for cell viability or phagocytosis assays, respectively. Leukocytes were stimulated with *CmNano* (100 μg/mL resuspended in distilled water) at 25 °C, with 85% relative humidity and 5% CO^2 atmosphere. Control samples consisted of leukocytes incubated with culture medium alone or 100 μL of distilled water. Leukocytes were collected at 24 h post-stimulation in triplicate for immunological assays. For gene expression, supernatant was discarded and leukocytes were resuspended in 1 mL Trizol reagent (Invitrogen, Thermo Scientific, Waltham, MA, USA), mixed well by vortexing for 1 min and stored at −80 °C for RNA extraction.

2.6. Cell viability

The resazurin assay was used to determine the effect of *CmNano* on leukocyte viability according to Riss et al. [21]. Briefly, HKL and PBL were dispensed in 96-well plates (90 μL 1.2×10^6 cells/mL) and incubated with 10 μL/well of *CmNano* (100 μg/mL) (final concentration of leukocytes, 1 x 10^6 cell/mL) and cultured overnight (25 °C and 5% CO_2). Leukocytes were stained with 10 μL resazurin solution (Sigma, St. Louis, MO, USA) and incubated at 25 °C and 5% CO_2 for 4 h. Fluorescence was measured in Varioskan™ Flash Multimode Reader (Thermo Scientific, Waltham, MA, USA) excitation at 530 nm and emission at 590 nm. Leukocytes without *CmNano* and those incubated with DMSO (10% final concentration) were used as controls. RPMI medium with *CmNano*, DMSO, and distilled water plus resazurin were used to subtract absorbance from immunostimulated leukocytes.

2.7. Immunological assays

2.7.1. Phagocytic activity

Phagocytosis activity was evaluated by using Vybrant™Phagocytosis assay kit (V-6694, Thermo Scientific, Waltham, MA, USA). After washing the cells, 100 μL of fluorescein-labeled *E. coli* BioParticles® (Thermo Scientific, Waltham, MA, USA) suspended in Hanks' balanced salt solution were added and incubated for 2 h. The suspension was then removed, and subsequently, 100 μL of trypan blue suspension was added and incubated for 1 min to quench the extra-cellular probe. After aspiration of trypan blue from experimental and control wells, fluorescence was measured at 484 (excitation) and 535 nm (emission) in a Varioskan™ Flash Multimode Reader (Thermo Scientific, Waltham, MA, USA). The phagocytosis response to the effector was expressed as:

Efficiency ratio (%) = Net Experimental Reading = Net Positive Reading x 100%

2.7.2. Respiratory burst

Respiratory burst activity of the leukocytes isolated from HKL and PBL was measured with the nitroblue tetrazolium (NBT) according to Kemenade et al. [22]. Leukocytes were incubated with NBT (1 mg mL^{-1}, Sigma, St. Louis, MO, USA). After 2 h of incubation, the reaction was stopped with 100 μL of methanol (70% v/v). The plates were air-dried and 120 μL of 2 M potassium hydroxide and 140 μL DMSO were added to each well. The optical density (OD) was measured at 655 nm in a microplate reader (iMark™ BioRad, Hercules, CA, USA).

2.8. Antioxidant assays

2.8.1. Superoxide dismutase (SOD) and catalase (CAT)

SOD activity was measured in leukocytes by the percentage reaction inhibition rate of enzyme with water soluble tetrazolium dye substrate (WST-1) and xanthine oxidase using a SOD assay kit (19160, Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Each endpoint assay was monitored at 450 nm, which is the absorbance wavelength for the colored product of the WST-1 reaction with superoxide. Lectures were conducted after 20 min of reaction time at 37 °C. Inhibition percentage was normalized by mg protein and displayed as SOD specific activity units.

CAT activity was assayed by the method of Clairborne [23]; the decrease in H_2O_2 absorbance was followed at 240 nm. One unit of enzyme activity is defined as the amount of enzyme required to degrade 1 mmol of H_2O_2 in 1 min.

2.9. Gene expression

Relative gene expression was analyzed in samples from immunostimulated leukocytes using real-time PCR and the 2^{DDCT} method according to Livak and Schmittgen [24]. Complementary DNA (cDNA) was synthesized from 1 mg of total RNA using the SuperScript III reverse transcriptase (Invitrogen, Thermo Scientific, Waltham, MA, USA) with an oligo-dT18 primer. For each mRNA, gene expression of cytosolic adapter protein (MyD88) and pro-inflammatory cytokines (IL-1β and TNF-α) was normalized by the elongation factor 1-alpha (ef-1α) expression in each sample (Table 1). Additionally, a threefold serially diluted cDNA (pooled) was included for each plate of each gene to evaluate the efficiency of qPCR reaction based on the standard curve method, using formulae E = $10^{(-1/\text{slope})}$ - 1 x 100. In all cases, each PCR was performed with triplicate samples.

Table 1
Sequences of the primers used for Longfin yellowtail *Seriola rivoliana*.

Gene	Gene abbreviation	Accesion No.	Primer sequence (5′-3′)	Product lenght
Interleukin-1β	*IL-1β*	KY860519	AGCCAGCAGAGACACTTAC TGGGTAAAGGTGGAAGTAG	131
Tumour necrosis factor α	*TNF-α*	KY860518	TTCATGCCTCTTAGCCACAGG CTCCGTCCGTCTCTGTAACTG	131
Piscidin	*Pisc*	KY860523.1	TCGTCCTGTTTCTTGTGTTGTC TGCTGTAGGTCTGTCATGCC	151
Myeloid differentiation factor 88	*MyD88*	KY860521	ATGAAGCGACGAAAAACCCC AAGACTGAAGATCCTCCACAATGTC	135
Elongation factor 1 α	*EF-1*	KY806112	TGGTGTTGGTGAGTTTGAGG CGCTCACTTCCTTGGTGATT	173

Table 2
Contents of total total phenolics, flavonoids and tannins of *C. mexicana*.

	Treatments	
	CmNano100	CmNano170
TPC[a] (mg g^{-1} DW)	54.8 ± 4.93[a]	49.8 ± 10.6[a]
TFC[b] (mg g^{-1} DW)	1.16 ± 0.0202[b]	0.95 ± 0.00[a]
CTC[c] (µmol/g^{-1} DW)	0.52 ± 0.10[a]	0.47 ± 0.23[a]

Means with different letter are statistically significant at 5% level probability.
 [a] Total phenolic content, TPC was expressed as milligrams of gallic acid equivalents/g dry sample.
 [b] Total flavonoid content, TCF was expressed as milligrams of quercentin equivalents/g dry sample.
 [c] Tannins content, CTC was expressed as milligrams of catechin equivalents (CE) per 100 g dry sample (mg/100 g).

2.10. Statistical analysis

All bioassays and measurements were performed in triplicate, and the mean ± standard deviation (SD) for each group and sampled time were calculated. A t-student or one-way analysis of variance (ANOVA) was performed to determine the effects of *CmNano100* and *CmNano170* on phytochemical contents, antioxidant capacity, and cellular antioxidant and immunological parameters using SPSS v.19.0 software (SPSS, Richmond, VA, USA). Means were separated by a Tukey multiple range test. Statistical analyses were made with the obtained data for each sample. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Phenolic, flavonoid and tannin total contents in Crataegus mexicana nanocapsulates

The quantity of phenolic compounds (TPC), flavonoid compounds (TFC), and tannin content (TTC) in *CmNano* are described in Table 2. Temperature (100 and 170 °C) unaffected the TPC or TTC; however, the TFC was higher ($p < 0.05$) in *CmNano100* respect to *CmNano170*.

3.2. Antioxidant capacity of Crataegus mexicana

The hydroxyl radical scavenging activity of *C. mexicana* was affected by the encapsulation at 170 °C, observing an increase in a concentration-dependent manner (Fig. 1a). In contrast, comparable hydroxyl radical scavenging activity of the BHT control was detected in *CmNano100*.

Ferric reducing antioxidant power (FRAP) in *CmNano* was also affected by the temperature at 170 °C, which was lower ($p < 0.05$) than that of *CmNano100* (Fig. 1b).

3.3. Crataegus mexicana does not cause cytotoxicity on fish leukocytes

Head-kidney (HKL) and peripheral blood (PBL) leukocytes of fish stimulated with *CmNano* showed higher viability than control after 24 h of incubation (Fig. 2). In contrast, cell viability was similar in HKL and PBL incubated with *CmNano100* or *CmNano170*.

3.4. Phagocytosis and respiratory burst of leukocytes stimulated by Crataegus mexicana

Phagocytosis efficiency was the highest and lowest ($p < 0.05$) in leukocytes (HKL or PBL) stimulated with *CmNano100* and *CmNano170*, respectively, compared to the control group (Fig. 3a).

The respiratory burst was higher ($p < 0.05$) in both HKL and PBL incubated with *CmNano100* or *CmNano170* with respect to that of the
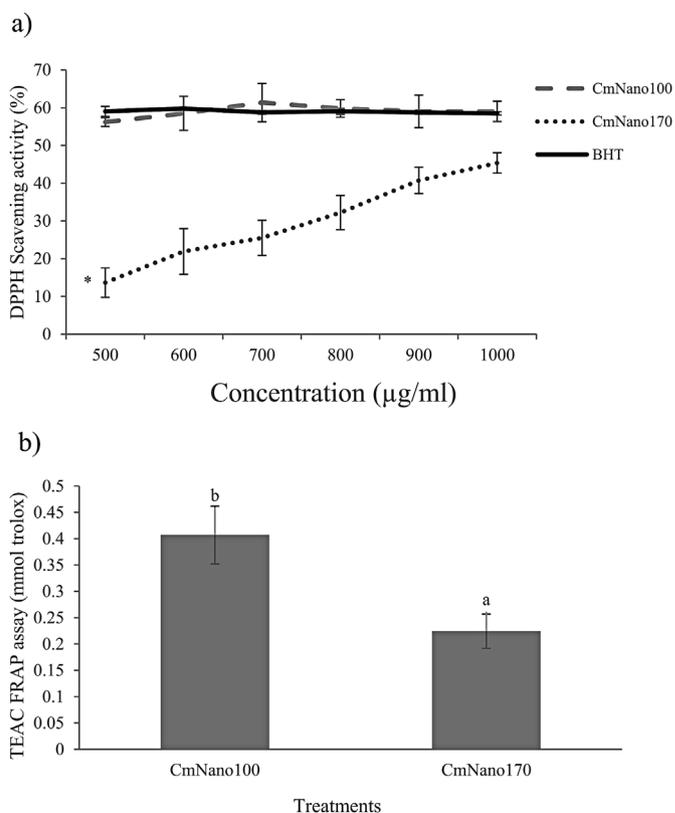

Fig. 1. a). Hydroxyl radical scavenging activity and b) ferric reducing antioxidant power (FRAP) of *Crataegus mexicana* (100 and 170 °C, *CmNano100* or *CmNano170*). Results are the mean ± SD of three separate experiments, each in triplicate. Different letters or asterisk indicate significant (p < 0.05) difference among groups.
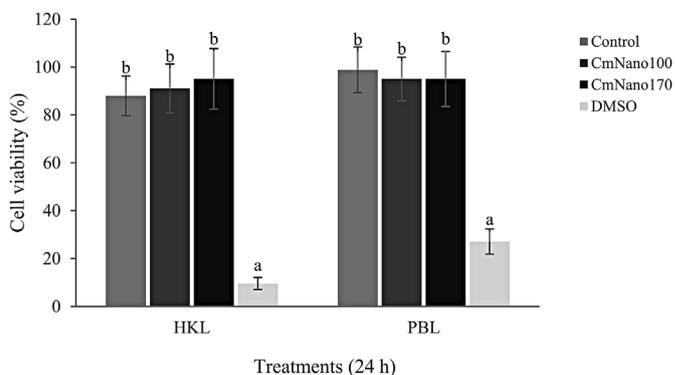

Fig. 2. Resazurin assay to assess cell viability of head-kidney (HKL) and peripheral blood (PBL) leukocytes stimulated with *Crataegus mexicana* (100 and 170 °C, *CmNano100* or *CmNano170*) at 24 h. Bars represent the mean ± SD (n = 9). Different letters indicate significant (p < 0.05) difference among groups.

control leukocytes (Fig. 3b).

3.5. Antioxidant enzyme activity increased in leukocytes treated with Crataegus mexicana

The activity of superoxide dismutase increased in both HKL and PBL incubated with *CmNano* compared to control groups (Fig. 4a). In contrast, the catalase activity was similar between HKL or PBL exposed to *CmNano* and the control leukocytes (Fig. 4b).
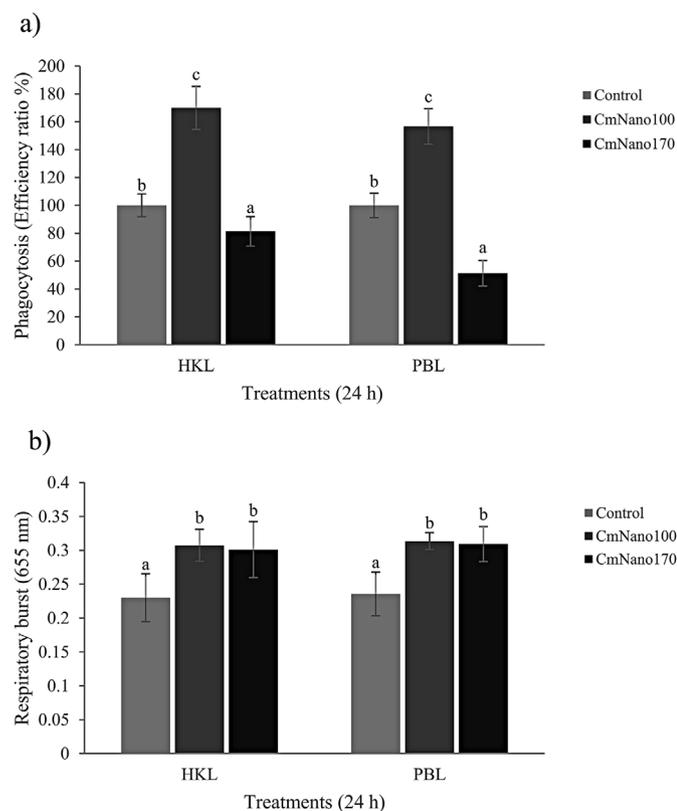
a)

b)

Fig. 3. a) Phagocytosis efficiency (%) and b) lysosomal respiratory burst activity of head-kidney (HKL) and peripheral blood (PBL) leukocytes stimulated with *Crataegus mexicana* (100 and 170 °C, *CmNano100* or *CmNano170*) at 24 h. Data are presented as mean ± S.D. Different letters denotes significant difference between treated groups (p < 0.05).

a)

b)

Fig. 4. a) Superoxide dismutase and b) catalase activities of head-kidney (HKL) and peripheral blood (PBL) leukocytes stimulated with *Crataegus mexicana* (100 and 170 °C, *CmNano100* or *CmNano170*) at 24 h. Values are expressed as mean ± SD. Different letters denotes significant difference between treated groups (p < 0.05).

3.6. Pro-inflammatory gene expression modulated by Crataegus mexicana in leukocytes

The expression MyD88 gene was similar among groups in HKL and down-regulated in PBL incubated with *CmNano* (100 or 170 °C) compared to control cells (Fig. 5a). The mRNA of IL-1β gene down-regulated in HKL and up-regulated in PBL incubated with *CmNano100* or *CmNano170* with respect to the control leukocytes (Fig. 5b). Finally, the TNF-α gene transcription was down-regulated in both HKL and PBL incubated with *CmNano* (100 or 170 °C) compared with the control groups (Fig. 5c).

4. Discussion

In the last year, the demand for immunostimulants based on herbal or medicinal plants as natural alternative remedies has increased in farm aquaculture to help boost immunological and antioxidant responses. Longfin yellowtail *Seriola rivoliana* is an important commercial fish in Baja California Sur, Mexico, and nutritional and immunological studies are needed as the industry grows. In Mexico, Hawthorn *Crataegus mexicana* is an important fruit with many medicinal applications [5]. The fruit pulp of *C. mexicana* is rich in antioxidants because of its high phenolic compound contents, but these antioxidants are vulnerable to different environmental changes [25]. An alternative to protect them is through nanoencapsulation using maltodextrin, which is commonly used in the food encapsulation industry to preserve the physicochemical properties [26]. This study found that the content of the main biologically active compounds in *C. mexicana*, such as polyphenols, flavonoids, and tannins was higher in *CmNano100* than *CmNano170*. Phenols, flavonoids, and tannins from plants are
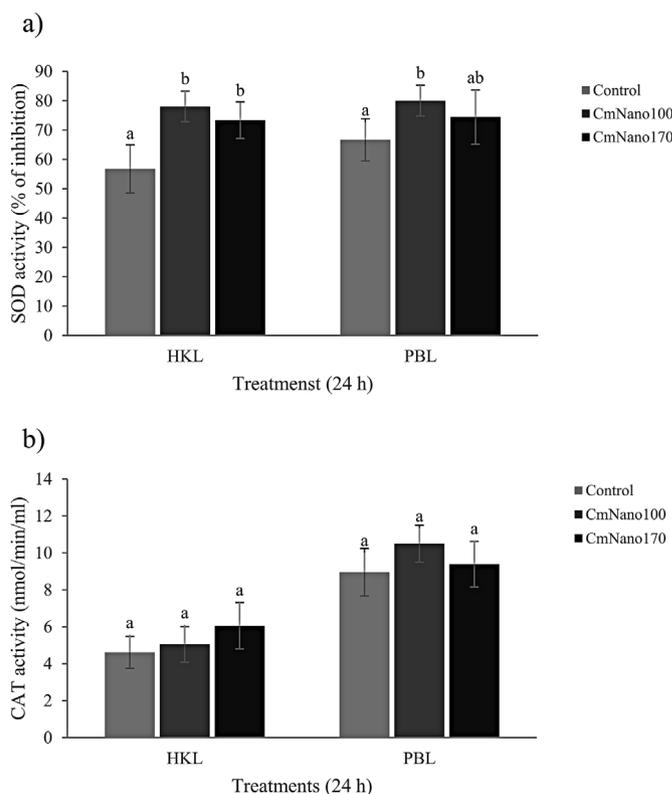
important antioxidants because of their phenolic hydroxyl chemical structure that results in redox properties [27–29]. In this study, *CmNano100* interestingly showed higher DPPH free radical scavenging and FRAP activities than those *CmNano170*. On this regard, several species of *Crataegus* have shown antioxidant activity, such as *C. aronia* [30], *C. pentaegyna* [31], and *C. folium* [32]. Therefore, reinforcement of endogenous antioxidants via intake of dietary antioxidants may be of great importance in attenuating the cumulative effects of free radical production [33,34].

In line with these findings, this study stimulated Longfin yellowtail *Seriola rivoliana* leukocytes with *CmNano* for 24 h to investigate its cellular antioxidant and immunological effects. In this experiment, *CmNano100* or *CmNano170* did not cause toxicity or death in head-kidney (HKL) or peripheral blood leukocytes (PBL). Thus, *CmNano* at 100 or 170 °C must be safe to leukocytes. Afterwards, the effects of *CmNano100* or *CmNano170* were studied on the main innate cellular immune activities, such as phagocytosis and respiratory burst activity in HKL and PBL. The main cellular activity of macrophages and neutrophils is the phagocytosis activity. Phagocytosis is a self-protective reaction against invading microorganisms [35]. Fish treated with medicinal plants or herbs have shown increased phagocytosis and respiratory burst activity [36–38]. The results in this study showed that phagocytosis activity was strongly enhanced in HKL and PBL stimulated with *CmNano100* after 24 h of incubation compared with the control group. Similarly, lysosomal respiratory burst activity by leukocytes increased after incubation with both experimental treatments of *CmNano100* or *CmNano170*. Those stimulation mechanisms in cells trigger diverse antimicrobial processes that use a wide variety of tools, including cellular activation after phagocytosis, production of oxidative radicals (ROS) and antioxidant enzymes to regulate ROS accumulation, and production of cytokines driving the inflammatory response, among
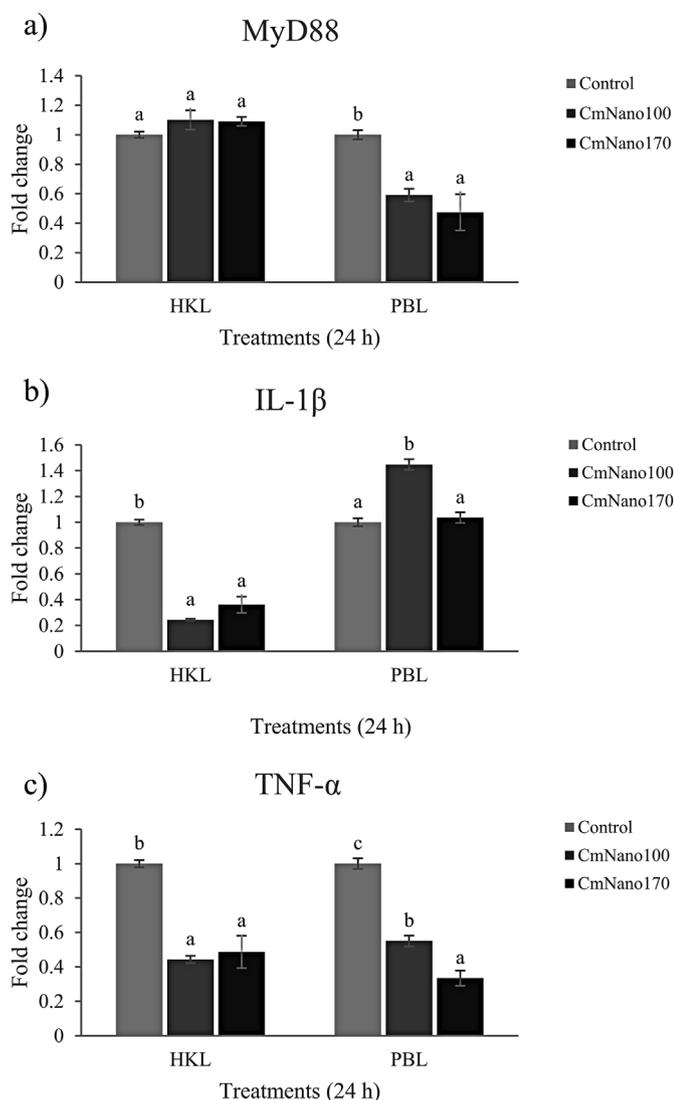

Fig. 5. Relative mRNA expression of cytosolic adapter protein a) MyD88 and pro-inflammatory cytokines, b) IL-1β and c) TNF-α measured by quantitative real-time PCR in head-kidney (HKL) and peripheral blood (PBL) leukocytes stimulated with *Crataegus mexicana* (100 and 170 °C, *CmNano100* or *CmNano170*) at 24 h. Data are presented as mean ± S.D. Fold increase relative to control. Different letters denotes significant difference between treated groups (p < 0.05).

other downstream effectors [39]. In this context, the fruit of *C. mexicana* has a high concentration of metabolites, which could participate for their several biological effects in the immune system [5,40,41]. Some metabolites may induce the expression of IL-1β, a proinflammatory cytokine that can stimulate the release of other cytokines capable of triggering macrophages, NK cells, and lymphocytes [1,36,42].

This study analyzed two important antioxidant enzymes produced by leukocytes to reduce the harmful impact of ROS excess, such as superoxide dismutase (SOD) and catalase. Remarkably, the SOD activity was strongly enhanced by the leukocytes stimulated with both *CmNano100* and *CmNano170*. In contrast, the catalase activity was slightly enhanced without significant differences compared with the control group. Biological systems can use two general processes to maintain the redox balance: (1) enzymatic conversion of ROS to less toxic or non-active ROS by antioxidant enzymes including SOD; and (2) direct scavenging of ROS by molecules from both endogenous (i.e. glutathione) and exogenous (i.e. food such as herbal or fruit bioactive

compounds, as mentioned above) origins [41].

Immunostimulants can induce proinflammatory responses [43], so this study evaluated gene expression level cytosolic adapter protein (MyD88) and proinflammatory (IL-1β, TNF-α) cytokines in leukocytes stimulated with *CmNano*. The *in vitro* study showed that *CmNano100* or *CmNano170* have potential anti-inflammatory effects in HKL and PBL. The MyD88 gene expression was unaffected or down-regulated whereas the IL-1β gene expression was down-regulated with the exception of PBL stimulated with *CmNano100*. Finally, the TNF-α gene expression was also down-regulated by *CmNano* compared to the control group. The adapter, myeloid differentiation protein-88 (MyD88) provides a structural platform for the recruitment of kinases and downstream effector molecules such as pro-inflammatory cytokines IL-1β and TNF-α [44]. Several studies have confirmed that certain toll-like receptor (TLR) agonists, such as lipopolysaccharide (LPS), are required to generate MyD88 signal transduction [45]. This study only analyzed the immunostimulant effect, and infection or TLR agonists will be required for further studies. Consistent with these observations, Kallassy et al. [46] observed a similar anti-inflammatory effect in a murine macrophage cell line using different extracts of *Crataegus azarolus* L. These authors confirmed that a robust anti-inflammatory effect was related with the antioxidant activity, which is in agreement with the results obtained here using *CmNano*.

In conclusion, *Crataegus mexicana* nanoencapsulated (*CmNano*) with maltodextrin at 100 °C was not cytotoxic, provided protection and maintained its phytochemical properties. *C. mexicana* is rich in polyphenols such as flavonoid and tannins. The immunobiological actions of *CmNano* in Longfin yellowtail (*S. rivoliana*) leukocytes demonstrated its immune, antioxidant, and anti-inflammatory potentials. The biological role of Hawthorn *C. mexicana* has not been explored in aquaculture yet, so this is the first information that could raise interest in applying it in the aquaculture industry. Further investigation is required to elucidate the immunostimulant effect of *CmNano* in a pathogen challenge experimentation.

Acknowledgments

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