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Assessment of chemical, biological and immunological properties of "Damiana de California" *Turnera diffusa* Willd extracts in Longfin yellowtail (*Seriola rivoliana*) leukocytes

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ABSTRACT

In Mexican herbal medicines or natural remedies, *Turnera diffusa* (Turneraceae) known as "Damiana de California", has ethnopharmacological relevance, including aphrodisiac, diuretic, and antimicrobial activities. To explore the immunological effect of infusion and methanolic extracts from Damiana de California, this study investigated its chemical, biological, antimicrobial and immunological properties in Longfin yellowtail *Seriola rivoliana* leukocytes. The analysis of chemical compounds revealed a considerable level of total phenolic and flavonoid contents in the infusion compared with methanolic extract. Furthermore, the antioxidant activity showed high hydroxyl radical scavenging activity in infusion extract compared with BHT positive control. Superoxide radical scavenging activity and ion chelation were higher in methanolic extract followed by infusion treatment. Interestingly, notable antimicrobial activity was observed in both extracts of *T. diffusa* against *Vibrio parahaemolyticus*. An *in vitro* study was performed using leukocytes of *S. rivoliana* treated with infusion or methanolic extracts at 12.5, 25 and 50 μg/mL for 24 h. Remarkably, infusion extract induced proliferation at any concentration but not the methanolic extract, which was diminished in a dose-dependent fashion. The immunostimulation study demonstrated that the phagocytosis activity increased in those leukocytes stimulated with methanolic extract but diminished the respiratory burst activity, in contrast to the activity observed in those leukocytes stimulated with infusion treatment. Finally, leukocytes incubated with the extracts and confronted with *V. parahaemolyticus* up-regulated the transcription of proinflammatory cytokine IL-1β gene in a dose response relationship. These findings suggest that the infusion treatment has potential therapeutic properties, promoting the antioxidant capacity and enhancing immune parameters in Longfin yellowtail *S. rivoliana*.

1. Introduction

In recent years, the interest in herbal or plant extracts have increased as alternative therapies that could effectively protect fish of diseases by stimulating their immune system. Moreover, natural medicinal products relatively non-toxic, environmentally friendly, and cost-effective, are often locally available, and can act against a broad spectrum of pathogens [1]. Herbs or plants are potential sources of phytochemicals (alkaloids, flavonoids, phenols, tannins) that are major bioactive compounds and natural antioxidants [2]. Phytochemicals act as reducing agents, metal ion chelators and scavengers of free radicals with great therapeutic potential such as antioxidant and immunomodulatory activities [3,4].

Turnera diffusa Willd or "Damiana de California" belongs to the Turneraceae family and it is a medicinal plant native of Baja California desert in Mexico [5,6]. Mexican indigenous populations used damiana leaves for the treatment of different illness, including sexual disfunction [7,8]. This small shrub is widely recognized for its medicinal properties among them, estrogenic, antibacterial and prosexual activities [6,9]. Zhao et al. [10] and Alcaraz-Meléndez et al. [11] showed a huge

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quantity of essential oils and flavonoids in *T. diffusa*, which could be responsible for its therapeutic properties. Antioxidant and phytochemical properties of *T. diffusa* have been reported for water-ethanol [9], methanolic [10,12] and essential oil extractions [11,13], but not for infusion preparations, which is the most consumed form. Tea-type infusion or water extracts is the method of obtaining bioactive compounds from plants, by keeping them immersed in cold or hot water [14]. Studies using aqueous extracts like infusion preparation are scarce, which have been traditionally used because of digestive and antispasmodic effects [15]. Actually, therapeutic applications of Damiana de California have gained an increasing interest for medical applications; however, no studies have been conducted to assess antioxidant composition and immunostimulant effects of methanolic and infusion extracts (the most consumed forms) of damiana in fish. Therefore, phytochemical compounds and antiradical capacity of infusion and methanolic extracts were studied. In addition, for the first time, the proliferation and immunostimulant effects of both (infusion and methanolic) extracts were evaluated in Longfin yellowtail *Seriola rivoliana* leukocytes.

2. Materials and methods

2.1. Preparation of infusion and methanolic extracts

Dry plants of "Damiana de California" *T. diffusa* Willd were collected in La Paz, Baja California Sur, Mexico. Damiana leaves were homogenized and milled to a fine powder (0.5- mm mesh).

For the infusion, 1 g of powder was dispensed to 100 mL of miliQ water, heated for 10 min, and let cool at 25 °C and sieved through Whatman No. 1 filter paper. Finally, infusion was lyophilized for 24 h.

For methanolic extract preparation [16], 1 g leaf powder in 10 mL of 100% methanol was incubated under agitation (28 °C, 250 rpm) for 24 h. The obtained extracts were filtered as indicated above and dried in a rotary evaporator at 40 °C for 6 h. Dried material was resuspended and analyzed according to the method for each chemical, antioxidant, antimicrobial and immunological determination as described below.

2.2. Fourier-transform infrared spectroscopy (FT-IR)

Infrared spectra of lyophilized samples were analyzed using a spectrophotometer (Cary 630, Agilent Technologies, USA) and recorded between 4000 and 650 cm^{-1}. Curve-fitting was performed with OriginPro 2016 software with default parameters, obtaining peak positions and area under the curves.

2.3. Phytochemical determination

2.3.1. Determination of total phenolic content

The total phenolic content of infusion and methanolic extracts was established according to the method described by Singleton et al. [17] with some modifications applied to a micromethod of Folin-Ciocalteu reagent. Optical density of extracts was recorded at 750 nm (Varioskan, Thermo Scientific, Waltham, MA, USA) [16]. A reference curve was constructed with gallic acid.

2.3.2. Total flavonoid content (TFC)

Flavonoid content was determined by the aluminum chloride colorimetric method [18]. A calibration curve was performed with quercetin from 0 to 1000 μg/mL [16].

2.4. Antioxidant properties of "Damiana de California" Turnera diffusa Willd

2.4.1. DPPH method

DPPH (2,2-Diphenyl-1-Picrylhydrazyl) radical scavenging effect was determined in infusion and methanolic extracts of *T. diffusa*, using the method reported by Brand-Williams et al. [19]. Butylated hydroxytoluene (BHT) was the positive control. Radical scavenging value was converted to percentage of efficient concentration (EC50), considering the quantity of the extract (mg) required to attain 50% activity per milliliter:

$$DPPH\ scavenging\ activity\ (\%) = \frac{[(A^0 - A^1)]}{A^0} * 100$$

Where A^0 and A^1 are the absorbances of control and sample, respectively, after 30 min of incubation.

2.4.2. Superoxide (O_2^-) radical scavenging action

This activity of *T. diffusa* infusion and methanolic extracts was determined according to the method reported by Martinez et al. [20]. Butylated hydroxyanisole (BHA) was the positive control, and the inhibition percentage was determined as follows:

$$Scavenging\ activity\ (\%) = \frac{[(A^0 - A^1)]}{A^0} * 100$$

Where A^0 and A^1 are the absorbances of blank and sample, respectively.

2.4.3. Iron(II) chelating effect

This analysis was performed using the method formerly reported by Canabady-Rochelle et al. [21]. Ferrous chloride ($FeCl_2$: 50 μL, 0.66 mM) was dispensed to 250 mL of *T. diffusa* (50–250 μg/mL) infusion or methanolic extract in methanol. Mixtures were incubated in shaking (200 rpm) at 35 °C for 15 min. Then 200 μL of 1.66 mM of ferrozine solution was dispensed and shaken vigorously and incubated for 10 min. Finally, reaction mixtures were centrifuged at 35 000 g for 5 min, and the absorbance of the supernatants was recorded at 562 nm. Ethylenediaminetetraacetic acid (EDTA) was the positive control, and the capacity of a sample to chelate ferrous ion Fe_2^+ was defined as follows:

$$Iron\ (II)\ chelation\ (\%) = \frac{[(A^0 - A^1)]}{A^0} * 100$$

Where A^0 was the absorbance of the blank and A^1 was the absorbance in the presence of the sample.

2.5. Bacterial activity of infusion and methanolic extracts of Turnera difussa against Vibrio parahaemolyticus

The *V. parahaemolyticus* strain used in this study was provided by Centro de Investigaciones Biologicas del Noroeste (CIBNOR, Mexico) from its bacterial collection. Briefly, the bacteria were cultured in tryptic soy broth (TSB, BD #211825) supplemented with 2.5% NaCl and incubated at 28 °C for 24 h. Then, *V. parahaemolyticus* cultures were centrifuged at 8000 g (4 °C) for 20 min. The supernatant was removed, and the bacterial pellet was suspended in sterile 0.9% phosphate buffered saline (PBS) to 1×10^6 cells/mL.

The microplate assay method was used for determining antibacterial activity of *T. difussa* extracts against *V. parahaemolyticus*. Each microplate well was filled with 100 μL of TSB and 20 μL T. *difussa* extracts at a concentration of 200, 400, 600 and 800 μg/mL. Then, 10 μL of *V. parahaemolyticus* suspension were inoculated (blank wells were filled with 20 μL of sterile deionized water) and incubated at 28 °C for 24 h. Bacterial growth was observed as turbidity determined at 600 nm before and after incubation by an ultraviolet (UV)–Vis spectrometer (iMark™, BioRad, Hercules, CA, USA). The antibacterial activity of *T. difussa* extracts against *V. parahaemolyticus* was determined in quadruplicate wells.

2.6. In vitro study

It is worth mentioning that for the *in vitro* studies using spleen leukocytes, both extracts (infusion and methanolic) were used in a low

concentration due to the high coloration of the extracts that interfered with the colorimetric assays. Infusion and methanolic extracts were dissolved in 1 M phosphate buffer solution (PBS, pH 7.4) and cell viability was determined according to Angulo et al. [22]. For the *in vitro* study, spleen samples of 9 healthy Longfin yellowtail *Seriola rivoliana* (30 ± 5 g mean body weight) were used. Briefly, spleen leukocytes were adjusted to 1.2×10^6 cells mL^{-1} (TC20, BioRad, Hercules, CA, USA) were dispensed (one mL) into cell culture plates (Sigma, St. Louis, MO, USA) plus 25 μL of infusion or methanolic extracts at a final concentration per well of 12.5, 25 and 50 μg/mL (5% CO_2, 25 °C for 24 h). Cells without extracts were used as blank. The cell viability and immune and antioxidant parameters were determined in three independent experimental tests and each test was performed in six wells for each treatment or control.

2.6.1. Cell proliferation assay

After 24 h of incubation, 10 μL (5 mg/mL) of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, St. Louis MO, USA) were dispensed [23]. The incubation was continued for 4 h. Absorbances were measured at 570 nm with microplate spectrophotometer.

2.6.2. Phagocytosis activity by neutral red

To determine the immunostimulant effect of the infusion and methanolic extracts on spleen leukocytes, phagocytosis activity was evaluated according to the methodology described by Wang et al. [24]. After cultivation for 24 h, leukocytes stimulated with both extracts, infusion and methanolic were removed, and washed two times with PBS. Then, 100 μL of neutral red solution (0.33% in DPBS) were dispensed followed by incubation for 4 h. Posteriorly, the neutral red solution was discharged, washed (twice) again with PBS. A solution of ethanol and acetic acid (cell lysate/1:1 ratio) was added and incubated for 1 h and the absorbance was recorded at 540 nm.

2.6.3. Oxidative radical production

The NBT (nitro blue tetrazolium) analysis was used to determine the radical oxygen production by leukocytes as described by Kemenade et al. [25]. Cells (100 μL) were placed in 96-well plates and NBT solution (1 mg/mL; Sigma, St. Louis MO, USA) was dispensed in darkness followed by an incubation for 2 h. The mixture was centrifugated and methanol (70% v/v) was added. Finally, 2 M KOH-DMSO was added. The absorbance was recorded at 655 nm.

2.6.4. Myeloperoxidase activity (MPO)

MPO was determined following Quade and Roth [26] methodology. Briefly, 20 μL of leukocytes were added in microtitre plates and 100 μL of TMB (3,3,5,5-Tetramethylbenzidine, 20 mM) and H_2O_2 (5 mM) were dispensed. The reaction was stopped with 50 μL of 4 M H_2SO_4. Absorbances were determined at 450 nm.

2.6.5. Antioxidant enzymes analysis

Leukocyte superoxide dismutase activity was determined with a SOD assay kit (Sigma, St. Louis, MO, USA) as described by Reyes-Becerril et al. [16]. This activity was expressed as the percentage of inhibition.

Leukocyte catalase activity was determined according to Clairborne [27] and described by Reyes-Becerril et al. [16], considering the reduction in absorbance of H_2O_2 registered at 240 nm (ε 40 M^{-1} cm^{-1}).

2.7. Pro-inflammatory cytokine IL-1β mRNA expression

To know the anti- or pro-inflammatory effect of *T. diffusa* (infusion and methanolic) extracts, a challenge using *V. parahaemolyticus* was performed following the methodology described by Angulo et al. [28]. Spleen leukocytes were stimulated with *T. diffusa* extracts and after 8 h of incubation, 20 μL comprising *V. parahaemolyticus* (1×10^8 cells mL^{-1}) were added. Sixteen hours later, cells were centrifuged (11 000 g, 20 °C, 1 min) and 1 mL of Trizol reagent (Invitrogen, USA) was dispensed for RNA purification. Relative gene expression was analyzed [29]. The expression of IL-1β (Accession No. KY860519) was normalized using the elongation factor 1-alpha (Accession No. KY806112) as reference gene in each sample. Detailed methods for RT-qPCR analyses are described in earlier reports [22].

2.8. Statistical analysis

Mean ± standard deviation (S.D.) of three replicates were calculated from all data and Student's *t*-test and ANOVA were used to discriminate the differences of extracts on antioxidant, proliferation, immunostimulation and gene expression. Tukey's multiple range test was used as post hoc analysis. All the analyses were run using SPSS v.21.0 program (Richmond, VA, USA).

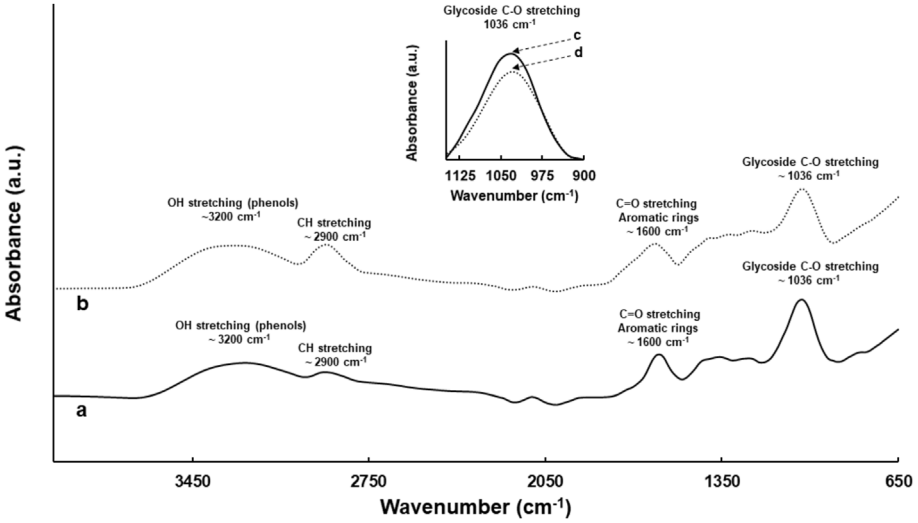

Fig. 1. FT-IR spectra of (a) infusion and (b) methanolic extracts of *Turnera diffusa* Willd. Inset: partial IR spectrum on the region from 1125 to 900 cm-1 of (c) infusion and (d) methanolic extracts.

3. Results

3.1. FT-IR analysis

In order to compare the lyophilized extracts, Fig. 1 depicts FT-IR curves that were normalized to unity based on the most intense band (as a reference band), which appears at 1036 cm^{-1} for both spectra. In both extracts (infusion extract, curve a; methanolic extract, curve b), similar characteristic signals are revealed: on the region at 3200 cm^{-1} the –OH vibrations attributed to phenols appear, a shoulder around of 2900 cm^{-1} is a characteristic signal observed due to the flexion of methylene groups. Towards 1600 cm^{-1} appears the peaks of –C=O groups, belonging to aromatic ring compounds from phenolic molecules. Interestingly, another band attributed to phenolic and antioxidant compounds (glycoside C–O stretching, and vibration region due to C–OH in phenols), appears in 1036 cm^{-1}, showing an increment on the area for the infusion spectrum (inset: curve c, area = 63.59 a.u.) compared to the methanolic extract (inset: curve d, area = 54.40 a.u.).

3.2. Total phenolic compounds of infusion and methanolic extracts in "Damiana de California" Turnera diffusa

The phytochemical assays are represented by the total phenolic contents (TPC) and total flavonoid contents (TFC), two key markers widely used to signify the general antioxidant activity in infusion and methanolic extracts. TPC of infusion and methanolic extracts were 9.25 ± 0.39 mg g^{-1} DW and 0.410 ± 0.0039 mg g^{-1} DW, respectively ($p < 0.05$, Fig. 2a). Similarly, the infusion sample had a significantly ($p < 0.05$) higher TFC value (0.404 mg g^{-1} DW) compared with the methanolic sample (0.0080 mg g^{-1} DW) (Fig. 2b).

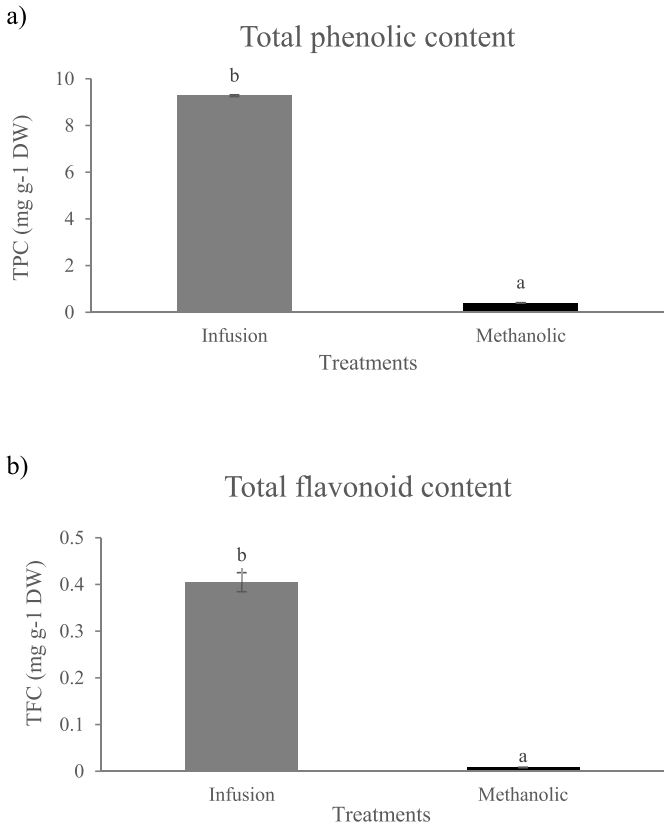

Fig. 2. Contents of total phenolics and total flavonoids of infusion and methanolic extracts from "Damiana de California" *Turnera diffusa* Willd Results are means ± standard deviation (SD) of three separate samples. Different letters denote significant differences between treated groups ($p < 0.05$).

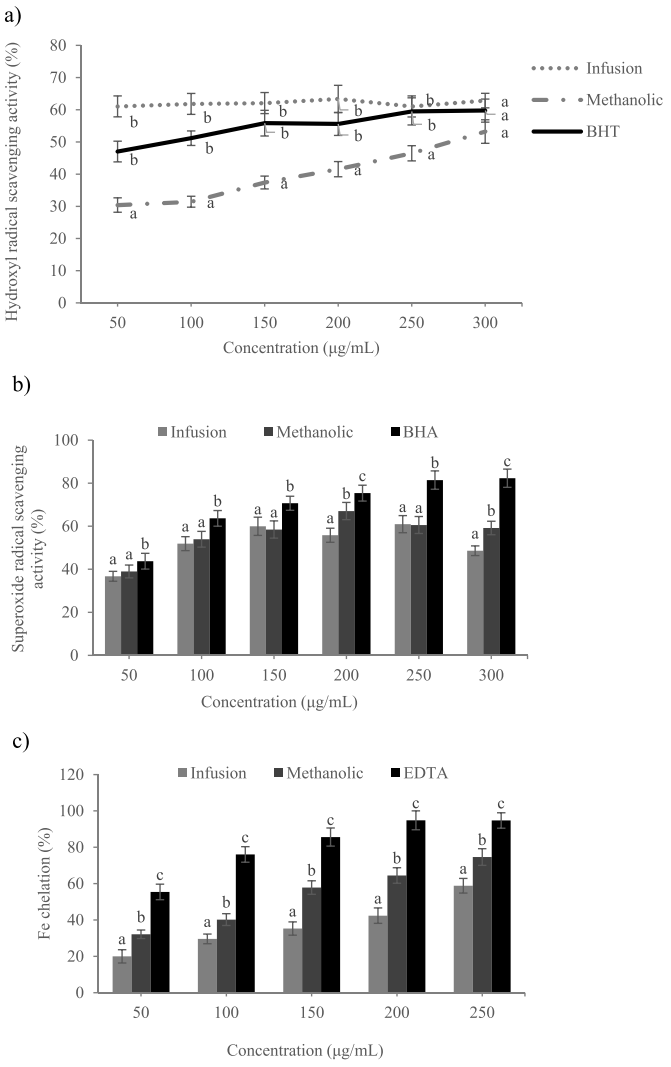

Fig. 3. Antioxidant activity of infusion and methanolic extracts from "Damiana de California" *Turnera diffusa* Willd (a) organic chemical compound 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activities of infusion and methanolic extracts and butylated hydroxytoluene (BHT). (b) Superoxide (O_2^-) radical scavenging activity of infusion and methanolic extracts and butylated hydroxyanisole (BHA). (c) Ferrous ion-chelating activities of infusion and methanolic extracts and ethylenediaminetetraacetic acid (EDTA). Results are means ± standard deviation (SD) of three separate samples. Different letters denote significant differences among treated groups ($p < 0.05$).

3.3. Total antioxidant capacity in "Damiana de California" Turnera diffusa

The hydroxyl radical scavenging activity of infusion extract measured by DPPH assay was significantly ($p < 0.05$) higher than the methanolic extract, and even higher than the positive BHT control but without significant differences (Fig. 3a).

The samples studied herein gave lower antiradical ability on superoxide radical scavenging activity than the positive BHA control; however, both extracts were dose-dependent from 50 to 200 where methanolic extracts of *T. diffusa* had the maximum superoxide radical scavenging activity at 200 µg/mL compared with the infusion sample ($p < 0.05$, Fig. 3b).

Interestingly, from the metal ion-chelating results, EDTA demonstrated high ferrous ion-chelating power of 94.7% when 200 µg/mL was used. Ferrous ion-chelating capacity of infusion and methanolic extracts from *T. diffusa* were increasing in a dose-dependent fashion where the methanolic extract showed a significantly ($p < 0.05$) higher ferrous ion-chelating capacity compared with the infusion extract (Fig. 3c).

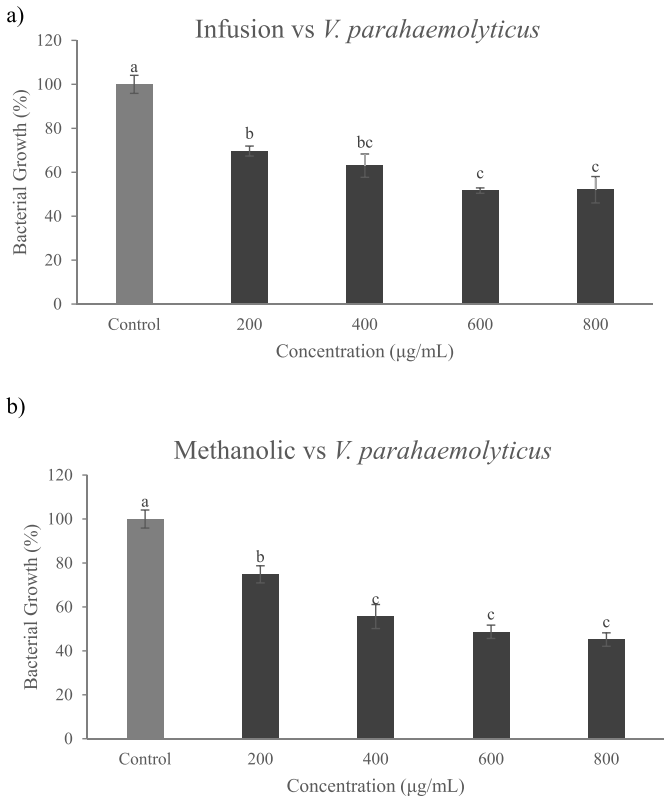

Fig. 4. Inhibition of bacterial growth (expressed as percentage) of a) infusion and b) methanolic extracts from "Damiana de California" *Turnera diffusa* Willd against *Vibrio parahaemolyticus*. Control represents medium with *V. parahaemolyticus*. The results are representative of at least three independent experiments and expressed as mean ± SD (n = 6). Different letters denote significant differences among treatment groups ($p < 0.05$).

3.4. Turnera diffusa extracts inhibited Vibrio parahaemolyticus growth

Our results showed that the bactericidal effects of both extracts of *T. diffusa* are very similar. Infusion and methanolic extracts reduced the growth of *V. parahaemolyticus* from 200 to 800 µg/mL by ~50% with respect to the control group (Fig. 4ab).

3.5. Cell proliferation activity by "Damiana de California" Turnera diffusa

Fig. 5a demonstrated the results of infusion and methanolic extracts (12.5, 25 and 50 µg/mL) on spleen leukocytes viability upon 24 h compared to the control group.

Interestingly we can observe an increase in the proliferation of spleen leukocytes treated with infusion extract ($p < 0.05$) (121, 126 and 112% at 12.5, 25 and 50 µg/mL, respectively). Leukocytes treated with methanolic extract decreased the viability in a dose-dependent manner (98, 90 and 83% at 12.5, 25 and 50 µg/mL, respectively).

3.6. Phagocytosis activity enhanced by "Damiana de California" Turnera diffusa

The effect of *T. diffusa* infusion and methanolic extracts was studied on phagocytosis activity of fish leukocytes. As shown in Fig. 5b, infusion and methanolic extracts could significantly ($p < 0.05$) enhance the phagocytosis of spleen leukocytes in all tested concentrations respect to control group. The effect of methanolic extract on phagocytosis showed higher ($p < 0.05$) activity in a dose-dependent manner compared with the infusion treatment.

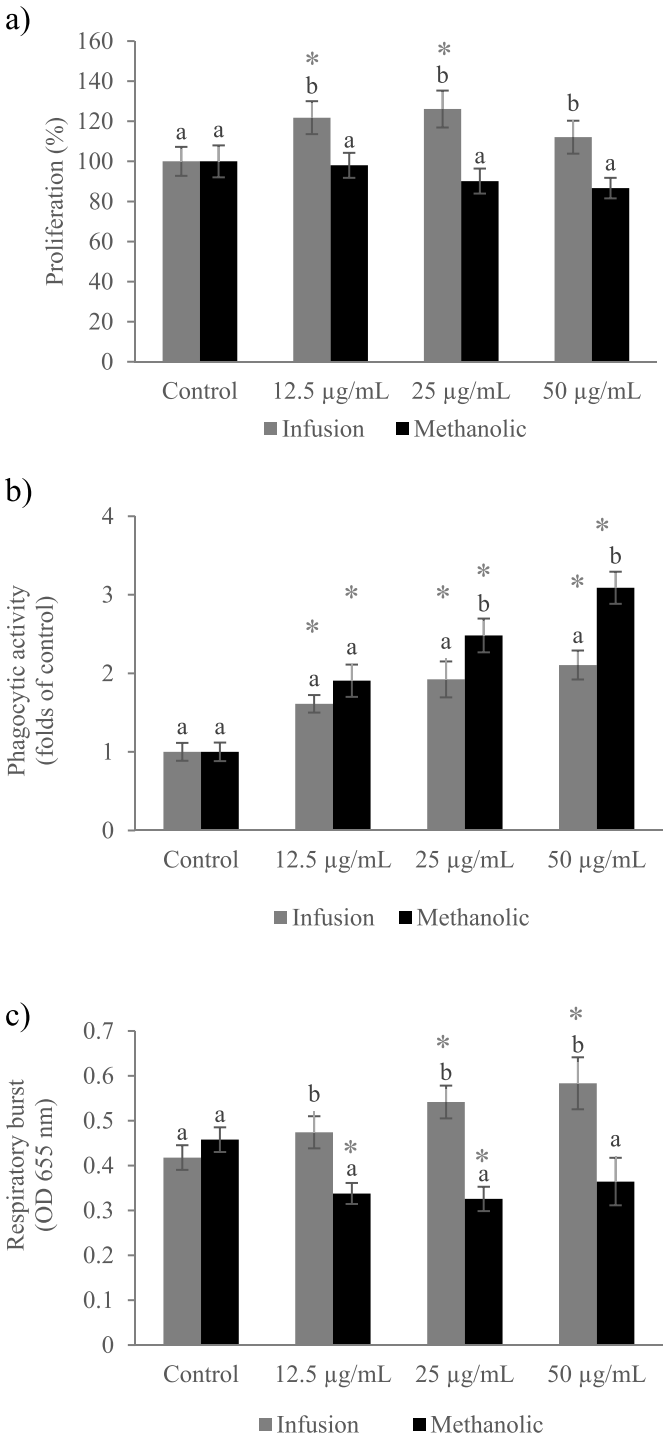

Fig. 5. Effects of infusion and methanolic extracts from "Damiana de California" *Turnera diffusa* Willd on (a) cell proliferation activity, (b) Phagocytosis ability, and (c) Respiratory burst in spleen leukocytes of *Seriola rivoliana*. Results are means ± standard deviation (SD) of three separate samples. Different letters denote significant differences among treated groups and asterisks on the data bars indicate significant differences between experimental group and the respective control group ($p < 0.05$).

3.7. Respiratory burst activity stimulated by "Damiana de California" Turnera diffusa

The respiratory burst was higher ($p < 0.05$) in leukocytes stimulated with the infusion at 25 and 50 µg/mL with respect to those of the control group. Indeed, respiratory burst activity in leukocytes incubated

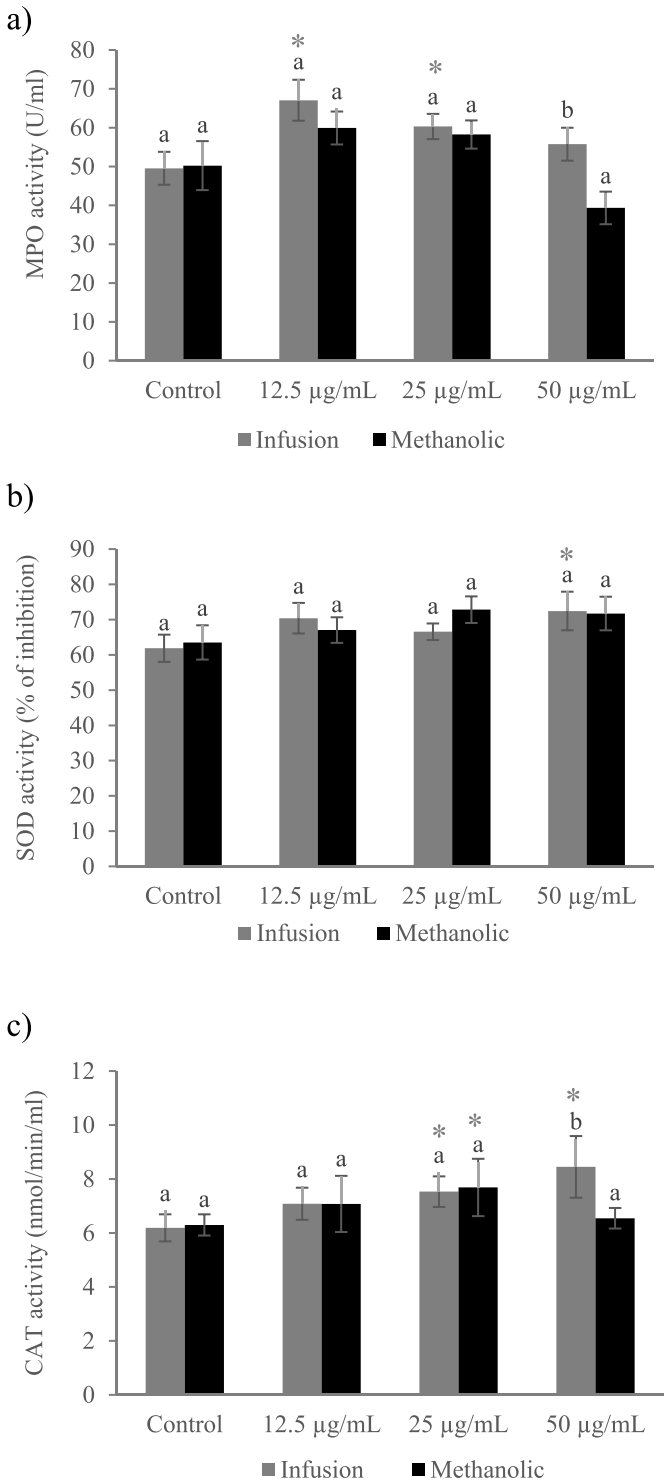

Fig. 6. Effects of infusion and methanolic extracts from "Damiana de California" *Turnera diffusa* Willd on (a) myeloperoxidase, (b) superoxide dismutase (SOD) and (c) catalase (CAT) activities in spleen leukocytes of *Seriola rivoliana*. Results are means ± SD of three separate samples. Different letters denote significant differences among treated groups and asterisks on the data bars indicate significant differences between experimental group and the respective control group ($p < 0.05$).

with infusion extracts was significantly ($p < 0.05$) higher at any concentration compared to leukocytes incubated with methanolic extracts (Fig. 5c).

3.8. Cellular enzymatic activity promoted by "Damiana de California" *Turnera diffusa*

Myeloperoxidase activity (MPO) increased ($p < 0.05$) in leukocytes incubated with infusion extract at concentrations 12.5 and 25 µg/mL in comparison with the control group (Fig. 6a). In addition, MPO activity was higher ($p < 0.05$) in leukocytes exposed to the infusion at 50 µg/mL compared with the methanolic group.

Superoxide dismutase (SOD) activity was higher ($p < 0.05$) in leukocytes treated (50 µg/mL) with infusion extract than that of control group (Fig. 6b). Catalase activity was higher ($p < 0.05$) in leukocytes incubated with infusion extract at higher concentrations (25 and 50 µg/mL) compared to the control leukocytes (Fig. 6c). In addition, catalase activity in leukocytes treated with infusion extract at 50 µg/mL was significantly ($p < 0.05$) higher than that of leukocytes treated with methanolic extract.

3.9. IL-1β gene expression

RT-qPCR analysis assessed the expression of IL-1β mRNA transcript in spleen leukocytes after stimuli with infusion or methanolic extracts of *T. diffusa* and then challenged with *V. parahaemolyticus*. Interestingly, leukocytes treated with infusion or methanolic leaf extracts and challenged with *V. parahaemolyticus* revealed an up-regulation of IL-1β gene in a dose-dependent manner compared with control or *V. parahaemolyticus* alone ($p < 0.05$). Co-treatment at 50 µg/mL of *T. diffusa* leaf extracts induced a higher up-regulation of IL-1β (Fig. 7ab) in *V. parahaemolyticus*-challenged leukocytes.

4. Discussion

Herbal or medicinal plants have shown to possess an abundant quantity of metabolites, such as polyphenols, flavonoids, amino acids and carbohydrates [30]. Principal phytochemical and antioxidant characterization of infusion and methanolic leaf extracts of "Damiana de California" *T. diffusa* Willd have been reported in this study. The antioxidant activity of phenolic compounds is attributed to their different molecular structures, concisely by the number and possible orientations of the hydroxyl groups, as well as the nature of the substitutions that the aromatic rings takes place. Thus, the most active antioxidant compound has more hydroxyl groups [31]. In this study, these bands have a similar behavior, but in the infusion spectrum the intensity in the band located at 1036 cm^{-1} is favored compared to the methanolic spectrum, probably by the polarity of the water [32]. It could indicate the presence of glycoside derivatives, such as arbutin that has been previously reported for *T. diffusa* [33]. Regarding to bioactive compounds, infusion samples had a major quantity of polyphenols and flavonoids than methanolic extract, which may be due to contact time and temperature at which the infusion was prepared, allowing a greater and better extraction of phenolic compounds. Several authors have observed that extraction conditions may affect the phytochemical compounds [34,35]. Martins et al. [36] observed a higher level of flavonoids and phenolic compounds in decoction extract than infusion extract of *Origanum vulgare* L. In line with several studies, antioxidant activity is one of the most valuable parameters of plant extracts, which is related with phenolic compounds and mostly flavonoids. The infusion extraction of *T. diffusa* had strong antioxidant activity using the DPPH radical scavenging method. DPPH is a stable free radical that accepts electrons or hydrogen radicals from donor compounds [37]. In this sense, the infusion extract of *T. diffusa* had more capacity for donating a hydrogen atom (hydroxyl radical scavenging activities) than positive BHT control. In addition, superoxide anion is the earliest reactive oxygen species produced. Superoxide anion scavenging activity was also evaluated for the first time in infusion and methanolic extracts of *T. diffusa* and compared with control BHA. The extracts showed high inhibitory activity (67% for methanolic and 60%

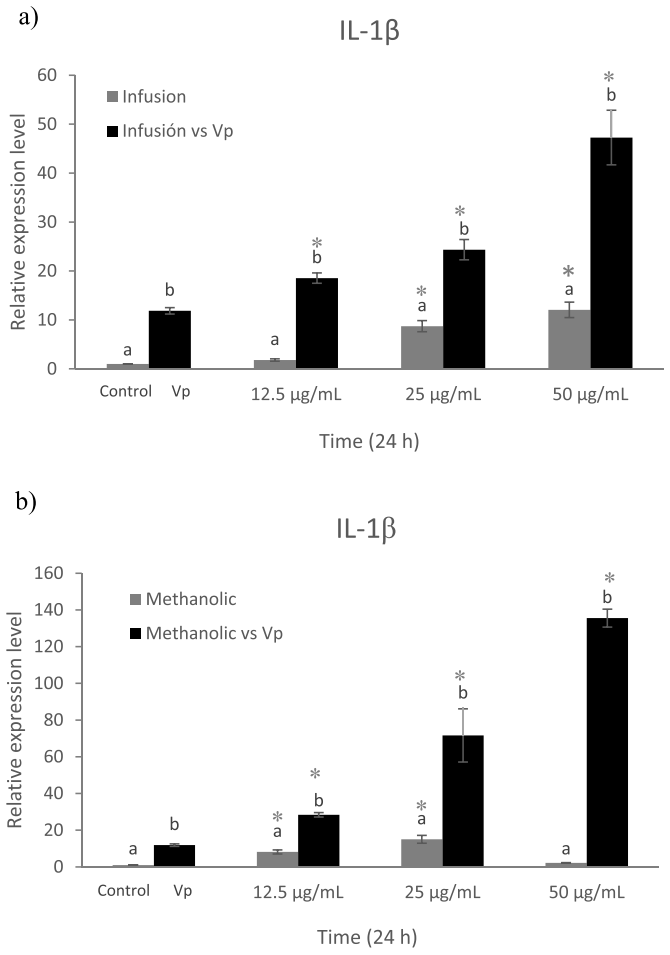

Fig. 7. Effect of "Damiana de California" *Turnera diffusa* Willd leaf extract on pro-inflammatory cytokine gene expression (IL-1β) in spleen leukocytes of *Seriola rivoliana*. (a) Infusion vs *Vibrio parahaemolyticus* (Vp) and (b) Methanolic vs *Vibrio parahaemolyticus* (Vp). Results are means ± standard deviation (SD) of three separate samples and EF-1α was used as a reference gene. Different letters denote significant differences between treated groups and asterisks on the data bars indicate significant differences between experimental group and the respective control group ($p < 0.05$).

for infusion) at 200–250 µg/mL, respectively. However, they showed lower superoxide anion scavenging effect than BHA (73–81% at 200–250 µg/mL).

The capacity to chelate metals is a mechanism to determinate the antioxidant activity. Ferrous iron (Fe^{2+}) is a pro-oxidant and considered as electron transfer antioxidant [38]. Substances with the capacity to bind Fe^{2+} may decrease its pro-oxidant effects and could rise its absorption and biological actions into the cells [39]. In this study, methanolic extract showed higher Fe^{2+} chelation activity compared to the infusion samples. The antioxidant capacity of natural extracts is influenced by several factors [33], and no well-defined relationship has been found between the antioxidant properties of extracts and their polyphenol content.

In aquaculture, several medicinal plants have demonstrated antimicrobial, antioxidant and immunostimulatory activities [40,41]. The antimicrobial activity of infusion and methanolic extracts of *T. diffusa* were evaluated by microdilution method against *V. parahaemolyticus*. The results showed antibacterial activity of both infusion and methanolic extracts of *T. diffusa* against *V. parahaemolyticus*, confirming one of their potential medicinal uses. Recently, Baez-Parra et al. [42] found that methanolic and hexanic extracts of *T. diffusa* var. diffusa and *T. diffusa* var. aphrodisiaca had antimicrobial activity against urinary tract pathogenic species (*Klebsiella pneumoniae*, *Enterococcus faeccalis*, *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*). In other study, Hernández et al. [43] showed antimicrobial activity of the hexanic and ethanolic extracts of damiana (*T. diffusa*) against gastrointestinal pathogens. As far as we know, this is the first study to formally demonstrate the antimicrobial effectiveness of *T. diffusa* for fish pathogens.

In the *in vitro* study, fish leukocytes were stimulated with infusion and methanolic extracts of *T. diffusa* (12.5, 25 and 50 µg/mL); proliferation, immune and antioxidant parameters were evaluated at 24 h post-incubation. In this study, a proliferative effect was observed in leukocytes stimulated with infusion samples at any concentration. The values obtained clearly demonstrated that infusion extract induce proliferation. In contrast, methanolic extract reduced the viability of leukocytes in a dose-dependent manner (98, 90 and 83% of viability) at 12.5, 25 and 50 µg/mL, respectively. On this regard, Koldas et al. [44] demonstrated that chemical compounds extracted from a given plant depend on the time of extraction, solvent used and the method of extraction. These important characteristics are associated with proliferation or cytotoxicity effects. Our results agreed with those of similar studies, such as ethanolic extracts of leaves from carob that showed dose-dependent cytotoxic activity [45]. In addition, Abdillahi et al. [46] proposed values of cytotoxicity for a given substance depending of the effect of cell viability as: no toxic (> 70%), weakly toxic (50%–70%) or toxic (< 50%). These results suggest that infusion rather than polar solvents could be a better strategy to obtain compounds from *T. diffusa* without causing cytotoxicity because it is of great interest to develop natural immunostimulators with low toxicity but high efficiency.

The immunostimulatory effect of "Damiana de California" *T. diffusa* infusion and methanolic extracts was analyzed on Longfin yellowtail *S. rivoliana* leukocytes. The phagocytic activity has been considered the most important cellular function of innate system against invading microorganisms, and its increase is related with immunostimulatory mechanisms [47]. In this study, phagocytic activity of leukocytes was enhanced upon stimulation with infusion and methanolic extracts. Fazio et al. [48] observed enhanced phagocytic activity seabream leukocytes incubated with *Lavandula* sp extracts. In contrast, Garcia-Beltran et al. [49] reported that only excessive quantities of *O. vulgare* leaf ethanolic extracts decreased phagocytosis, whereas aqueous or ethanolic leaf extracts at appropriate doses enhanced this activity. During the phagocytosis, phagocytes increased their oxygen consumption through the NADPH oxidase, which generated superoxide anion and hydrogen peroxide (reactive oxygen species, ROS) in a process called the respiratory burst, and consequently antioxidant enzymes were also produced [50]. Curiously, in this study, a high respiratory burst activity was observed in cells treated with infusion in a dose-dependent fashion compared with the control group, while a depressing effect was observed with methanolic extract. Professional phagocytes play an important role in the clearance of microbial pathogens, for example, via mechanisms involving the production of oxygen radicals or nitrogen radicals, such as nitric oxide [51]. Therefore, it is possible that methanolic extract diminish the respiratory burst activity while increased nitric oxide production. However, this hypothesis must be tested. Curiously, similar to the present results were reported by Garcia-Beltran et al. [49] where the respiratory burst activity on leukocytes gradually diminished by methanolic extract of *O. vulgare* after 24 h of stimulation. In contrast, in rainbow trout (*Oncorhynchus mykiss*) the effect of the methanolic extract of black cumin (*Nigella sativa*) elevated the respiratory burst activity in treated groups compared to the control group [52]. Additionally, fish leukocytes produced enzymes, such as myeloperoxidase, superoxide dismutase and catalase to counteract the excess of ROS. Azurophilic granules of neutrophilic granulocytes stored the proinflammatory enzyme myeloperoxidase, during the respiratory burst, this enzyme or hemoprotein catalyzes the formation of hypochlorous acid from hydrogen peroxide, a toxic compound for invasive bacteria [53]. Similarly, catalase uses hydrogen peroxide generated from the respiratory burst as substrate to convert it into water and

oxygen. Once again, an enhancement of myeloperoxidase and catalase activities was observed in leukocytes stimulated with infusion extracts in comparison with the control group.

The anti-inflammatory and antioxidant properties of diverse extracts of *Turnera* spp. have been associated to numerous secondary metabolites [9,54,55]. Therefore, this study evaluated gene expression level of proinflammatory cytokine IL-1β in leukocytes stimulated with infusion and methanolic extracts at different concentrations and challenged with *V.parahaemolyticus*. Interleukin-1β is a key regulator involved in the inflammatory cell reaction [56]. This study demonstrated that stimuli with both infusion and methanolic leaf extracts of *T. diffusa* significantly increased pro-inflammatory IL-1β gene expression in spleen leukocytes against infection of *V. parahaemolyticus*. Interestingly, IL-1β is considered a biomarker of zebrafish sepsis provoked by *V. parahaemolyticus* infection [57], highlighting that this cytokine is critical to control this bacterial disease [53]. *In vivo* and *in vitro* studies for herbal extracts have suggested that cytokine modulation may provide the mechanism of action for many of their therapeutic effects [58]. Garcia-Beltran et al. [49] observed an immunostimulant activity in fish leukocytes and bactericidal effects of infusion and polar extracts (at different concentrations) against several bacterial pathogens, including species of the *Vibrio* group. Thus, different extracts of *T. diffusa* could increase the pro-inflammatory cytokine IL-1β production to help leukocytes kill bacteria by inflammatory cascades, including the production of other cytokines, lipid mediators, and adhesion molecules [59].

5. Conclusion

For the first time, this study provides the effect of Damiana de California *T. diffusa* infusion and methanolic extracts on chemical, biological, and immunostimulant properties using leukocytes of Longfin yellowtail *S. rivoliana.* The chemical ATR-FTIR analysis revealed the presence of different bioactive compounds, which could be related with the *T. diffusa* antioxidant capacity. Clearly, infusion extract is the treatment that had more phytochemical compounds and DPPH activity. Infusion and methanolic extracts at > 200 μg/mL had a strong antibacterial effect against *V. parahaemolyticus*. The *in vitro* immunological assay results were strongly correlated with total phenolic contents in leukocytes stimulated with infusion treatment at 25 μg/mL. Interestingly, leukocytes incubated with *T. diffusa* extracts and challenged with the bacteria *V. parahaemolyticus* enhanced the pro-inflammatory gene expression of IL-1β in a dose-depended manner, which gave *T. diffusa* interesting bactericidal promoting properties. Future studies should be focused on antimicrobial activities of *T. diffusa*. Furthermore, our knowledge should be extended to isolate and identify the specific compounds responsible for distinct bioactivities in the extracts. This first work with Damiana de California extracts help us to continuous with a posterior study, where Damiana will be evaluated *in vivo* experiments for future application as additive or immunostimulants for aquaculture industry.

CRediT authorship contribution statement

Martha Reyes-Becerril: Conceptualization, Writing - original draft. **Perla Ginera:** Investigation. **Jorge Silva-Jara:** Investigation. **Adriana Macias:** Investigation. **Carlos Velazquez-Carriles:** Investigation. **Lilia Alcaraz-Meléndez:** Methodology. **Carlos Angulo:** Writing - review & editing.

Declaration of competing interest

The authors declare no competing financial interest.

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