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Survival of White Spot Syndrome Virus–Infected *Litopenaeus vannamei* Fed with Ethanol Extract of *Uncaria Tomentosa*

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Abstract

With the present project, the phytochemical composition of *Uncaria tomentosa* cultivated in Chapecó (Brazil) was analyzed and then the potential of using this local plant extract to protect by oral feeding virus-infected *Litopenaeus vannamei* shrimp was also investigated. For this study, five treatments were tested: uninfected shrimp fed with pelleted feed (T_C), WSSV-infected shrimp fed with pelleted feed (T_1), infected shrimp fed with pelleted feed with 1% ethanol extract of *U. tomentosa* (EEUT) (T_2), infected shrimp fed with pelleted feed with 2% EEUT (T_3), and infected shrimp fed pelleted feed with 4% EEUT (T_4). The chemical analysis of EEUT showed the presence of alkaloids, flavonoids, sterols, terpenes, coumarins, and tannins and also a high *in vitro* free radical-scavenging activity as well as high total phenolic content. Shrimp fed with 2 and 4% EEUT (T_3 and T_4) showed a survival probability significantly higher than T_1 and T_2 treatments and no clinical symptoms of WSSV infection. Immunological assay also showed a positive phenoloxidase activity effect on shrimp fed with EEUT.

KEYWORDS

***Litopenaeus vannamei*, plant extracts, *Uncaria tomentosa*, white spot syndrome virus**

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Uncaria tomentosa (Willd.) D.C. (Rubiaceae) also known as "cat's claw" has been used to treat several human diseases such as dysentery, rheumatism, diabetes, cancer of the urinary and digestive tract, cirrhosis, gastritis, inflammation, tumors, fevers, and abscesses (Ndagijimana et al. 2013). In fact, important biological activities such as anti-inflammatory, antioxidant, radical scavenging activity, antiviral, and immunomodulation effects have been linked to *U. tomentosa* pentacyclic oxindole alkaloids (Gonçalves et al. 2005; Reis et al. 2008). Alcoholic preparations have shown higher concentration of total phenolic compounds and antioxidant activity than aqueous extractions (Krishnaiah et al. 2011). With the intensification of shrimp farming, viral diseases such as white spot syndrome virus (WSSV) have become increasingly common. In Brazil, WSSV has been detected in south and northeastern regions compromising *Litopenaeus vannamei* farming activity (Nogueira 2013). A viable option to preventively treat this disease is the use of plant extracts with antiviral activity (Balasubramanian et al. 2008a, 2008b). *Uncaria tomentosa* extracts may play an important role in maintaining the health of aquatic organisms during their growth cycle. Medina-Beltrán et al. (2012) reported that powders of *Echinacea purpura* and *U. tomentosa* sprayed onto the feed of *L. vannamei* had antiviral activity against WSSV. Nonetheless, there is still a lack of knowledge on the long-term effects as well as on the homogenization of the extract preparation and shrimp administration (Reverter et al. 2014). The objectives of the present work were (1) to study the chemical composition and antioxidant activity of ethanol extract of *U. tomentosa* and (2) to investigate by bioassays the ability of ethanol extract of *U. tomentosa* to protect *L. vannamei* shrimps against WSSV.

Materials and Methods

Plant Material and Extraction Procedure

Dried sliced bark of *U. tomentosa* (200 g; Chá Chileno®, Chapecó, SC, Brazil) was macerated in 2 L of ethanol (Vetec, Rio de Janeiro, Brazil) at room temperature for 5 d following the protocol described by Calixto and Yunes (2001). The ethanol extract obtained from *U. tomentosa* (EEUT) was filtrated (45 µm, Whatman filter paper; Sigma-Aldrich Co., St. Louis, MO, USA) and concentrated under vacuum at 40 C. The resulting plant extract (23.57 g) was stored under refrigeration for all experiments.

Thin-Layer Chromatography

The silica-coated aluminum plates (0.25 mm, 60F245; Merck, Darmstadt, Germany) were used as stationary phase and the identification of each major chemical group present in EEUT was performed using the following solvents (Tomazelli Junior et al. 2017): for terpenes and sterols, a mixture of 70% hexane to 30% of ethyl and *p*-anisaldehyde was used as mobile phase and reagent, respectively. Then, the precoated plates were heated at 100 C for 10 min for their visualization of these components. For alkaloids, 95% chloroform with 5% methanol was used as mobile phase and the present spots were detected after spraying with Dragendorff's reagent. For flavonoids and tannins, two mixtures of 95% chloroform, 5% methanol, 70% hexane, and 30% of ethyl acetate were used as mobile phases, respectively. In this case, the spots were visualized with 3% of ferric chloride. Finally, for coumarin analysis, 95% of chloroform and 5% of methanol were used as mobile phase and the spots were visualized under UV light (360 nm) after being immersed in 5% solution of KOH in ethanol (Calixto and Yunes 2001).

Identification and Quantification of Phenols and Flavonoids by HPLC-DAD

High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) analyses were performed using a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, a SPD-M20A diode array detector (DAD) (Shimadzu), and LC solution 1.22 SP1 software (Shimadzu). The reverse-phase chromatographic analyses were performed under gradient conditions using

C18 column (4.6 × 150 mm; Macron, Center Valley, PA, USA) packed with 5-µm diameter particles. Mobile phase was a mixture of methanol (A; Merck) and 5% aqueous formic acid (B; Merck). The gradient of concentrations was applied over 60 min at a flow rate of 1 mL/min as follows: first 5–15% A over 10 min, then a range from 35 to 50% A over 15 min and finally the concentration of A changed from 50 to 100% over 10 min (Gonçalves et al. 2005).

Chromatographic profiles were recorded at 254 nm for gallic acid; at 280 nm for catechin and epicatechin; at 327 nm for caffeic, chlorogenic, and ellagic acids; and at 356 nm for quercetin, quercitrin, kaempferol, and luteolin. Samples and mobile phase were filtered (0.45 µm; Millipore, Austin, TX, USA) and then degassed by ultrasonic bath. Stock standard solutions were prepared at concentrations ranging from 0.030 to 0.250 mg/mL using HPLC mobile phase. Chromatography peaks were confirmed by comparing their retention time with standards of reference and by DAD spectra (200–600 nm). All chromatography operations were performed at room temperature and in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses (σ) and the slope (S) using three independent analytical curves. The LOD and LOQ were calculated as 3.3 and 10σ/S, respectively (Boligon et al. 2013).

Total Phenolic Content

Total phenolic content was measured by Folin–Ciocalteu assay (Singleton and Rossi 1965). EEUT (100 µL) was added to 1 mL of 10-fold-diluted Folin–Ciocalteu reagent (Merck). After agitation, the mixture was kept at room temperature for 5 min. Next, 1 mL of 10% sodium carbonate solution (Vetec) was added. After 90 min, absorbance was measured at 725 nm and gallic acid was used as standard for the calibration curve. Total phenolic content was expressed as mg gallic acid equivalents (GAE) per g of sample in dry weigh t (mg/g).

Trolox Equivalent Antioxidant Capacity

Trolox equivalent antioxidant capacity (TEAC) of EEUT was measured using the methodology described by Re et al. (1999). The ABTS radical (2, 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) was generated by mixing 25 mL of 7 mM ABTS (Sigma-Aldrich Co.) with 440 µL of 140 mM potassium persulfate (Sigma-Aldrich Co.) and allowed to react for 12–16 h in the dark at room temperature. An aliquot of EEUT (30 µL) was mixed with 3 mL of the ABTS radical at dark conditions and absorbance was measured at 734 nm. At the same time, a trolox calibration curve with 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox; Sigma-Aldrich Co.) was prepared for a concentration range of 0–500 µM, and the inhibition percentage obtained for the sample was interpolated to calculate the concentration in trolox equivalents (µM). Each sample was analyzed in triplicate.

Viral Inoculum Preparation

WSSV-infected *L. vannamei* with prominent white spots were collected from shrimp farms located in the county of Laguna (Santa Catarina, Brazil). Their infection was confirmed by polymerase chain reaction (PCR) analysis following the protocol described by Hameed et al. (2003). Potentially WSSV-infected shrimps were mixed with Tris–NaCl buffer (Sigma-Aldrich Co.), centrifuged at 670*g* for 20 min at 4 C, and the solution obtained was filtered (0.45 µm; Sartorius) and frozen with liquid nitrogen. A sample of the viral inoculum was quantified by real-time PCR (qPCR) by Laboratory of Biomarkers of Aquatic Contamination and Immunology (LABCAI, Federal University of Santa Catarina, Santa Catarina, Brazil) obtaining 10^6 viral particles per µL.

Collection and Maintenance of Experimental Shrimp

Litopenaeus vannamei shrimp (weight 24.17 ± 2.07 g) were collected from growout ponds of the Laboratory of Marine Shrimps (LCM; Federal University of Santa Catarina,

TABLE 1. In vivo *determination of antiviral activity of ethanol extract of* U. tomentosa *against white spot syndrome virus (WSSV) inoculated in* L. vannamei *shrimp.*

Treatments	Biomass (g)[1]	Dilution of the viral inoculum	Ethanol *U. tomentosa* extract artificial pellet feed (%)	Confirmation of WSSV-infection – PCR
T_C	23.47 ± 1.98	10^{-2}	–	–
T_1	23.93 ± 2.01	10^{-2}	–	+
T_2	24.38 ± 2.07	10^{-2}	1	+
T_3	24.75 ± 2.12	10^{-2}	2	+
T_4	24.34 ± 2.20	10^{-2}	4	+

PCR = polymerase chain reaction.
[1]Mean and standard deviation.

Santa Catarina, Brazil) and maintained in a round 100-L fiberglass tank (Tomazelli Junior et al. 2017). Natural seawater was used in all the experiments and shrimp were fed with artificial pelleted feed (Nicoluzzi® with 32% crude protein, Santa Catarina, Brazil) reaching feeding levels of 0.36 g animal/d. Temperature, pH, dissolved oxygen (DO), and total ammonia nitrogen (TAN) were recorded daily at 0800 h, 1600 h, and midnight (Tomazelli Junior et al. 2017). Salinity was measured with a salinometer (COD 3953; Alfakit, Florianópolis, Brazil), DO was measured with an oxygen meter (AT 160 SP; Alfakit), and TAN was estimated according to the method proposed by Koroleff (1976).

Test Diet Preparation

EEUT was incorporated at three different concentrations 1, 2, and 4%, (calculated on the total amount fed daily to shrimp) to commercially available artificial pelleted feed (32% of crude protein; Nicoluzzi). For coating, a protocol applied in a previous study (Tomazelli Junior et al. 2017) and adapted from Balasubramanian et al. (2008a) was used. EEUT solutions was mixed with the food pellets and incubated at room temperature for 30 min to allow the absorption of the plant extract and then coated with 0.5% food grade unflavored gelatin dissolved in water and gently mixed with the food pellets. The feed was then dried and stored at room temperature until given to shrimp. The same process was repeated for the shrimp control feed without adding the plant extract.

Experimental Design

One hundred twenty-five shrimp were distributed in five treatments in triplicate. *Litopenaeus vannamei* shrimp were placed in 40-L high-density polyethylene. Each tank was divided into two compartments with a perforated polyethylene division that allowed water to flow. In one of the compartments, 10 shrimp were placed to perform the survival analysis and in the second compartment 15 shrimp were placed for hemolymph analysis. Five treatments were applied in this study (Table 1): uninfected shrimp fed with pelleted feed (T_C), WSSV-infected shrimp fed with pelleted feed (T_1), WSSV-infected shrimp fed pelleted feed with 1% EEUT (T_2), WSSV-infected shrimp fed pelleted feed with 2% EEUT (T_3), and WSSV-infected shrimp fed pelleted feed with 4% EEUT (T_4). Infections (batches T_1–T_4) were carried out by intramuscular injection in the second abdominal somite with 100 µL of the dilution 10^{-2} viral inoculum previously prepared. T_C was injected with 100 µL of Tris–NaCl solution (Sigma-Aldrich Co.). Shrimp were fed three times a day at 0800, 1600, and 1200 h to satiety throughout the experiment. The shrimp were examined twice a day to look for clinical signs of WSSV disease; to check their feeding behavior; and also to count, remove, and study dead shrimp.

Hemolymph Collection for Immunological Analysis

Hemolymph was taken from 15 shrimp (3 shrimp per each tank) at 0, 24, 48, and 72 h, using 1-mL sterile syringes with 21G needle, and were

cooled at 4 C (Tomazelli Junior et al. 2017). The coagulated hemolymph was frozen at −20 C and was repeatedly centrifuged at 10,000*g* for 10 min to obtain the serum that was aliquoted and stored at −20 C for later use in other immunological analyses (Maggioni et al. 2004).

Agglutinating Activity

Litopenaeus vannamei serum samples (50 µL each) were serially diluted in TBS-1 (50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, 5 mM $MgCl_2$, and pH 7.4) and placed in 96-microwell plates with concave bottom (Techno Plastic Products AG, Trasadingen, Switzerland). An aliquot of 50 µL of 2% dog erythrocytes solution in TBS-1 was placed in each well and the microwell plates were placed in a humidified chamber and incubated at room temperature and saturated conditions for 2 h. Control samples were prepared using TBS-1 instead of *L. vannamei* serum. Serum agglutination titer was linked to the highest dilution that was capable of agglutinating erythrocytes (Maggioni et al. 2004).

Phenoloxidase Activity

The presence of phenoloxidase enzyme (PO) and its zymogenic form proPO in the shrimp serum was determined spectrophotometrically at 490 nm after the oxidation of the enzyme substrate L-dihydroxyphenyl-alanine (L-DOPA; Sigma-Aldrich Co.) as described by Perazzolo and Barracco (1997). Briefly, serum samples were serially diluted in TBS-2 (10 mM Tris, 336 mM NaCl, 5 mM $CaCl_2$, 10 mM $MgCl_2$, and pH 7.6), and 50 µL of these solutions were preincubated with an equal volume of the 1 mg/mL enzyme inducer trypsin (Sigma Chemical Co.) for 15 min at 20 C in 96-microwell plates (flat bottomed; Techno Plastic Products AG). For control samples, trypsin and serum were replaced by TBS-2. After incubation, 50 µL of 3 mg/mL L-DOPA (Sigma Chemical Co.) was added to the wells, and the formation of DOPA-chrome pigment was monitored after 5 and 10 min at 490 nm. One unit of the specific activity from PO was equivalent to the variation of 0.001 in the absorbance/min/mg of protein (Söderhäll and Häll 1984).

Statistical Analysis

Data analyses were performed by using the free software R version 3.1.1 (R Core Team 2014) and the add-on packages stats and survival (Therneau 2012). Tukey's test was used to obtain paired comparisons among sample means. The Kaplan–Meier method was used to measure the fraction of subjects living for a certain amount of time after treatment was applied (Goel et al. 2010), and the Cox semiparametric model was used to show the differences between treatments (Cordón-Lagares et al. 2016). Level of significance was set at $P < 0.05$.

Results and Discussion

Phytochemical Screening

Thin-layer chromatography analysis of EEUT showed the presence of alkaloids, flavonoids, sterols, terpenes, coumarins, and tannins. Moreover, the HPLC profile showed the main presence of 12 chemical compounds: gallic acid (retention time 9.95 min, Peak 1), catechin (14.87 min, Peak 2), chlorogenic acid (19.36 min, Peak 3), caffeic acid (23.71 min, Peak 4), epicatechin (28.04 min, Peak 5), ellagic acid (31.07 min, Peak 6), rutin (35.01 min, Peak 7), quercitrin (43.96 min, Peak 8), quercetin (46.57, Peak 9), kaempferol (57.11 min, Peak 10), luteolin (64.38 min, Peak 11), and apigenin (73.92 min, Peak 12) (Fig. 1 and Table 2). In fact, more than 200 chemical components from different families of organic products such as indole alkaloids, triterpenes, flavonoids, and phenylpropanoids have been isolated from the genus of *Uncaria* (Keplinger et al. 1999; Sandoval et al. 2002; Zhang et al. 2015). It is well known that flavonoids and phenolic acids play an important role as antioxidants for human beings (Omale and Okafor 2008). Moreover, flavones and catechins have been identified as two of the most powerful flavonoids for protecting the human body against reactive oxygen species (Nijveldt et al. 2001; Tapas et al. 2008). In our EEUT, two flavones were found, apigenin (9.59 mg/g) and luteolin (6.62 mg/g)

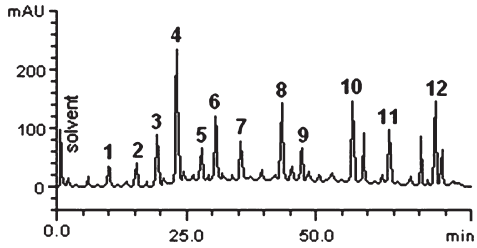

FIGURE 1. *Chromatographic profile of the ethanol extract of* U. tomentosa *high-performance liquid chromatography at 327 nm: gallic acid (retention time* (t_R), $t_R = 9.95$ *min; Peak 1), catechin* ($t_R = 14.87$ *min; Peak 2), chlorogenic acid* ($t_R = 19.36$ *min; Peak 3), caffeic acid* ($t_R = 23.71$ *min; Peak 4), epicatechin* ($t_R = 28.04$ *min; Peak 5), ellagic acid* ($t_R = 31.07$ *min; Peak 6), rutin* ($t_R = 35.01$ *min; Peak 7), quercitrin* ($t_R = 43.96$ *min; Peak 8), quercetin* ($t_R = 46.57$; *Peak 9), kaempferol* ($t_R = 57.11$ *min; Peak 10), luteolin* ($t_R = 64.38$ *min; Peak 11), and apigenin* ($t_R = 73.92$ *min; Peak 12).*

TABLE 2. *Components of ethanol extract of* U. tomentosa.

Compounds	Extract (mg/g)	LOD (μg/mL)	LOQ (μg/mL)
Gallic acid	3.27 ± 0.02^{a}	0.016	0.053
Catechin	3.41 ± 0.04^{a}	0.009	0.029
Epicatechin	4.08 ± 0.03^{d}	0.014	0.046
Quercetin	4.13 ± 0.01^{d}	0.013	0.043
Rutin	6.49 ± 0.01^{b}	0.024	0.076
Chlorogenic acid	6.57 ± 0.01^{b}	0.023	0.072
Luteolin	6.62 ± 0.03^{b}	0.029	0.097
Ellagic acid	8.36 ± 0.02^{e}	0.027	0.089
Quercitrin	9.41 ± 0.02^{f}	0.008	0.026
Kaempferol	9.57 ± 0.03^{f}	0.018	0.059
Apigenin	9.59 ± 0.01^{f}	0.021	0.069
Caffeic acid	14.95 ± 0.01^{c}	0.011	0.034

LOD = limit of detection; LOQ = limit of quantification.

Results are expressed as mean ± SDs of three determinations.

Means with different superscripts in the same row are significantly different at $P < 0.05$ (Tukey's test).

(Table 2). These substances are strong antioxidants and have free-radical scavenging capacity that displays specific anti-inflammatory and antimicrobial activities that are also assigned to apigenin, kaempherol, quercetin, quercitrin, and rutin (Cushnie and Lamb 2005; Seelinger et al. 2008). Sandoval et al. (2002) found catechin (8.47 mg/g), epigallocatechin, epicatechin, and epigallocatechin gallate in a freeze-dried *U. tomentosa* extract. Several researchers have reported that flavonoids added to shrimp feed seem to protect the shrimp body against reactive oxygen and nitrogen species that make part of the major defense system currently recognized in crustaceans (Han et al. 2007; Aguirre-Guzmán et al. 2009). For instance, Hsieh et al. (2008) showed that rutin improved the biochemical, immunological, and hematological parameters of *L. vannamei* shrimps infected with *Vibrio alginolyticus*.

TEAC and Total Phenolic Content of EEUT

EEUT used for this research had a TEAC value of 3.610 ± 0.054 mmol/g. This result showed the high antioxidant capacity of EEUT when compared to extracts obtained from other plants such as *Sargentosa cuneata* (0.265 ± 0.046 mmol/g) and *Faxinus rhynchophylla* (0.166 ± 0.034 mmol/g) (Soobrattee et al. 2005; Li et al. 2008; Krishnaiah et al. 2011). In addition, Rufino et al. (2010) studied TEAC of 18 nontraditional tropical fruits from Brazil and found the highest TEAC values for *Camu camu* and *Juçara* 1.270 ± 0.338 and 0.606 ± 0.142 mmol/g, respectively. Total phenolic concentration (TPC) found in EEUT was 554.60 ± 0.015 mg/GAE/g. Pilarski et al. (2006) found TPC values of 111 and 292 mg/GAE/g for aqueous and 50% alcoholic bark extracts of *U. tomentosa*, respectively. The differences of TPC values between the studies may be explained by the different plant extract procedure and the percentage used. During the phagocytosis process, highly toxic molecules such as reactive oxygen and nitrogen species are produced to destruct the invading pathogens. Nonetheless, these molecules can also cause serious damage to host tissues. One way to avoid this auto aggression is having antioxidants of external origin (Barracco et al. 2007). Therefore, plant extracts with high TEAC and TPC values can be used to raise healthy penaeid shrimp.

Evaluation of the Agglutinating Ability of Hemolymph

At 48 h, Treatments T_2 ($P = 0.034$) and T_4 ($P = 0.034$) showed significant differences with

TABLE 3. *Average of the serum agglutination titer (log_2(x)) in each level of treatments and time of the shrimps fed supplemented diets with ethanol extract of* U. tomentosa *and white spot syndrome virus challenged.*

	Time (h)			
Treatments	0	24	48	72
T_C	9.00 ± 0.0^{aA}	$10.33 \pm 0.58^{bA,B}$	$10.33 \pm 0.58^{bA,B}$	10.66 ± 0.58^{bA}
T_1	10.33 ± 1.52^{aA}	10.66 ± 0.58^{aA}	12.00 ± 0.0^{aA}	10.66 ± 0.58^{aA}
T_2	$9.66 \pm 0.58^{c,A}$	$11.00 \pm 0.0^{a,bA}$	12.00 ± 0.0^{aA}	$10.33 \pm 0.58^{b,cA}$
T_3	10.00 ± 0.0^{bA}	$11.00 \pm 0.0^{a,bA}$	$11.66 \pm 0.58^{aA,B}$	$10.66 \pm 0.58^{a,bA}$
T_4	$10.33 \pm 0.58^{a,bA}$	9.33 ± 0.58^{bB}	12.00 ± 1.0^{aA}	$10.66 \pm 0.58^{a,bA}$

Lowercase letters are treatments within each level of time and capital letters are time within each level of treatment. Means with different superscripts in the same row are significantly different at $P < 0.05$ (Tukey's test).

TABLE 4. *Phenoloxidase activity (unit/min/mg) in each level of treatments and time of the shrimps fed supplemented diets ethanol extract of* U. tomentosa *and white spot syndrome virus challenged.*

	Time after challenge (h)			
Treatments	0	24	48	72
T_C	$104.70 \pm 12.11^{bA,B}$	51.62 ± 0.210^{cA}	$116.51 \pm 6.77^{bA,B}$	174.57 ± 13.95^{aA}
T_1	$104.60 \pm 21.47^{aA,B}$	77.93 ± 20.27^{aA}	80.16 ± 2.49^{aB}	63.46 ± 13.97^{aC}
T_2	$104.44 \pm 26.29^{a,bA,B}$	68.61 ± 14.87^{bA}	$99.05 \pm 19.93^{bA,B}$	$152.02 \pm 17.59^{aA,B}$
T_3	91.78 ± 12.03^{aB}	91.60 ± 27.57^{aA}	$106.37 \pm 25.58^{aA,B}$	134.73 ± 11.71^{aB}
T_4	148.79 ± 18.60^{aA}	82.64 ± 17.41^{bA}	124.56 ± 3.20^{aA}	125.34 ± 6.35^{aB}

Lowercase letters are treatments within each level of time and capital letters are time within each level of treatment. Means with different superscripts in the same row are significantly different at $P < 0.05$ (Tukey's test).

higher agglutination titer (Table 3), but generally no significant differences were detected between the treatments applied (Table 3). These results showed that the immune system of shrimp fed with EEUT was not able to increase the production capacity and replacement of lectins by hemocytes and hepatopancreas throughout the infection process.

Phenoloxidase Activity

Significant differences were found between T_C, T_1, and T_2 treatments after 24 h (Table 4). PO activity significantly decreased with values ranging from 51.62 (T_C) to 91.60 (T_3) unit/min/mg followed by a significant increase in POA values after 72 h (Table 4). Specifically, at 72 h, POA values of T_C, T_2, T_3, and T_4 were significantly higher than the PO activity value of T_1 (63.46 unit/min/mg, infected shrimps fed with pelleted feed without EEUT). When an increase in PO activity values in shrimp serum is produced, a protective effect is reported. Therefore, shrimp fed with 1, 2, and 4% EEUT were protected against WSSV. This also indicated the ability of the shrimp fed EEUT to maintain proPO and PO at higher levels, maintaining development of the phenoloxidase cascade. Other authors have also detected increased PO activity values after feeding penaeids with a mixture of antiviral and immunomodulatory plant extracts (Medina-Beltrán et al. 2012; Peraza-Gómez et al. 2014).

In Vivo *Determination of Antiviral Activity*

The survival curves for Treatments T_1, T_2, T_3, and T_4 are shown in Figure 2. The Cox semiparametric model pointed out the differences between the treatments applied (Table 5). Treatments T_2, T_3, and T_4 were compared with T_1, or positive treatment, where shrimp were infected with WSSV and were not fed with EEUT. Treatments T_3 ($P = 0.027$) and T_4 ($P = 0.024$) showed higher probability of survival and were significantly different from T_2 ($P = 0.075$). To calculate the relative risk, value 1 was considered as the occurrence of mortality. Values higher than 1 indicated risk factor (with higher probability to die than the positive control

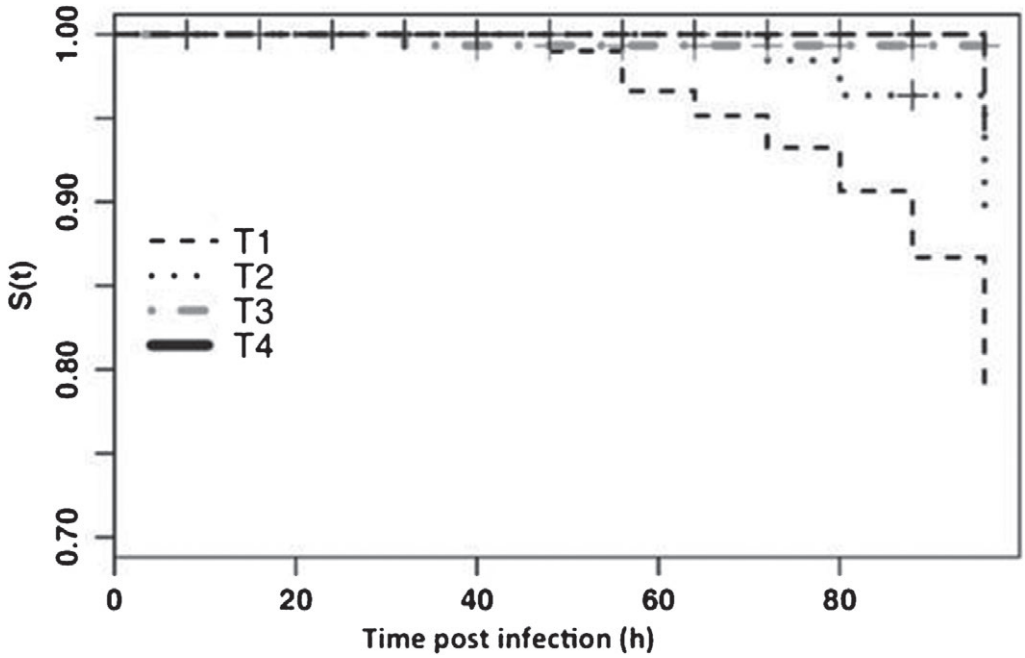

FIGURE 2. *Estimated Kaplan–Meier survival curves of uninfected shrimp fed only with commercial feed (T_C), white spot syndrome virus-infected shrimp fed only with commercial feed (T_1), infected shrimp fed with commercial feed supplemented with 1% ethanol extract of* U. tomentosa *(EEUT) (T_2), infected shrimp fed with commercial feed supplemented with 2% EEUT (T_3), and infected shrimp fed commercial feed supplemented with 4% EEUT (T_4) and challenged by 96 h.*

and values lower than 1 indicated protection factors (less probability to die than the positive control). Thus, for the relative risk of mortality, there were no significant differences between Treatments T_1 and T_2, but significant differences were found when Treatments T_3 and T_4 were compared with Treatment T_1 (Table 5). Therefore, the risk indices indicated that adding EEUT was a protection factor for the shrimp health for T_3 and T_4 treatments. In addition, for T_3 and T_4 treatments a survival rate of 94% was also detected and no clinical signs of viral infection such as decrease in food intake, reddish ends, and apathy. Moreover, for the T_C treatment, there was no mortality or signs of viral infection. In the course of the bioassays, it was observed that the intestine of shrimp was always found full after being fed. Occasionally, there was some small amount of pellets not eaten by the shrimp in the early hours after infection in Treatments T_C, T_3, and T_4. In these treatments the shrimp continued to eat normally until the end of the experiment.

TABLE 5. *Relative risk for* L. vannamei *mortality estimated by the semiparametric Cox model, followed by intervals with 95% CI.*

		CI (95%)	
Covariate	Relative risk	LL	UL
T_C	–	–	–
T_2	0.299	0.079	1.275
T_3	0.096	0.013	0.771
T_4	0.091	0.011	0.730

CI = confidence interval; LL = lower limit; UL = upper limit; – = not applicable.

Conclusions

The EEUT is a source of phenolic acids and flavonoids and may be used to raise healthy penaeid shrimp. Treatments with 2 and 4% of EEUT ended up with the absence of clinical signs of WSSV, and the animals continued to feed normally. The survival probability of these treatments was significantly higher than T_1 (positive control) and T_2 (1% EEUT), showing protection against WSSV symptoms.

Immunological assay also showed a positive POA effect on shrimp fed with EEUT. The results of this work support the importance of using EEUT to protect shrimp against WSSV. Further experiments need to be performed to evaluate the effects of different fractions of EEUT.

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Literature Cited

Aguirre-Guzmán, G., J. G. Sánchez-Martínez, A. I. Campa-Córdova, A. Luna-González, and F. Ascencio. 2009. Penaeid shrimp immune system. Thai Journal of Veterinary Medicine 39:205–215.

Balasubramanian, G., M. Sarathi, C. Venkatesan, J. Thomas, and A. S. Sahul Hameed. 2008a. Studies on the immunomodulatory effect of extract of *Cyanodon dactylon* in shrimp, *Penaeus monodon*, and its efficacy to protect the shrimp from white spot syndrome virus (WSSV). Fish and Shellfish Immunology 25:820–828.

Balasubramanian, G., M. Sarathi, C. Venkatesan, J. Thomas, and A. S. Sahul Hameed. 2008b. Oral administration of antiviral plant extract of *Cynodon dactylon* on a large scale production against white spot syndrome virus (WSSV) in *Penaeus monodon*. Aquaculture 279:2–5.

Barracco, M. A., L. M. Perazzolo, and R. D. Rosa. 2007. Imunologia de crustáceos com ênfase em camarões. Laboratório de Imunologia Aplicada à Aqüicultura, Universidade Federal de Santa Catarina, Florianópolis, Brazil.

Boligon, A. A., A. C. Feltrin, and M. L. Athayde. 2013. Determination of chemical composition, antioxidant and antimicrobial properties of *Guzuma ulmifolia* essential oil. American Journal of Essential Oils and Natural Products 1:23–27.

Calixto, J. B. and R. A. Yunes. 2001. Plantas medicinais sob a ótica da química medicinal moderna. Argos, Chapecó, Brazil.

Cordón-Lagares, E., F. Garcia-Ordaz, and J. J. García del Hoyo. 2016. The determinants that cause small-scale vessels to exit fishing: the case of the Spanish small-scale purse seine fishery. Fisheries Research 181:155–162.

Cushnie, T. P. and A. J. Lamb. 2005. Antimicrobial activity of flavonoids. International Journal of Antimicrobial Agents 26:343–356.

Goel, M. K., P. Khanna, and J. Kishore. 2010. Understanding survival analysis: Kaplan–Meier estimate. International Journal of Ayurveda Research 1:274–278.

Gonçalves, C., T. Dinis, and M. T. Batista. 2005. Antioxidant properties of proanthocyanidins of *Uncaria tomentosa* bark decoction: a mechanism for anti-inflammatory activity. Phytochemistry 66:89–98.

Hameed, A. S., G. Balasubramanian, S. S. Musthaq, and K. Yoganandhan. 2003. Experimental infection of twenty species of Indian marine crabs with white spot syndrome virus (WSSV). Diseases of Aquatic Organisms 57:157–161.

Han, X., T. Shen, and H. Lou. 2007. Dietary polyphenols and their biological significance. International Journal of Molecular Sciences 8:950–988.

Hsieh, T. J., J. C. Wang, C. Y. Hu, C. T. Li, C. M. Kuo, and S. L. Hsieh. 2008. Effects of rutin from *Toona sinensis* on the immune and physiological responses of white shrimp (*Litopenaeus vannamei*) under *Vibrio alginolyticus* challenge. Fish and Shellfish Immunology 25:581–588.

Keplinger, K., G. Laus, M. Wurm, M. P. Dierich, and H. Teppner. 1999. *Uncaria tomentosa* (Willd.) DC. Ethnomedicinal use and new pharmacological, toxicological and botanical results. Journal of Ethnopharmacology 64:23–34.

Koroleff, F. 1976. Determination of nutrients. Pages 117–181 *in* K. Grasshoff, editor. Methods of seawater analysis. Verlag Chemie, Weinhein, Germany.

Krishnaiah, D., R. Sarbatly, and R. Nithyanandam. 2011. A review of the antioxidant potential of medicinal plant species. Food and Bioproducts Processing 89:217–233.

Li, H. B., C. C. Wong, K. W. Cheng, and F. Chen. 2008. Antioxidant properties in vitro and total phenolic contents in methanol extracts from medicinal plants. LWT - Food Science and Technology 41:385–390.

Maggioni, D. S., E. R. Andreatta, and E. M. Hermes. 2004. Evaluation of some hemato-immunological parameters in female shrimp *Litopenaeus vannamei* submitted to unilateral eyestalk ablation in association with a diet supplemented with superdoses of ascorbic acid as a form of immunostimulation. Aquaculture 241:501–515.

Medina-Beltrán, V., A. Luna-González, J. A. Fierro-Coronado, Á. I. Campa-Córdova, V. Peraza-Gómez, M. C. Flores-Miranda, and J. N. Gutiérrez Rivera. 2012. *Echinacea purpurea* and *Uncaria tomentosa* reduce the prevalence of WSSV in whiteleg shrimp (*Litopenaeus vannamei*) cultured under laboratory conditions. Aquaculture 358–359:164–169.

Ndagijimana, A., X. Wang, G. Pan, F. Zhang, H. Feng, and O. Olaleye. 2013. A review on indole alkaloids isolated from *Uncaria rhynchophylla* and their pharmacological studies. Fitoterapia 86:35–47.

Nijveldt, R., E. van Nood, D. E. van Hoorn, P. G. Boelens, K. van Norren, and P. A. van Leeuwen. 2001. Flavonoids: a review of probable mechanism of action and potential applications. American Journal of Clinical Nutrition 74:418–425.

Nogueira, P. 2013. Um enfoque imunológico sobre infecção viral em *Litopenaeus vannamei* coletados de fazendas de cultivo no Nordeste do Brasil. Universidade Federal Do Ceará, Fortaleza, Brazil.

Omale, J. and P. N. Okafor. 2008. Comparative antioxidant capacity, membrane stabilization, polyphenol composition and cytotoxicity of the leaf and stem of *Cissus multistriata*. African Journal of Biotechnology 7:3129–3133.

Peraza-Gómez, V., A. Luna-González, J. M. González-Prieto, A. Fierro-Coronado, and H. A. González-Ocampo. 2014. Protective effect of microbial immunostimulants and antiviral plants against WSSV in *Litopenaeus vannamei* cultured under laboratory conditions. Aquaculture 420–421:160–164.

Pilarski, R., H. Zielinski, D. Ciesiolka, and K. Gulewicz. 2006. Antioxidant activity of ethanolic and aqueous extracts of *Uncaria tomentosa* (Willd). DC. Journal of Ethnopharmacology 124:18–23.

Re, R., N. Pellegrini, Proteggente, A. Pannala, M. Yang, and C. Rice-Evans. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine 26: 1231–1237.

Reis, S. R., L. M. Valente, A. L. Sampaio, A. C. Siani, M. Gandini, E. L. Azeredo, L. D'Avila, J. L. Mazzei, M. Henriques, and C. F. Kubelka. 2008. Immunomodulating and antiviral activities of *Uncaria tomentosa* on human monocytes infected with dengue virus-2. International Immunopharmacology 8:468–476.

Reverter, M., N. Bontemps, D. Lecchini, B. Banaigs, and P. Sasal. 2014. Use of plant extracts in fish aquaculture as an alternative to chemotherapy: current status and future perspectives. Aquaculture 433:50–61.

Sandoval, M., N. N. Okuhama, X. J. Zhang, L. Condezo, J. Lao, F. M. Angeles, R. A. Musah, P. Bobrowski, and M. J. S. Miller. 2002. Anti-inflammatory and antioxidant activities of cat's claw (*Uncaria tomentosa* and *Uncaria guianensis*) are independent of their alkaloid content. Phytomedicine 9:325–337.

Seelinger, G., I. Merfort, U. Wölfle, and C. M. Schempp. 2008. Anti-carcinogenic effects of the flavonoid luteolin. Molecules 13:2628–2651.

Singleton, V. L. and J. A. Rossi. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture 16:144–158.

Söderhäll, K. and L. Häll. 1984. Lipopolysaccharide-induced activation of prophenoloxidase activating system in crayfish haemocyte lysate. Biochimica et Biophysica Acta 797:99–104.

Soobrattee, M. A., V. S. Neergheen, A. Luximon-Ramma, O. I. Aruoma, and T. Bahorun. 2005. Phenolics as potential antioxidant therapeutic agents: mechanism and actions. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 579: 200–213.

Tapas, A. R., D. M. Sakarkar, and R. B. Kakde. 2008. Flavonoids as nutraceuticals: a review. Tropical Journal of Pharmaceutical Research 7:1089–1099.

Therneau, T. 2012. Survival: a package for survival analysis in S. R package version 2.36-12. https://cran.r-project.org/web/packages/survival/index.html

Tomazelli Junior, O., F. Kuhn, P. J. Mendonça Padilha, L. R. Mota Vicente, S. Winckler da Costa, B. Corrêa da Silva, D. Dias Schleder, A. A. Boligon, J. Scapinello, C. Nunes Nesi, J. Dal Magro, and S. De Lamo Castellví. 2017. Effect of *Cynodon dactylon* extract on white spot virus-infected *Litopenaeus vannamei*. Aquaculture International 25:1107–1122.

Zhang, Q., J. J. Zhao, J. Xu, F. Feng, and W. Qu. 2015. Medicinal uses, phytochemistry and pharmacology of the genus *Uncaria*. Journal of Ethnopharmacology 173:48–80.