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Efficacy of a pharmaceutical preparation based on glycyrrhizic acid in a challenge study of white spot syndrome in white shrimp (Litopenaeus vannamei)

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A B S T R A C T

There is a lack of preventive and therapeutic drug-based treatments for the shrimp viral disease known as white spot syndrome (WSSV). Thus a challenge study inducing WSSV in juvenile white shrimp (Litopenaeus vannamei) was established, setting 4 groups: challenged — not treated and unchallenged, untreated control groups and two experimental ones (E1 and E2) both treated with diammonium glycyrrhizic acid, extracted from licorice with added vitamins and oligoelements, and as in-feed medication. Group E1 received diammonium glycyrrhizic acid included in their daily feed, starting 17 days before challenge with WSSV and maintaining the treatment for further 5 days after the end of the trial, which was set on day 18. Group E2 received this medication as group E1 throughout the trial, but starting 1 day before the challenge with WSSV. The group with highest surviving median values was E1, amounting two times the survival median in comparison with the control groups (P = 0.007). Also a statistical difference was found in terms of survival means in favor of group E1 as compared to group E2. Macroscopic and histopathological findings revealed lesions compatible with WSSV and similar mortality in the challenged untreated group. These findings were highly reduced or inexistent in mortality analyzed from groups E1 as well as in the unchallenged — untreated control group and greatly reduced in group E2. Considering the apparent high efficacy observed and that glycyrrhizic acid and mineral and vitamin components added as treatment, and taking as an advantage that this preparation has been regarded as nutraceuticals, it is here proposed that large scale trials should be conducted to evaluate the effects here observed in commercial and larger scale shrimp farms.

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1. Introduction

In spite of difficulties, shrimp production is an important and profitable food producing industry. Even though, in many countries cultured shrimp production has been severely hindered by various viral diseases i.e., white spot syndrome — WSSV (Inouye et al., 1994; Rosenberry, 2001). First signs of this disease, such as sudden reduction in food consumption and red discoloration, are followed by a sharp increase in mortality in shrimp farms over the next 3–10 days, even reaching 100% mortality (Peinado-Guevara and López-Meyer, 2006). First description of WSSV appears to have been from an outbreak in Taiwan in 1992 (Chen, 1995; Chou et al., 1995). This disease seems to have spread world-wide, except perhaps to Australia. It has been suggested that world weather changes have contributed to the dissemination of this disease (Sonnenholzner et al., 2002). As the name of the disease suggests, the main signs of WSSV are 0.5–2.0 mm white spots in the interior part of the shell, appendices, uropods, telson, pereiopods, pleopods and cuticle of the abdominal segments (Takahashi et al., 1994). The color of shrimps becomes pale red and the lymphoid organ becomes turbid (Takahashi et al., 1994), and it has been described as hypertrophic (Vidal et al., 2001). Diseased shrimps become lethargic, and they show erratic swimming and lack of appetite and die during the next three days.

Viusid® (from Catalysis, S.A. de C.V., Mexico) is the proprietary name preparation based on diammonium glycyrrhizic acid, extracted from licorice with added vitamins and oligoelements. It has been claimed that this drug preparation stimulates production of gamma interferon in human beings (Sugawara, 1986). Glycyrrhizic acid possesses antiviral activity in vitro and in vivo interfering with both DNA and RNA replications, hence interfering with replication of a wide range of viruses, including herpes, influenza A and B, hepatitis B, coronavirus, and SARS (Badam, 1994; Chen, 1995; Durand et al., 1997; Lee et al., 2007; Lin, 2003; Pompei et al., 2009). Glycyrrhizic acid has also demonstrated to be capable of impeding virion eclosion from its capsid (Pompei et al., 2009), apparently due to a dose-dependant inhibition
of kinase-P phosphorylation (Chavali et al., 1987). Additionally, it has been shown to interfere with arylamine N-acetyltransferase bacterial activity, hence exhibiting antibacterial effects at least vs Streptococcus spp., Haemophilus spp., and Klebsiella spp. (Krausse et al., 2004; Lo et al., 1996; Tanaka et al., 2001).

There are no biological or chemical effective treatments to treat WSSV. According to Le Moullac et al. (1998), body defense mechanisms in shrimp are greatly based on the number of circulating hemocytes in their hemolymph, and it has been observed better clinical responses to viral diseases in shrimp with high number of hemocytes. Hence, stimulation of their immune system may be a way to increase shrimp body defense mechanisms, particularly before they face the viral challenge. Thus, considering the apparent immune-modulator and antiviral activities of glycyrrhizinic acid, it was set as the aim in this study to assess this preparation for its potential protective effects in a laboratory controlled challenge with WSSV.

2. Material and methods

This study was carried out with a total of 960 juvenile white shrimp (Litopenaeus vannamei) obtained from a farm free of WSSV as confirmed by PCR analysis 5 days prior to the beginning of the trial. The study lasted for 18 days. Shrimps had a mean weight of 5 g at the beginning of the trial and were randomly distributed in four groups with four replicates each. Thus each replicate was carried out with 60 shrimps and the groups were distributed as follows:

1. Control untreated-challenged group (CUCH), fed with drug-free food throughout the trial and challenged with WSSV on day zero.
2. Control untreated-unchallenged group (CUUCH), fed with drug-free food throughout the trial but not challenged and dosed with saline solution.
3. Experimental 1 (E1), fed as E1, but starting 17 days before challenge as above with WSSV and maintaining the treatment for further 5 days after.
4. Experimental 2 (E2), fed standard diet plus Viusid® throughout the trial and starting 1 day before the challenge with WSSV, as group CUCH.

Once groups were formed five shrimps from each group were randomly selected and their hemolymph obtained and measured to set basal values of total hemocyte counts by direct counting with the Neubauer chamber. Then, additional samples from five shrimps per group were obtained for hemocyte count on days 6, 12 and 18.

Shrimps were maintained in 1000 L tanks with continuous flow of brackish water at an approximate rate of 10 L/h. Temperature was kept at 23–25 °C with a thermostat (LED 200 W Dymax), pH was approximately 7.6–7.8 (Aqualytic, Germany), and continuous aeration was provided at 6.79–6.56 mL/min. Animals were fed twice a day with commercial shrimp drug-free pellets (Camaronina Purina®, Sonora, México), having: 35% protein min, 9% fat min, 3/32 in pellets and considering a 3% feed intake per day with respect to the biomass as established by Alday-Sanz (2010). Lack of ecdysis in shrimp was ensured before initiation of this trial.

Glycyrrhizinic acid was incorporated to pelleted shrimp-feed as liquid Viusid® (Catalysis Spain, distributed by Dermaceutical México, S.A. de C.V. Mexico City). To achieve this, 540 mL of the commercial preparation was diluted in 100 L of demineralized water. Then pellets were dressing-sprayed on big trays. Feed was allowed to dry at room temperature for 8 h, stored in paper bags and fed to shrimps.

### Table 1
Experimental distribution of control groups and treatments with glycyrrhizic acid.

| Group | Shrimp tank number | Mean shrimp weight (g) | Mean dose of glycyrrhizic acid per shrimp (dose = 20 μL/g) | Inoculum with WSSV |
|-------|-------------------|------------------------|----------------------------------------------------------|--------------------|
| CUCH  | 1                 | 11.8                   | 236                                                      | Yes                |
|       | 2                 | 8.6                    | 173                                                      | Yes                |
|       | 3                 | 9.6                    | 191                                                      | Yes                |
|       | 4                 | 10.2                   | 204                                                      | Yes                |
| CUUCH | 5                 | 9.3                    | Saline solution                                          | No                 |
|       | 6                 | 8.6                    | Saline solution                                          | No                 |
|       | 7                 | 9.9                    | Saline solution                                          | No                 |
|       | 8                 | 9.1                    | Saline solution                                          | No                 |
| E1    | 13                | 10.8                   | 216                                                      | Yes                |
|       | 14                | 10.3                   | 187                                                      | Yes                |
|       | 15                | 10.0                   | 199                                                      | Yes                |
|       | 16                | 10.5                   | 209                                                      | Yes                |
| E2    | 9                 | 9.4                    | 188                                                      | Yes                |
|       | 10                | 11.5                   | 230                                                      | Yes                |
|       | 11                | 9.6                    | 192                                                      | Yes                |
|       | 12                | 9.9                    | 197                                                      | Yes                |

**CUCH** = control untreated challenged.

**CUUCH** = control untreated unchallenged.

**E1** = Experimental 1.

**E2** = Experimental 2.

**WSSV** = white spot syndrome virus.

![Fig. 1. Estimated probability surviving for each group. Log-rank test $\chi^2 = 12.1; P = 0.007.$](image-url)
2.1. Sampling for histopathology

All dead shrimps were fixed with Davidson’s solution (330 mL ethyl alcohol plus 220 mL formalin, 115 mL glacial acetic acid and 335 mL distilled water). Three milliliters of this solution was injected into the shrimps in five spots: lateral and anterior of hepatopancreas, posterior and anterior to the abdomen, and in the ventral sinus of the shrimp. Then, they were submerged in Davidson’s solution for at least 3 days before processed for histopathological analysis as customary.

2.2. Inoculum

Inoculum was prepared as described by Lo et al. (1996), taking infected tissues from whole shrimp specimens freeze–stored at −80 °C, from an outbreak in Sonora State, México in 2005. Briefly, whole shrimp specimens were homogenized in buffer TN 1 × (20 mM Tris–HCl, 0.4 M NaCl, pH 7.4), at a rate of 0.3 g of tissue per mL of TN buffer, using a Polytron PT 1200 homogenizer (Kinematica®, U.S.A.). Homogenates were first filtered through gauze pads to remove solids and the fluid phase was centrifuged in 1.5 in microcentrifuge (Eppendorf®, 5415, USA) at 13,000 × g during 2 min. The supernatant was removed and filtered again through 0.45 μm HV filters (Durapore®, USA). The infective filtrate was pooled and distributed in 1.5 mL/L aliquots in cryotubes (Corning, USA) with screw topped for preservation in liquid nitrogen, thus maintaining homogeneity of infecting aliquots.

Using WSSV infective filtrate, lethal dose 90% (LD90) had been previously established in our laboratory setting as follows: one aliquot of the infecting material was diluted 1:10 in TN buffer solution and 0.02 mL/g of shrimp was inoculated to shrimp by catheter to obtain a cumulative mortality mean of 20, 60 and 90% by the third day. Titer of the virus specimen was homogenized in buffer TN 1× (20 mM Tris, pH 80 °C, cance−hypertrophied nuclei of the ectodermic cells with heterophilic nuclear inclusion bodies within the nucleus. These lesions comply with white spot syndrome. There were no microscopic lesions related to WSSV in shrimp treated with glycyrrhizic acid as in group E1, or in the untreated-unchallenged group. Group E2 did not present necrotic cells, and the nuclear inclusions were considerably less abundant. The lymphoid organ showed karyorrhexis in some cells and unrelated to WSSV.

2.3. Statistical analysis

Sample size was calculated for log-normal distribution test on medians with at least 0.80 power of the test and 0.05 of significance level the Ho: medians of the treated groups are equal to median of the control group with the study size program1; the resultant is n = 36 but we raise to 40.

The analysis of the data was carried out through the cumulative Kaplan–Meier functions of probability with log-rank analysis (Goel et al., 2010). Results are presented as means and medians for the survivals in each group and the Kaplan–Meier graph of the probability of cumulative survivals.

3. Results

The group with highest surviving median values was E1 (see Fig. 1 and Table 2), amounting two times the survival median in comparison with the CUCH and CUUCH groups (P = 0.007). Also, a statistical difference was found in terms of survival means in favor of group E1 as compared to group E2.

Total basal hemocyte counts had mean values that ranged from 9752 ± 1402 to 11288 ± 1398 ± 1SD with no statistically significant difference among groups. As the trial progressed values in all groups except the CUCH group, showed a steady decay in the mean number of total hemocytes. However, no statistically significant difference among them was detected (see Table 3).

Macroscopic lesions observed in dead shrimps in the glycyrrhizic acid-treated group (E1) were limited to some shrimps with antenna and uropods lightly reddened. Other changes were unnoticed. Group E2 showed reddening of antenna, full gut and some shrimps were flocid, slightly darkened and grooved, as if affected with a very mild form of WSSV. Lesions in shrimp challenged with WSSV and not treated (CUUCH group) were characterized by reddening of antenna, and guts were found empty and most of them grooved, as characteristic of WSSV. For the CUUCH group macroscopic lesions were not specific and unrelated to WSSV.

Histological results from mortality showed that CUCH shrimp had hypertrophied nuclei of the ectodermic cells with heterophilic nuclear inclusions that were also found in antennal gland cells and in the epithelial cells of the stomach. In this latter organ some few cells were clearly necrotic. The lymphoid organ showed karyorrhexis in some cells and basophilic inclusion bodies within the nucleus. These lesions comply with white spot syndrome. There were no microscopic lesions related to WSSV in shrimps treated with glycyrrhizic acid as in group E1, or in the untreated-unchallenged group. Group E2 did not present necrotic cells, and the nuclear inclusions were considerably less abundant.

4. Discussion

The white spot syndrome virus has a wide range of hosts and the non-peneid shrimps can be infected as well, such as crab, river lobsters and freshwater shrimps (Chou et al., 1995). Hence it is therefore difficult to contain it or avoiding outbreaks in most commercial shrimp farms. It is a very virulent pathological agent and causes higher percent mortalities (Sahul Hameed et al., 2001). To date, no full effective preventive measures to keep a farm isolated or useful pharmacological cures have been proposed. In this context, this report is, to the best of our knowledge, the first successful pharmaceutical preparation capable of preventing or at least minimizing mortality caused by WSSV.

Table 2
Survival means and medians with the standard errors and 95% confidence intervals of surviving days in the four groups of study.

| Group       | Mean   | Median   |
|-------------|--------|----------|
|             | Mean estimation | Standard error | Confidence interval 95% | Median estimation | Standard error | Confidence interval 95% |
|             | Lower limit | Upper Limit |                       | Lower limit | Upper Limit |                       |
| CUUCH       | 3.733±     | 0.258     | 3.228                 | 4.239     | 2.000       | NE.                   |
| CUCH        | 2.663±     | 0.168     | 2.333                 | 2.993     | 2.000       | 0.261                 |
| E1          | 2.954±     | 0.078     | 2.800                 | 3.107     | 3.000       | 0.129                 |
| E2          | 4.432±     | 0.103     | 4.230                 | 4.634     | 4.000       | 0.184                 |
| Global      | 3.475±     | 0.079     | 3.320                 | 3.630     | 3.000       | 0.122                 |

NE: non-estimable. a,bDifferent literals denote differences (P = 0.007).

CUUCH = Control untreated challenged.
CUCH = Control untreated unchallenged.
E1 = Experimental 1.
E2 = Experimental 2.

1 Olofsson B. Creostat HB 2001–2007.
The resistance mechanism of shrimps to WSSV and other viruses is incompletely understood. Various studies on how shrimp survives to viral infections state that the individual immune processes play a key role, such as infiltration, phagocytosis and encapsulation of viruses within a given tissue (Durand et al., 1997; Momoyama et al., 1994). In shrimp, the lymphoid organ is an integral part of the circulatory system, and can act as virus-particle filter (Bell and Lightner, 1988). Pazir et al. (2011) describe intra-nuclear bodies in lymphoid organ cells of diseased shrimps (L. vannamei) diagnosed with WSSV in farms of the Persian Gulf. The lesions there described comply closely with the ones observed in this trial, including karyorrhexis in some cells in the lymphoid organ as well as basophilic inclusion bodies within the nucleus. Yet, these formation spheres also occur in at least six different viral infections on penaeid shrimps (Bonami et al., 1992). Hence, this finding is hardly pathognomonic of WSSV.

Results obtained from the total counting of hemocytes did not reveal an immune-linked effect of glycyrrhizic acid in the E1 and E2 groups. Yet, it showed that there is a clear decline in their numbers as the disease progresses. Nevertheless, a comprehensive analysis could reveal further information. Thus, our observations are not conclusive; important variations in the number of hemocytes in various studies have been reported (Kim et al., 1999; Sonnenholzer et al., 2010), and also variation in hemocyte apoptosis has been described in shrimp with resistance to WSSV virus (Granja et al., 2003). Hence, further research is needed to clarify the role of hemocytes in WSSV surviving shrimps.

In conclusion, there appears to be high efficacy to augment survival of shrimps affected with WSSV in experimental groups. Also, glycyrrhizic acid plus mineral and vitamin components in the commercial preparation added as in-feed treatment to shrimps has been regarded as a nutraceutical preparation by Badam, L., 1994. In vitro studies of the effect of glycyrrhizin from the Indian Glycyrrhiza glabra Linn on some RNA and DNA virus. Indian J. Pharm. 26 (3), 194–199.

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Table 3

| Group      | Basal values | Day after challenge |
|------------|--------------|---------------------|
| CUUCH      | 10253 ± 1251 | 6                   |
| CUUCH      | 11284 ± 1398 | 7                   |
| E1         | 9752 ± 1402  | 8                   |
| E2         | 11265 ± 1435 | 9                   |