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Impact of phytocee™, a phytogenic feed additive on survivability of *Litopenaeus vannamei* shrimps under WSSV and salinity stress challenges

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Abstract
Objective: The present study was aimed to evaluate the impact of Phytocee™ (PHY) on survivability of *Litopenaeus vannamei* shrimp challenged with white spot syndrome virus (WSSV) and salinity shock stress (SSS).
Methods: In experiment 1, shrimps were distributed into two groups *viz* AAM500 (AAM, ascorbic acid monophosphate) and AAM500+PHY500, and its impact on survival rate in normal shrimps without any challenge was recorded. In experiment 2, four groups *viz* AAM500, AAM500+PHY1000, AAM250 and AAM250+PHY1000 were subjected to WSSV challenge after 28 days of feeding. In experiment 3, two groups *viz* AAM500 and AAM500+PHY1000 were exposed to SSS after 42 days. The mortality (%) and median lethal time (MLT) were recorded in experiment 2 and 3.
Results: PHY500 produced a better survivability in normal shrimps, and PHY1000 displayed a lesser mortality (%) and higher MLT in shrimps challenged with WSSV and SSS.
Conclusions: This study demonstrated the adaptogenic potential and anti-stress activity of Phytocee ™, hence it could be recommended to use safely in shrimps for better disease resistance and survivability.

Keywords: Phytocee™, *Litopenaeus vannamei*, WSSV challenge, salinity stress, survivability

Introduction
Shrimp is profitable and globally cultivated crustacean species, and presently Pacific whiteleg shrimp (*Litopenaeus vannamei*) is the principal species cultivated in India and across the globe. In India, shrimp farming contributes to the 70% of seafood collected from 1.20-million-hectare of saline water that is spread across the nine maritime states [1]. However, variations in environmental conditions are the potential stressors to the animals, resulting in limited growth, increased vulnerability to infectious diseases and even high mortalities in extreme cases [2, 3]. Though the shrimp culture techniques and field managemental practices have significantly improved in the recent years, concerns of infectious diseases and mortality are still a threat to the industry. It has been a well-known fact that invertebrates possess two internal homeostasis protection systems *viz* innate immunity system and environmental stress responding system, together play a pivotal role in the protection of cells against biotic and abiotic stresses. Shrimps are invertebrates which are extremely sensitive to environmental variations. Furthermore, shrimp as an aquatic habitat are exposed to wide variety of stresses like sudden temperature and salinity shifts, heavy metal toxicity, viral infection and various other pathogenic microorganisms. Shrimps can combat such stress conditions only with the help of their innate immunity [4, 5] as they lack adaptive immunity and ability to produce immunoglobulins [6].
The shrimp-culture industry around the world has faced the problem of epizootics caused by more than 20 viruses [7]. Of these, white spot syndrome virus (WSSV) has caused 100% mortality in many farms with consequent severe economic losses [8], which would happen in 4-7 days in lab and 10-14 days in the ponds [9]. Although WSSV has been extensively studied in the past two decades and several detecting protocols have been well established [10]. The solution for virus treatments is still not available. Till now, good managemental practices have been considered as the only way to prevent viral outbreaks [11]. However, number of plants from both terrestrial and marine origin have been tested against viral diseases to judge their immunostimulant efficacy [12]. In addition, herbal preparations significantly contribute in stress

management, stimulation of appetite, and more importantly stimulation of immune system even at low concentrations [13]. Research from our laboratory have also shown that Phytocee™ has anti-stress, antioxidant and immunomodulatory potential through modulating the hypothalamic-pituitary-adrenal axis in broiler chickens. With this background, the present study was conducted to evaluate the adaptogenic and immunomodulatory effect of phytogenic feed additive, Phytocee™ by determining the survivability rate and median lethal time (MLT) of *Litopenaeus vannamei* shrimp using WSSV challenge and salinity stress challenge models.

Materials and Methods

Test material used

The phytogenic feed additive, Phytocee™ is formulated by Natural Remedies Pvt. Ltd., Bengaluru, India and is composed of *Emblica officinalis*, *Withania somnifera*, and *Ocimum sanctum*. The formulation was standardized to contain a known quantity of polyphenols and gallic acid.

Study design and experimental setup

A total of 610 specific pathogen free *Litopenaeus vannamei* shrimps procured from Shrimp Improvement Systems, Florida, USA, were randomly distributed among the three experiments (Table 1) and reared under standard conditions. However, three days before starting the disease challenge trial (experiment 2), shrimps were transferred from feeding trial facility to disease challenge facility and housed in 10L individual infection units for acclimatization. All the shrimps were fed with respective pelleted feed prepared according to a formula proprietary to IMAQUA. Uneaten food and waste materials were removed daily before feeding. The water quality parameters such as dissolved oxygen (≥4 mg/L), temperature (27±1.0°C), pH (7.80 – 8.50), total ammonia nitrogen (<0.05 mg/L) and nitrites (≤0.8 mg/L) were measured every day and salinity (20 ppt) was measured every 3 days.

Table 1: Study design and experimental setup

Group	Treatment		Shrimp per group (X replicates)	Feeding Period
	AAM (g/ton feed)	Phytocee™ (g/ton feed)		
Experiment 1: Assessment of survivability under normal conditions				
AAM500	500	-	210 (70 X 3)	42 Days
AAM500+PHY500	500	500	210 (70 X 3)	
Experiment 2: Assessment of mortality and median lethal time under WSSV challenge				
AAM500	500	-	30 (10 X 3)	28 Days
AAM500+PHY1000	500	1000	30 (10 X 3)	
AAM250	250	-	30 (10 X 3)	
AAM250+PHY1000	250	1000	30 (10 X 3)	
Mock Control	-	-	10	
Experiment 3: Assessment of mortality and median lethal time under salinity stress challenge				
AAM500	500	-	30 (10 X 3)	42 Days
AAM500+PHY1000	500	1000	30 (10 X 3)	

Experimental procedure

In experiment 1, *Litopenaeus vannamei* shrimps were fed for 42 days with respective experimental diets *viz* control group (AAM500) and treatment group (AAM500+PHY500) without any experimental challenge according to experimental setup in Table 1. After 28 days of feeding, the disease challenged trial (experiment 2) was performed by oral inoculation of shrimp with WSSV [14] Thai-1 strain according to the procedure 'feed inoculation' as described by Van Thuong *et al.* [15]. In order to assess the effect of the experimental products on the shrimp resistance to stress, shrimps were exposed to osmotic stress (salinity shock from 20 to 0 ppt) after 42 days of feeding trial (experiment 3).

Observations

The survival rate was recorded to determine the impact of Phytocee™ on survivability of shrimps under normal condition free from challenges (experiment 1). In experiment 2, the mortality (%) and MLT were observed for all the groups for 7 days post-inoculation. In experiment 3, the number of dead shrimp and time of death were recorded every 20 mins for 6 hours in each group, which was further used to calculate the mortality (%) and MLT (minutes).

Statistical analysis

The data were expressed as mean and standard error of the mean (S.E.M).

Results

Experiment 1: Assessment of survivability under normal conditions

The survival (%) of Phytocee™ supplemented group was comparatively higher when compared to control group containing AAM (500 g/ton). The survival (%) exhibited by treatment groups AAM500 and AAM500+PHY500 was 79.73 ± 2.74 and 87.37 ± 3.85 respectively (Figure 1).

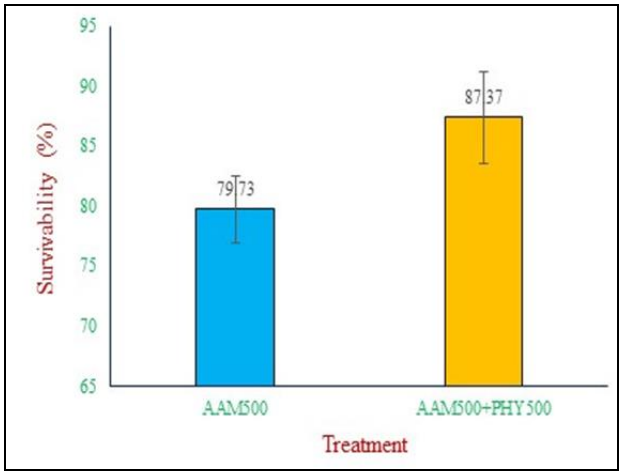

Fig 1: Impact of Phytocee™ on survivability of *Litopenaeus vannamei* shrimp without challenge

Values are expressed as mean ± S.E.M; n=3; AAM, Ascorbic acid monophosphate; PHY, Phytocee™

Experiment 2: Assessment of mortality and MLT under WSSV challenge

As expected, there was no mortality observed in mock control groups. Supplementation of Phytocee™ (1000 g/ton) along with basal diets containing AAM250 and AAM500 reduced the mortality (%) in shrimps challenged with WSSV. Among all groups, AAM500+PHY1000 produced the least mortality (43.33%) which is below 50%, hence MLT was not calculated for this group. AAM250+PHY1000 produced the lesser mortality and higher MLT as compared to the respective control group containing AAM250. The treatment groups AAM500, AAM500+PHY1000, AAM250 and AAM250+PHY1000 exhibited the mortality (%) was 50.00 ± 1.49, 43.33 ± 0.80, 66.67 ± 0.80 and 56.67 ± 1.67, respectively, and the MLT was 144, 84 and 96 hours respectively (Table 2).

Table 2: Effect of Phytocee™ on mortality and MLT of *Litopenaeus vannamei* shrimp challenged with WSSV

Treatment	Mortality (%)	MLT (hpi)
Mock control	00.00 ± 0.00	-
AAM500	50.00 ± 1.49	144
AAM500+PHY1000	43.33 ± 0.80	-
AAM250	66.67 ± 0.80	84
AAM250+PHY1000	56.67 ± 1.67	96
Values are expressed as mean ± S.E.M; n=3; AAM, Ascorbic acid monophosphate; PHY, Phytocee™; MLT, Median lethal time; WSSV, White spot syndrome virus; hpi, Hours post-inoculation		

Experiment 3: Assessment of mortality and MLT under salinity stress challenge

In salinity stress experiment, the mortality (%) and MLT (minutes) were improved in the Phytocee™ supplemented group as compared to basal diet group containing AAM500. The AAM500 and AAM500+PHY1000 groups exhibited the mortality (%) was 66.67 ± 1.67 and 50.00 ± 1.05 respectively and the MLT (minutes) was 260 and 340 respectively (Figure 2 & 3).

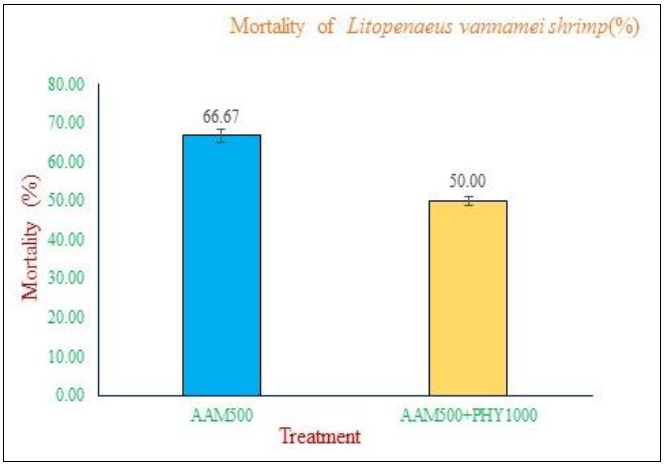

Fig 2: Impact of Phytocee™ on mortality of *Litopenaeus vannamei* shrimp under salinity stress challenge

Values are expressed as mean ± S.E.M; n=3; AAM, Ascorbic acid monophosphate; PHY, Phytocee™

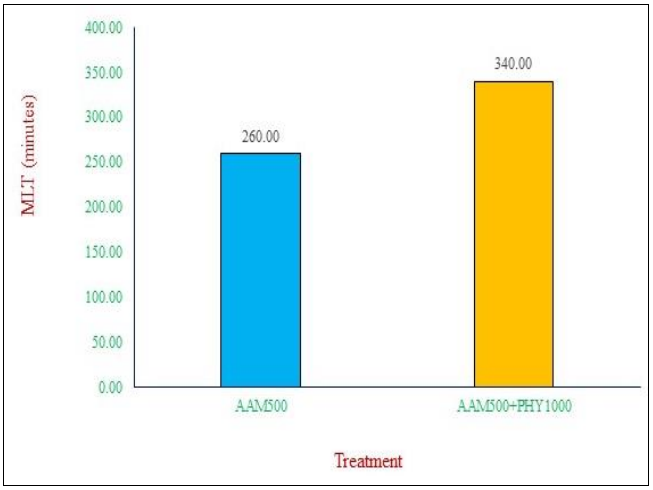

Fig 3: Impact of Phytocee™ on MLT of *Litopenaeus vannamei* shrimp under salinity stress challenge

Values are expressed as mean ± S.E.M; n=3; MLT, Median lethal time; AAM, Ascorbic acid monophosphate; PHY, Phytocee™

Discussion

The defence mechanisms of crustaceans are less developed when compared to finfish and other vertebrates. More specifically, crustaceans have no adaptive memory, hence, they do not have the ability of producing immunoglobulins. Since shrimp lacks an adaptive immune system, the innate immune system and environmental stress responses are the only defensive mechanism to combat infectious diseases and environmental stresses. Adaptogens are the natural immunomodulators that enhances the confrontation ability of aquatic animals against pathogens through augmentation of non-specific defence mechanism, and many literature reported the use of phytobiotics in aquaculture to stimulate the activity of components of immune system [16-18] Agarwal and Singh reported immunomodulating effects of various Indian medicinal plants in mammalian models [19].

The present study was designed and conducted to ascertain the adaptogenic and immunomodulatory effect of a phytogenic feed additive, Phytocee™ by evaluating its impact on survivability under normal conditions, and mortality and MLT in *Litopenaeus vannamei* shrimps exposed to WSSV and salinity stress challenges. In the current study, Phytocee™ when given in basal diet containing AAM500 resulted in better survivability in shrimps reared under conditions free from challenges and the survivability is 7.64% higher than normal control containing AAM500 (87.37% vs 79.73%). Besides, Phytocee™ at 1000 g/ton feed was found to display better protection against WSSV challenge as compared to AAM500 and the mortality observed in AAM500+PHY1000 was 7% lesser than control group. When Phytocee™ supplemented with AAM250, there was about 10% better survival or lesser mortality in comparison with control group fed with basal diet containing AAM250 (mortality 56.67 vs 66.67%). AAM was found to produce a dose-dependent reduction in mortality, AAM500 having produced a better protection against WSSV challenge as compared to AAM250 (mortality 50% vs 66.67%). Among all treatment groups, AAM500+PHY1000 group had shown the least mortality (43.33%) in WSSV challenge study. Similarly, AAM500+PHY1000 had also exhibited a better protection and lesser mortality against salinity stress challenge in

shrimps and the mortality was 16.67% lesser when compared to control group, AAM500 (mortality 50% vs 66.67%). These results indicate the Phytocee™ supplementation complements AAM in augmentation of shrimp immune response and thus providing a better protection against WSSV viral challenge and salinity stress challenge. The better survivability of Phytocee™ supplemented shrimps through better resistance to WSSV challenge and salinity stress challenges observed in the present study was supported by Immanuel *et al.* and Citarasu who reported the supplementation of herbal extracts to post-larvae enhanced the survivability and augmented the production of *Penaeus indicus* [20, 21]. It was also reported that supplementation of mixture of Chinese herbs caused the augmentation of nonspecific immunity in fishes and shrimp [22, 23]. In addition, Yu *et al.* demonstrated the improvement in survival rate of *Litopenaeus vannamei* following supplementation of medicinal herbs added diets [24].

Phytocee™ treated shrimps was found to display a higher MLT both in WSSV and salinity stress challenge models. The shrimps fed Phytocee™ along with AAM250 had shown a better resistance to mortality when subjected to WSSV challenge (MLT 96 hrs vs 84 hrs). AAM500+PHY1000 had shown the best resistance to WSSV challenge among all treatment groups as there was the least mortality (43.33%). In salinity challenge study, Phytocee™ has also produced a better MLT that is 80 minutes more than respective control group (340 minutes vs 260 minutes). These results signify that Phytocee™ supplementation improves the resistance and endurance of shrimps as a result they could face the challenges better and survive for a longer period time. This result agreed with the findings of Divya *et al.* who reported the supplementation of Phytocee™ improved the swimming time by 1.7-fold and reduced the immobility time in rats subjected to chronic variable stress [25], implying its endurance enhancing, anti-stress and adaptogenic potential. Various research studies conducted by Chandrasekaran *et al.*, Joseph *et al.* and Selvam *et al.* also supported the adaptogenic role of Phytocee™ through its immunomodulatory, anti-stress and antioxidant activities exerted by water soluble polyphenolic compounds present in it [26- 28].

W. somnifera, one of the vital ingredients of Phytocee™ is known to enhance the swim endurance, reduce the stress induced cortisol concentration and improve the immunity in animals [29] and increases the survivability duration of shrimp challenged with WSSV [30]. *O. sanctum*, another ingredient in Phytocee™ is observed to boost defence mechanism against various infectious diseases through augmenting the immune responses in nonstressed and immunosuppressed animals [31-33]. Similarly, the phytoconstituents present in the leaves of *O. sanctum* such as water-soluble phenolic compounds, eugenol, methyl eugenol and caryophyllene are known to have potential immunostimulant properties [34]. Oral supplementation of *E. officinalis*, another ingredient in Phytocee™ caused enhancement in the immune system and subsided the microbial infection in the goldfish *Carassius auratus* [35]. Jayathirtha and Mishra reported the supplementation of *E. officinalis* in the diet of Tilapia resulted in the augmentation of specific and nonspecific immune system and provided the protection against *Aeromonas hydrophila* infection [36].

The beneficial effects of Phytocee™ could be attributed to the collective effect of its individual ingredients *viz E. officinalis*, *O. sanctum* and *W. somnifera* which are rich in polyphenols, withanolides and triterpenoids. These phytochemical constituents are earlier proven to be responsible for antistress/adaptogenic effect [37-40]. These results also confirmed that Phytocee™ supplementation improves the resistance and endurance of shrimps through improving the energy status and immunity of shrimps as whole, hence results in better protection and survival as they are better prepared to face challenges.

Conclusion

Phytocee™ has adaptogenic potential through enhancing the endurance and immune response of shrimps and consequently provides better resistance and protection against WSSV and salinity stress challenges resulting in higher survivability and MLT. Besides, Phytocee™ supplementation improves the energy status, endurance and immunity of shrimps and complements AAM in augmenting the immune response for better protection and survival during challenge conditions. Hence, Phytocee™ can be recommended to use safely along with AAM in *Litopenaeus vannamei* shrimp culture for better disease resistance and survivability. However, further investigations are required at molecular level to elucidate the concrete mechanism of action of adaptogenic and immunomodulatory property of Phytocee™ in shrimps.

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