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Effect of marine plant (*Excoecaria agallocha*) extract against luminescence disease causing *Vibrio harveyi* during shrimp larviculture

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ABSTRACT

Crude extract of *Excoecaria agallocha* was tested for antagonism against *Vibrio harveyi* and an inhibitory zone of 9.9 ± 0.4 mm was observed. When *V. harveyi* was grown in Luria Bertani (LB) agar with crude extract of plant, the growth of microorganisms decreased from 2.946 to 1.782 OD in 5 days. In control, *V. harveyi* growth increased from 3.290 to 3.592 OD. When *V. harveyi* was treated with the plant extract, proteolysis, lipolysis, phospholipase and thermonuclease activity and bio-luminescence was weak. Similarly, crude bacteriocin OD values showed a decreasing trend from 1.937 to 1.452 OD as compared to the control from 2.073 to 1.739 OD. The disruption of protease production ranged from 0.010 to 0.121. Exopolysaccharide production of *V. harveyi* decreased from 2.791 to 2.691 OD as compared to the control (3.387 to 3.23 OD). The reduction in the cumulative percentage mortalities in the treatment tank was 4 to 14 % due to the addition of crude extract of *E. agallocha* when compared to the control. The results suggested that the crude extract of *E. agallocha* can be used as a non-antibiotic agent to control shrimp disease caused by *V. harveyi* (**Keywords :** *Excoecaria agallocha, Vibrio harveyi,* bio-luminescence, virulence factors, shrimp larviculture).

INTRODUCTION

Aquaculture industry in India and other parts of the world has been facing serious problems due to the outbreak of diseases caused by *Vibrio spp.* Global estimates of disease losses by the World Bank was approximately US $ 3 billion / year. *Vibrio harveyi* are gram negative, bio-luminescent bacterium, omnipresent in the marine environment and are considered as the cause for a number of diseases in the wild and grow-out aquaculture systems through quorum sensing (Haldar *et al.,* 2011; Bassler *et al.,* 1994). Quorum sensing has been implicated in the control of bacterial behaviours such as secretion of virulence factors, biofilm formation, bioluminescence production, conjugation, sporulation, and swarming motility (Nanting *et al.,* 2009). *V. harveyi* is the causative agent of luminous disease on post larvae of shrimps (80 to 100 % mortality). As control measures, the farmers used antibiotics in a disorganized way, therefore, *Vibrios* have turned more resistant against antibiotics used in aquaculture. Mass mortality was reported among the *Penaeus monodon* post larvae owing to multiple antibiotic resistance of *V. harveyi* (Karunasagar *et al.,* 1994). Therefore, the quest for alternative methods to control infection caused by antibiotic-resistant *Vibrios* becomes imperative for the sustainable development of aquaculture. Hence, in place of antibiotics, alternative bio-inhibitors can be used to control antibiotic resistant marine *Vibrios*. Several mangrove plants have been reported to have potential bioactive substances which could be utilized as bactericide, fungicide and they also contain some chemical compounds of pharmaceutical value (Kathiresan *et al.,* 2013). *Excoecaria agallocha* (vernacular name - Thillai), commonly known as milky mangrove, belong to the plant family *Euphorbiaceae* and contains various bioactive components such as diterpenoids, triterpenoids, flavonoid, phorbol esters, etc. (Nusrat *et al.,* 2008). These plants of tropical and sub - tropical regions grow in the saline intertidal zones of sheltered coast lines. Hence, a study was undertaken to explore the antagonistic property of *E. agallocha* against *V. harveyi* in the postlarvae of shrimp, *P. monodon*.

MATERIALS AND METHODS

Isolation of microorganism

V. harveyi isolated from the water sample collected from Muttukadu Experimental Station (MES) of CIBA at Chennai was identified. The identification of *V. harveyi* was carried out in selective agar (VHSA), various biochemical tests and stored in VHSA slant at 4 °C (Yusminah *et al.,* 2002).

Collection of plant and extract preparation

E. agallocha were collected from Pichavaram mangrove forest, Tamil Nadu, India (Latitude 11º 27' N; Longitude 79 º 47' E), shade dried at room temperature until they were brittle, pulverised with a mechanical grinder and stored in an airtight container. Then, 40 g of plant powder was extracted with 200 mL of ethyl acetate (boiling point 76 - 78 °C) using soxhlet apparatus.

Antimicrobial assay by 'Well diffusion method'

The crude extracts of *E. agallocha* leaf were subjected to antimicrobial assay using the agar well diffusion assay (Olurinola *et al.,* 1996). Luria Bertani (LB) agar medium (100 mL) was prepared, inoculated with 100 µL of *V. harveyi* (1.8 OD), mixed gently and poured into sterile petri dishes. Appropriate number of wells (6 mm each) were made in the solidified agar. A drop of soft agar (0.85 %) was used to seal the base of each well. The wells were filled with extracts of various concentrations of *E. agallocha* (200 - 400 µg) after dissolving in dimethylsulfoxide (DMSO).The inoculated plates were incubated at 28 ºC for 24 h. Zone of inhibition (mm) was measured and tabulated.

Effect of plant extract on the growth of V. harveyi and its virulence factors

In two petri plates with 100 µL of LB broth, one marked as control (without crude extract of plant) and the other marked as treatment (with crude extract of plant) were inoculated with 1mL of *V. harveyi* (1.8 OD). In the treatment plate was added the plant extract (200 µg mL^{-1} of medium) and incubated at 28 °C in a shaker incubator (100 rpm) for 5 days. Growth and luminescence of *V. harveyi* in both the treatment and control was monitored for 5 days by using spectrophotometer (OD at 600 nm) and luminometer (Victor TM - X3, Perkin Elmer, USA). From both the control and treatment were taken 2 mL of broth every day and tested for various virulence factors *viz.,* proteolytic activity (Brock *et al.,* 1982), lipolytic activity (Al-Wali *et al.,* 1998), phospholipase activity (Al-Wali *et al.,* 1998), thermonuclease activity (Manilal *et al.,* 2010), crude extracellular protein (Bacteriocin) (Lavanya and Subhashini, 2013) and exopolysaccharide (EPS) (Bramhachari and Dubey, 2006). The cells obtained from the broth were spot-inoculated onto nutrient agar separately with 10 % skimmed milk for proteolytic test, 2 % tributyrin for lipolytic test, 5 % egg yolk for phospholipase test and 1 % DNA for thermonuclease activity (Manilal *et al.,* 2010). The plates were then incubated at 37 °C for 24 h. The enzymatic activity of the bacteria (control and treatment) was detected by observing the presence of clear zones around the spot. Determination of virulence protease activity from *V. harveyi* treated with crude extract of *E. agallocha* was performed using the azocasein method (Brock *et al.,* 1982). The supernatant obtained from the broth (treatment and control) was tested for protease activity. One unit (U) of protease activity was defined as micromole of substrate converted per minute under standard assay conditions. For estimating the crude extracellular protein (Bacteriocin) (Lavanya and Subhashini, 2013), the cell suspension from LB broth (treatment and control) was centrifuged at 5000 × g for 10 min. The cells were then separated, supernatant was collected and used for the estimation of crude extracellular protein by Lowry's method (Lowry *et al.,* 1951). The cell suspension was obtained from the LB broth and the EPS production pattern was observed at 520 nm (Bramhachari and Dubey, 2006).

Adherence ability of V. harveyi against crude extract of E. agallocha

Salt aggregation test (SAT)

The cells obtained from the broth (treatment and control) were washed with 2.5 % NaCl, suspended in sodium phosphate buffer (pH 6.8) and OD was adjusted to 1.0 at 420 nm. Then, 50 µL of this suspension was mixed with equal volume of various molarities of ammonium sulphate (0.05 to 4.0 mol L^{-1}) in a 96 welled plate, kept at room temperature and observed for agglutination. The SAT value was recorded as the lowest molarity of ammonium sulfate that resulted in

visible bacterial aggregation on evaluation of bacterial cell surface hydrophobicity. Strains with SAT values > 4.0 mol L^{-1}, 2.0 - 4.0 mol L^{-1}, 1.0 - 2.0 mol L^{-1} and 0.0 - 1.0 mol L^{-1} were designated as no, low, moderate and high hydrophobicity, respectively (Guo *et al.,* 2012).

Bacterial adhesion to hydrocarbons (BATH) assay

The broth culture was washed and suspended in 50 mM sodium phosphate buffer (pH 7.4) by adjusting the OD to 0.4 at 660 nm (A_0). The suspension was treated with xylene (2.5:1 v/v), mixed and kept at room temperature for 20 min. The OD of the aqueous phase was read at 660 nm (A_1). The percentage of bacteria adhering to hydrocarbon (A) was calculated using the following formula:

$$A = \frac{A_0 - A_1}{A_0} \times 100$$

Cells with % adhesion ≥ 70 %, 50 - 70 % and < 50 % were arbitrarily classified as high, moderate and low hydrophobicity, respectively (Guo *et al.,* 2012).

Treatment of E. agallocha against V. harveyi in shrimp larviculture

Plastic tanks (30 L) were washed with 1 % $KMnO_4$ solution, filled with 10 L of 18 ± 1 PSU (Practical Salinity Unit) seawater and covered. Disease free postlarvae (PL 15 days) of *P. monodon* procured from commercial shrimp hatchery were acclimatized in 18 % seawater for 3 days at 28 ± 1°C with continuous aeration prior to the experiment. In each experimental tanks were stocked 450 postlarvae (PL) with an average body weight of 18 - 19 mg. Control tank was inoculated with 10 mL of *V. harveyi* (1.8 OD) and the treatment tank was inoculated with 10 mL of *V. harveyi* (1.8 OD) along with crude extract of *E. agallocha* (200 µg mL^{-1}). The experiment was conducted for 30 days without exchanging water and the PL were fed twice a day. The dead PL were collected and the cumulative percentage mortality was ascertained from the tanks. Water quality parameters like salinity, temperature and pH were measured using salinometer, thermometer and pH meter, respectively (Kannappan *et al.,* 2012). Enumerations of total heterotrophic bacteria (THB) and *V. harveyi* count were carried out in both the treatment and control every five days for 30 days in trypticase soya agar (TSA) and *V. harveyi* selective agar medium (Harris *et al.,* 1996).

Statistical analysis

All the experimental values were average of three replications expressed with SD. Cumulative percentage mortality (CPM) was calculated by the formula (Kannappan *et al.,* 2012) :

$$\text{Cumulative percentage} = \frac{\text{Cumulative frequency}}{\text{Total number of observations (n)}} \times 100$$

RESULTS AND DISCUSSION

The crude marine plant (*E. agallocha*) extract showed anti-bacterial activity against *V. harveyi* with an inhibitory zone of 9.9 ± 0.4 mm (excluding the well), whereas no inhibition was observed using DMSO (80 %) as control against *V. harveyi*. Further, various concentrations of *E. agallocha* extract demonstrated anti-bacterial activity (Table 1). The minimum inhibitory concentration (MIC) of crude extract of plant was 200 µ g of 1mg mL^{-1} of *E. agallocha* extract against 100 µ L of 1 × 10^8 cfu mL^{-1} of *V. harveyi* by 'well diffusion assay'. The crude extract treatments inhibited the growth of *V. harveyi* in LB broth under aerobic conditions. The growth of *V. harveyi* varied from 2.946 to 1.782 OD and 3.290 to 3.592 OD from 1st to 5th day in the treatment (with crude extract) and in the control, respectively (Figure 1a) and the difference was 1.81 OD. In case of treatment, bio-luminescence has been very low to moderate, but in case of control the

Table 1. Antimicrobial activity of *E. agallocha* (crude extract) against *V. harveyi*

Antibiogram	Concentration (µg)	Zone of Inhibition (mm)
Crude extract of plant	200	3.9 ± 0.3
	250	4.5 ± 0.2
	300	6.1 ± 0.2
	350	8.1 ± 0.3
	400	9.9 ± 0.4
Positive control (Tetracycline)	100	21 ± 0.1
Negative control (80 % DMSO)	50 (µL)	ND

Values are mean ± SD for three observation; ND - not detected; DMSO - Dimethyl sulfoxide

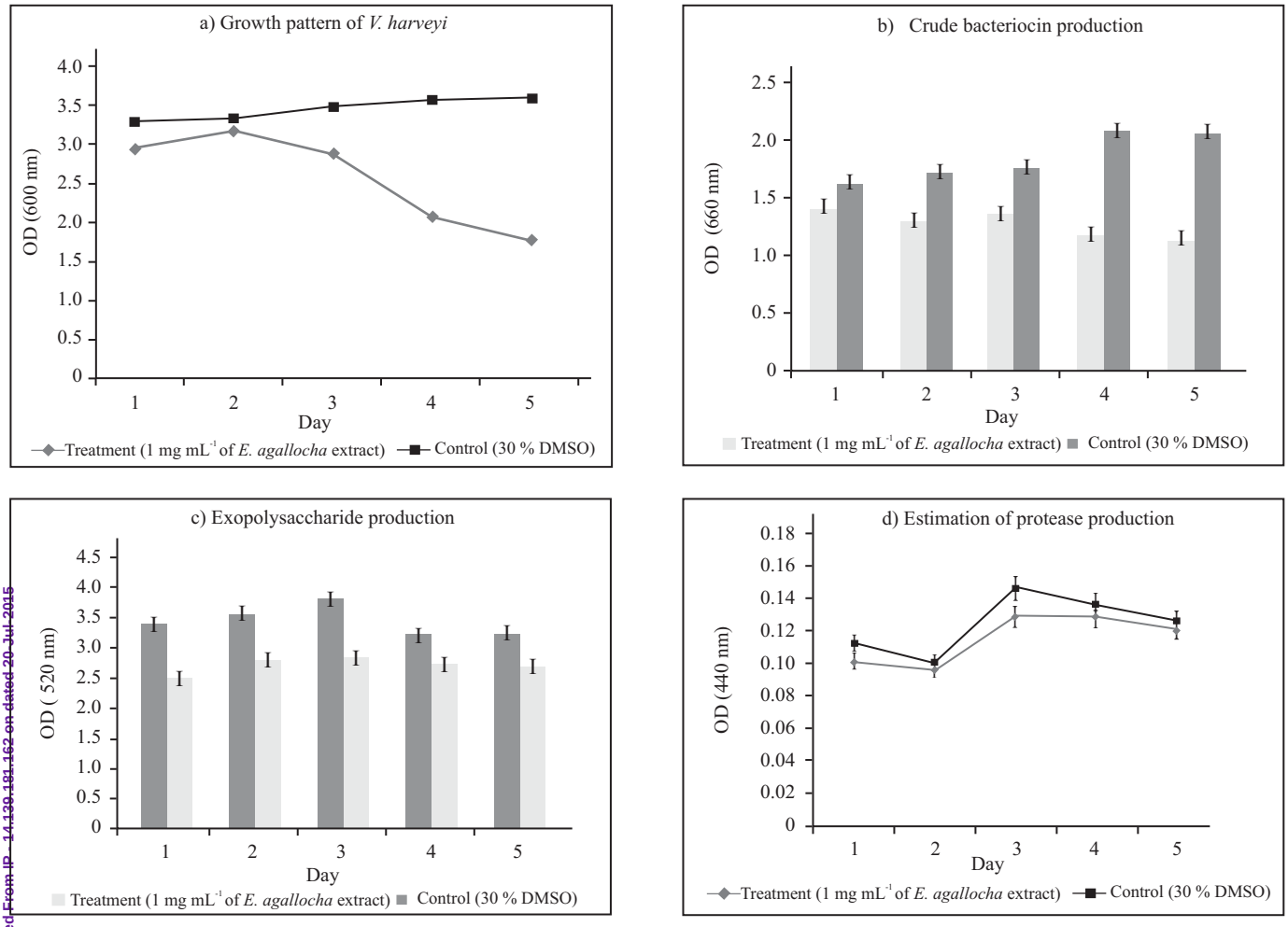

Figure 1. Effects of *E. agallocha* (crude extract 1 mg mL^{-1}) against the growth and virulence factors of *V. harveyi* (a - Growth pattern of *V. harveyi*; b - Crude extracellular protein (Bacteriocin) in *V. harveyi*; c - Exopolysaccharide production in *V. harveyi*; d - Protease production in *V. harveyi*)

luminescence was very strong. According to Liu *et al.* (1996) and Natrah *et al.* (2011), key virulence factors such as bio-luminescence, proteolysis, lipolysis and hemolysis might play a leading role in the pathogenicity of *V. harveyi* in *P. monodon* and *P. japonicus*. When *V. harveyi* was treated with plant extract, proteolysis, lipolysis, phospholipase and thermonuclease activity was observed to be weak, however, in the control, activities were very strong (Table 2), which further confirmed that the bioactive component present in the marine plant *E. agallocha* inhibited the virulence factors of *V. harveyi* and reduced its pathogenicity against shrimp larvae.

The OD values of crude bacteriocin in the treatment varied from 1.937 to 1.452 when compared to that of the control (2.073 to 1.739 OD) (Figure 1b). Disruption of protease production was observed in *V. harveyi* treated with *E. agllocha* extract (Figure 1c). The OD values of exopolysaccharide production of *V. harveyi* in the treatment (with plant extract) decreased (2.791 to 2.691) as compared to the control values (3.387 to 3.23 OD) (Figure 1d). According to Montgomery and Kirchman (1994) bacterial adherence to the external surfaces and tissues of a host is an essential initial step in the infection of a host and subsequent occurrence of disease. It is widely accepted that hydrophobicity and biofilm production are major factors in the adhesive process and survival of pathogens in cells (Zoueki *et al.,* 2010). Hydrophobic microorganisms are capable of adhering to the oil/water

Table 2. Effect of *E. agallocha* (crude extract) against *V. harveyi* and its virulence factors

Observation	Luminescence of *V. harveyi*		Proteolytic activity		Phospholipase activity		Lipolytic activity		Thermonuclease activity	
Day	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control
1	+	+++	+	+++	+	++++	++	++++	+	++++
2	+	+++	+	+++	+	++++	++	++++	++	++++
3	+	+++	+	+++	++	++++	++	++++	++	++++
4	+	++++	+	+++	++	++++	++	++++	++	++++
5	+	++++	++	++++	++	++++	++	++++	++	++++

Control - *V. harveyi* with 30 % of DMSO; Treatment - *V. harveyi* treated with crude extract of *E. agallocha*
Activity of *V. harveyi* (+ = low or weak; ++ = moderate; +++ = high; ++++ = very high)

interface and utilizing oil components as a source of energy for growth and metabolism (Marshall, 1991). In the present study, the SAT and BATH values of the control revealed the *V. harveyi* cells to be more hydrophobic in nature. However, the SAT values of 1.25 - 2 mol L^{-1} in the treatment demonstrated the potential of the crude extract of *E. agallocha* to alter the high hydrophobic nature of *V. harveyi* cells to moderately hydrophobic (Table 3). Possibly, more extract of plant would have changed the bacteria to weakly hydrophobic.

The BATH values of the treatment in all the five days were 58.76 to 26.82 % as against the control values of 72 to 70 % (Table 3). These results revealed the moderate hydrophobicity of *V. harveyi* cells treated with the plant extract. The control group showed more than 70 % cell adhesion due to its hydrophobic nature, whereas, in the treatment, the *V. harveyi* cells demonstrated a cell adhesion of 28.82 %. Results of the adherence tests reflected the ability of *E. agallocha* extract to reduce the highly hydrophobic nature of *V. harveyi*.

Table 3. Adherence ability of *V. harveyi* against crude extract of *E. agallocha*

Observation	Salt aggregation test (SAT) (mol L^{-1})		Bacterial adhesion test (BATH) (%)	
Day	Treatment	Control	Treatment	Control
1	1.25	2	58.76	72.14
2	2	1.5	29.42	67.12
3	1	3	24.71	74.49
4	1.7	2	26.82	43.66
5	2	1.5	59.07	70

SAT values > 4.0 mol L^{-1} - no hydrophobicity; 2.0 - 4.0 mol L^{-1} - low hydrophobicity; 1.0 - 2.0 mol L^{-1} - moderate hydrophobicity; 0.0 - 1.0 mol L^{-1} - high hydrophobicity

BATH values ≥ 70 % - high hydrophobicity; 50 - 70 % - moderate hydrophobicity; < 50 % - low hydrophobicity

During the challenge study, the water quality parameters such as salinity, pH, and temperature were kept constant without much variation between the control and treatment tanks (Table 4). The initial load of *V. harveyi* used in both treatment and control tanks was 5.5×10^5 CFU mL^{-1}. The present work demonstrated the promising results of survival enhancement in *P. monodon* larviculture treated with *E. agallocha* extract which showed 60.4 % reduction in the cumulative percentage of mortality, whereas, it increased in the control up to 74.4 %. The difference in the cumulative percentage mortality between the treatment and control was 14 % (Table 4). The growth of the *V. harveyi* reduced to 9.9×10^2 CFU mL^{-1} in the treatment tank during the 30th day as compared to its respective control (3.5×10^3 CFU mL^{-1}). The difference in the growth reduction in the treatment tank was 10^5 to 10^2 CFU mL^{-1}, whereas in the control it reduced from 10^5 to 10^3 CFU mL^{-1}. Total heterotrophic bacteria (THB) load decreased from 4.3×10^5 CFU mL^{-1} to 9.6×10^2 CFU mL^{-1} in the 30th day but in the control, it decreased from 6.9×10^4 CFU mL^{-1} to 1.2×10^3 CFU mL^{-1}. These results revealed that a concentration of 2 g of plant extract used was able to antagonize 10^5 to 10^2 CFU mL^{-1} of *V. harveyi* and THB in the treatment tank. Suryati and Hala (2002) also reported that the crude extract of *E. agallocha* also protected the shrimp from other pathogens such as *V. alginoliticus, V. costicola, V. leiognathi, V. metschnikovii, V. mimicus, V. splindidus, V. tubiashi.* The results suggested that the crude extract

Table 4. Cumulative percentage mortality pattern of shrimp postlarvae against *V. harveyi* treated with *E. agallocha* extract

Days of Larvi-culture	Cumulative percentage mortality		Treatment tank		Control tanks		Average weight of postlarvae (mg)		Water quality parameters for treatment and control tubs			
	Treatment tank with *V. harveyi*	Control tank with *V. harveyi*	THB Count (CFU mL^{-1})	*V. harveyi* count (CFU mL^{-1})	THB Count (CFU mL^{-1})	*V. harveyi* (CFU mL^{-1})	Treatment	Control	Temperature (°C)	Salinity (PSU)	pH in control tubs	pH in treatment tubs
1st	Nil	Nil	4.3×10^{5}	5.5×10^{5}	6.9×10^{4}	5.5×10^{5}	18.5±2	17.7±3	28±1.0	20±0.5	8.30±0.2	8.30±0.2
5th	Nil	4	3.8×10^{4}	1.6×10^{4}	2×10^{4}	6.2×10^{4}	64.5±5	58.8±9	30±1.0	20±0.5	8.40±0.2	8.40±0.2
10th	9.2±0.3	12±0.5	5.9×10^{3}	8.9×10^{3}	8×10^{3}	5.7×10^{3}	130.3±8	127.3±7	31±1.0	20±0.5	8.20±0.2	8.30±0.2
15th	18.6±0.5	21±0.8	4.5×10^{3}	5.7×10^{3}	4×10^{3}	3.0×10^{3}	165.6±10	162.8±13	29±1.0	20±0.5	8.30±0.2	8.40±0.2
20th	34.0±0.8	46 ±1.2	2.2×10^{3}	1.2×10^{3}	1.5×10^{3}	1.7×10^{3}	213.9±11	205.9±12	30±1.0	21±0.5	8.20±0.2	8.30±0.2
25th	46.5±0.4	58±1.1	1.0×10^{3}	1.1×10^{3}	1.3×10^{3}	2.0×10^{3}	259.3±14	254.2±16	28±1.0	21±0.5	8.10 ±0.2	8.20±0.2
30th	60.4±0.9	74.4±1.3	9.6×10^{2}	9.9×10^{2}	1.2×10^{3}	3.5×10^{3}	298.6±23	286.9±21	28±1.0	21±0.5	8.30±0.2	8.30±0.2

PSU - Practical Salinity Unit; THB - Total Heterotrophic Bacteria
Values are average of three determinations with standard deviation

from *E. agallocha* can be used as a non-antibiotic agent to control shrimp disease caused by *V. harveyi.*

CONCLUSION

In aquaculture (shrimp and fish farming), inappropriate administration of synthetic antimicrobial agents to control *V. harveyi* may lead to development of resistance among the bacteria. Synthetic antibacterial agents have not been commonly accepted as aquaculture therapeutics agents. Therefore, it can be concluded that the bioactive compounds present in the marine plant *E. agallocha* to be a better alternative as an antibacterial agent against disease-causing *V. harveyi* in shrimp farming. This alternative bio-agent does not cause any microbial resistance or impart any residual effect to the aquaculture system. Furthermore, the bio active components derived from the marine plant, *E. agallocha* were much economical than antibiotics.

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