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Potential of marine macroalgae against halomonas species isolated from the epithelial surface of infected adult *Hippocampus kuda* (yellow seahorse)

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Abstract

An impediment to successful rearing of seahorses *Hippocampus kuda* (yellow seahorse) are microorganisms that serve as causative agent of diseases leading to high mortality and low survival rates in tanks. Antibiotics are used to treat such diseases but improper usage of antibiotics may promote resistant pathogens. The aim of this study was to isolate and identify bacteria species from infected seahorses with signs of ulcerative dermatitis through DNA barcoding via 16s rDNA bidirectional gene sequencing and determine marine algae with potential antibacterial activity in order to find alternate source for treatment. Results show three (3) strains of Halomonas species based on morphological characterization and DNA barcoding. Ethanol extraction was used to produce varying concentrations of algal extracts and were tested against the Halomonas species using Kirby-Bauer disc diffusion method. The zones of inhibition exhibited by the three different extracts against strains of Halomonas were not comparable to broad spectrum commercialized antibiotic Tetracycline (positive control) but showed great potential if percent concentration of extracts were to be increased. Thus, marine algae used in this study: *Ulva intestinalis* and *Sargassum crassifolium* can be a potential natural source of antibacterial compounds against pathogenic micoorganisms that pose a threat to seahorse aquaculture in general.

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Introduction

According to the International Union for Conservation of Nature (IUCN) many species of seahorses are vulnerable and threatened (Lin *et al*., 2007). The high demand of seahorses for traditional Chinese medicine, excessive "bycatch" of seahorses, habitat loss, and pollution to the sea (Cartagena, 2014) greatly contribute to rapid depletion of this species. Due to the huge gap between the supply and demand of the seahorses and its high price in the international market, it stressed the need for an increase and improvement for rearing efforts for the seahorses (Bruckner *et al*., 2005).

Rearing these species are quite difficult because they are very sensitive to certain temperature changes (Sheng *et al*., 2006) and very susceptible to marine pathogens that could lead to diseases and even death of the seahorses (Raj *et al*., 2010; Balcazar, 2010). The limited knowledge on rearing these species could possibly result to low survival rate of cultured seahorses and thereby affecting the supply in the market.

Since *Hippocampus kuda* is one of the most commonly traded species in Southeast Asian region, attempts were made in mass rearing for this particular species. But, due to its high susceptibility to marine pathogens like *Vibrionaceae, Pseudomonadaceae, Moraxellaceae, Alcaligenaceae, Enterococcaceae, and Bacillaceae* this suggests that bacterial infections are the main problem in mass producing *Hippocampus kuda,* these marine pathogens are very common in aquatic animals. Also, there are numerous studies such as *Vibrio* showing causing diseases in seahorses having signs of white patches, lethargy swim, pale tail and skin (Binh *et al*., 2016), herewith, outbreaks of diseases in the tanks could lead to the death of all the seahorses in the tank. The pathogens are introduced if untreated seawater is introduced to the tank (Regunathan and Wesley, 2004). Most of the time bacterial pathogens are the culprit for diseases in rearing tanks, the identification of bacterial species and quest for safe and alternative treatment remained a predicament thus, this study.

Literature show Vibrio bacteria commonly isolated from aquatic organisms. Among Vibrio species that are pathogens on marine fish species include *Vibrio anguillarum, V. ordalii, V. harveyi, V. splendida, V. orientalis, V. fischeri.* Studies reported Vibrio causing diseases in seahorses, the first signs were anorexia, lethargy swim, pale tail and fin, white patch, deep skin ulcers (Vincent and Clifton-Hadley, 1989; Alcaide *et al.,* 2001). However, it still remained obscure if Vibrio species are the only bacteria present that are potentially pathogenic behind the mortality of adult seahorses. Hence, part of this study was to identify isolated bacteria species from infected seahorses with symptoms of ulcerative dermatitis through DNA barcoding via 16s rDNA bidirectional gene sequencing.

Moreover, using commercial antibiotics for treating the seawater and for treating diseases in tanks may lead to many side effects (Vatsos and Rebours, 2015); including toxicity to the reared specimens, release of the residues of the antibiotics into the environment, and could cause resistance of bacteria species, marine pathogens. These can bring risk to other animals and also humans (Cavallo, 2013). This leads to the increasing demand of finding alternate antimicrobial substances that can be very effective in controlling diseases and also have minimal side effects. Due to the growing incidences of drug-resistant pathogens, attention has been drawn to the antimicrobial property of plants and their metabolites (Ncube *et al*, 2007).

In this respect, marine plants offer potential source of antibiotics. Marine algae or seaweeds received a lot of attention lately and are considered as a great source material for unique natural products with pharmacological and biological activities (El Shafay *et al*., 2016). Studies have shown that seaweeds are source of unique and different array of secondary metabolites and considered as potential source of marine medicinal and antimicrobials (Manilal *et al*., 2009; Manivannan, 2011). The secondary metabolites

in algae have made them the primary material for antimicrobial medicine. Secondary metabolites from green, brown, and red algae could be a potential bioactive compound that could inhibit bacteria (Smit, 2004). Common available green and brown marine algae are *Sargassum crassifolium and Ulva intestinalis* respectively.

In addition, the antibacterial activity of seaweeds is generally assayed using extracts in various organic solvents, like acetone, methanol–toluene, ether, ethanol, and chloroform-methanol. Several extractable compounds, such as cyclic polysulfides and halogenated compounds, are toxic to microorganisms and therefore responsible for the antibiotic activity of some seaweeds. However, an antibiotic assay of extracts in organic solvents probably does not reflect adequately the antibacterial activity of marine algae under natural conditions. In earlier investigations, phenolics were found to be released into seawater from fucoid thalli, and these served as antifouling substances (Liao *et. al.*, 2003).

Nowadays, marine algae researchers have been working toward the development of novel techniques that are efficient in terms of yield, time, cost, and environment friendly. There are different methods that can be used to obtain these compounds. Initially, a suitable extraction technique should be selected.

For extraction, various mechanical, chemical, biological methods can be used. A key issue is the selection of the appropriate solvent for the extraction. The type of solvent (regarding its polarity) will lead to the extraction of different groups of compounds, which will have various final use (Michalak and Chojnacka, 2015). In this study, ethanol was the solvent used for extracting secondary metabolites from marine macroalgae.

The product of using ethanol as the solvent for extraction contains high hydrophilic compounds which comprise very polar neutral, acidic, and basic compounds, sugar, amino acids, nucleotides, and as well as polysaccharides (Yahya *et al.*, 2018).

Herewith, this study isolated and identified bacteria species from infected seahorses with symptoms of ulcerative dermatitis through DNA barcoding via 16s rDNA bidirectional gene sequencing and at the same time determine potential antibacterial activity of common marine algae *Sargassum crassifolium and Ulva intestinalis* in order to find alternate source for treatment of infected seahorses. Important findings may serve as baseline data necessary for successful aquaculture and conservation strategies.

Materials and methods

Sampling area and sample specimens

Seahorse samples were readily available in the laboratory at Premier Research Institute of Science and Technology (PRISM) and were donated as live bycatch adult samples from fishermen, placed on rearing tanks for months and were infected by bacteria showing signs of ulcerative dermatitis that ultimately led to mortality. Hence, such opportunity was taken advantage to isolate and identify bacteria species from infected seahorses through DNA barcoding via 16s rDNA bidirectional gene sequencing and at the same time determine potential antibacterial activity of common marine algae. The seahorses were reported to come from Tubod, Lanao del Norte, Philippines. Identification of samples was done through illustrated keys, Guide to the identification of Seahorses (Lourie *et al.*, 2004) and consultation of experts.

Isolation and purification of bacterial isolates

A sterile cotton swab was used to collect bacteria from the epithelial surface of the infected seahorse (Fig. 1). The cotton swab was dipped into the vials with marine broth which was prepared aseptically prior to the experiment. The vials were incubated at room temperature for 24 hours. After the incubation period, the vials were stored in the refrigerator for streak plate method in order to have pure isolates from the bacterial culture. After the incubation, serial dilution was done to achieve 0.5 McFarland Turbidity Standard, roughly $1.5x10^8$ cells/ml. This was done in order to avoid too numerous count of colonies or too few to count number of colonies. Spread plate method

using a sterile cotton swab was used on Marine Agar to obtain isolated colonies. The plates were then incubated for 24 hours at room temperature. Isolated and distinct bacterial colonies were picked and were inoculated in a new agar plate with Marine Agar as its medium for the four-quadrant streak method to obtain pure cultures. Cultures were then maintained for morphological tests.

Morphological cell characterization and colonies

Colonies were examined under the stereomicroscope. Colony morphology characterization was based on form, margin, color, elevation and surface of the whole colony. The colony shape includes circular, punctiform, irregular, filamentous, and rhizoid. The bacterial margin includes entire, undulate, filamentous, umbinate, erose and curled. For the colonies' elevation are flat, raised, convex, pulvinate, and umbinate. Surface colonies can be classified as concentric, smooth, dull, and shiny (Fig. 2). Descriptions were compared to Bergey's Manual of Determinative Bacteriology (usually named Bergey's Manual) that portrays most of bacterial species (Garritty *et al*., 2004).

For the cell characterization, gram stain technique or gram staining, (Gram's method), was done to distinguish and classify bacterial species into Gram-positive or Gram-negative bacteria by applying a primary stain (crystal violet) to a heat-fixed smear of a bacterial culture from the agar slant to the microscopic slide followed by the addition of iodide, which binds to crystal violet and traps it in the cell followed by rapid decolorization with ethanol or acetone and lastly counterstaining with safranin.

DNA extraction and barcoding

Three distinct colonies were identified based on the morphological characterization. These were then sub-cultured in fresh marine broth, incubated overnight in preparation for DNA extraction. Bacterial DNA was extracted using Quick Bacteria Genomic DNA Extraction Kit (Dongsheng BIOTECH) and following the manufacturer's instructions. After which, the extracted genomic DNA were sent to Macrogen, Inc. Laboratory in Korea for DNA barcoding using bidirectional 16s rDNA gene sequencing. PCR amplification was done using the paired primers 27F (5'-AGATTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Deobagkar *et. al*, 2012). PCR amplification was carried out within the standard PCR conditions: Pre-denaturation of 94°C for 1 min; followed by 30 cycles of denaturation at 94°C for 30 sec; Annealing at 55°C for 30 sec; Extension at 72°C at 1 min.; Termination of 72°C for 10 min. and cooling temperature at 4°C. Obtained 16s gene sequences were aligned by Sequencer 4.1.4 and results were compared with GenBank-NCBI reference sequences using BLAST. (www.ncbi.nlm.nih.gov/blast/).

Sampling and collection of seaweeds

Seaweed, *Ulva intestinalis* was handpicked from the sandy bottom in intertidal zone at Tubigan, Initao, Misamis Oriental, Philippines and the seaweed S*argassum crassifolium* was collected by scuba diving and by handpicking from the upper intertidal zone which was exposed to strong water movement at Brgy. Buru-un, Iligan City, Philippines (Fig. 3).

Algae samples were cleaned of epiphytes and other marine organisms by using fresh water and necrotic parts were removed. The samples were further rinsed with distilled water to remove any associated debris. The freshly cleaned seaweeds were air-dried for 7 days in order to have a moisture content of at least 12-20% and the samples were grinded.

$$\text{Moisture Content (\%)} = \frac{W2 - W3}{W2 - W1} \times 100$$

where,

W1= weight of container

W2= weight of container + sample before drying

W3= weight of container with sample after drying

A total of 196.77 grams was obtained for the *Sargassum crassifolium* which had a moisture content of 19.44%; *Ulva intestinalis* weighed 199.36 grams with 12.6% moisture. The grinded seaweeds were soaked in 500 mL of ethanol for 72 hours. Extraction was done using a soxhlet apparatus. The

extraction solvent was evaporated at controlled temperature.

Phytochemical screening

Suitable amount of sample was extracted using 80% ethanol. The extracts were evaporated to incipient dryness respectively and tested for the presence of secondary metabolites such as flavonoids, tannins, saponins, phenolics, steroids, alkaloids and anthraquinones. These were carried out according to the common phytochemical methods described by Baleta *et al.*, 2017.

Preparation of Different Concentration of Algal Extracts

The percent concentration of two seaweed extracts was calculated as follows: 19.6% for *Sargassum crassifolium* and 9.6% for *Ulva intestinalis*. The 19.6% concentration was set to be the pure ethanolic extract of *S. crassifolium* and 9.6% concentration was set to be the pure ethanolic extract of *U. intestinalis*.

Subsequent dilutions were made from the pure extract to obtain the different percentage dilutions. Three different concentrations of the ethanolic extracts of *U. intestinalis* and *S. crassifolium* were prepared: 25%, 50%, and 100%. Twenty-five percent concentration was prepared by mixing 250 microliters of the seaweed extract and750 microliters of distilled water. For the 50% concentration, 500 microliters of the seaweed extract was diluted by 500 microliters of distilled water.

Preparation of test organisms

The bacterial isolates from the epithelial surface of the seahorse, *Hippocampus kuda* after identification were subcultured and served as the test organisms. There were grown on an appropriate media, Marine Agar and were incubated overnight at room temperature. Pure cultures were further seeded in a lawn in Mueller-Hinton agar plates for the disk diffusion assay.

Kirby-Bauer disk diffusion test

Kirby-Bauer disk diffusion test was done where, bacterial suspension of the three (3) isolated strains of bacteria from the epithelial surface of infected seahorse served as test microorganisms. The bacterial suspension was set to 0.5 McFarland standard and Mueller-Hinton agar plates were seeded with a lawn of the three (3) test organisms for the antibacterial screening using sterile cotton swabs and employing aseptic techniques. Antibiotic disk tetracycline (30 µg) was used as the positive control while the solvent ethanol served as the negative control. A volume of 30µl of each concentration (25%, 50%, 100%) of *Ulva intestinalis* and *Sargassum crassifolium* ethanolic extracts were dispensed in sterilized blank discs of around 6mm in diameter and placed in the agar surface aseptically along with the negative and positive control. The assay was performed in triplicates. The resulting plates were incubated at 30°C for 24hrs and maximum of 72 hrs. Observations from 24 to 72 hrs were recorded. Photographs were then taken for all the plates examined with the ruler that served as a scale. Photographs were processed and analyzed using UTHSCA Image tool software to measure the diameter of each zone of inhibition in millimeters. Mean diameter in mm and standard deviation were generated and recorded by the software (Bauer *et al.*, 1966; Hudzicki, 2009).

Statistical analysis

Paleontological Statistics (PAST) software was used to analyze data. Statistical difference between groups of the seaweed extracts tested into different strains of bacterial species using one-way ANOVA. Tukey's pairwise comparison (post hoc test) was done to check which concentration of algal extracts was highly significant with $p<0.05$ were considered significant.

Results and discussion

Isolation and Identification of bacteria from infected seahorse

Three different species of Halomonas were isolated from the epithelial surface of infected dead seahorses. Symptoms and signs of ulcerative dermatitis such as the hyperaemia of the ventral aspect of the caudal tail to the vent or as multifocal epidermal ulcerations throughout the body were comparable to the study of

LePage, (2012) as observed. Identification was done based on colony morphology on the isolates and DNA barcoding was also performed. Table 1 and Fig. 4 show the morphology and characteristics of the three bacterial strains. Fig. 5 shows the gram-stain reaction of the three bacterial strains. The three bacterial isolates were gram negative, rod-shaped cells. These bacterial strains yielded a light brown to cream colored, circular, opaque colonies, with an entire margin.

Table 1. Colony morphology characteristics of three bacterial isolates (epithelial surface).

Bacterial Isolates	Characteristics						
	Shape	Color	Elevation	Margin	Surface	Opacity	Size
H-1	Circular	Cream	Convex	Entire	Concentric	opaque	Small
H-2	Circular	Cream	Convex	Entire	Smooth	opaque	Medium
H-3	Circular	Cream	Convex	Entire	Concentric	opaque	Large

The findings were similar to Huval *et al.* (1995). Results reveal isolates belong to the genus Halomonas which is described as aerobic, gram-negative bacteria, halotolerant microorgransims that can inhabit a wide range of salinity and the cells shapes are rod-shaped, straight or curved (only 4 species have this kind of shape) (Kim *et al.,* 2013). Halomonas is a genus of the family Halomonadaceae, and a member of Gammaproteobacteria. These Gammaproteobacteria have 16 orders of which are important group of bacteria, ecologically, medically, and scientifically (Garrity *et al.,* 2005).

Table 2. Phytochemical screening of the ethanolic extracts of *Ulva intestinales* and *Sargassum crassifolium*.

	Alkaloids	glycosides	flavonoids	saponins	steroids	tannins
U. intestinalis	(+)	(-)	(+)	(+++)	(+++)	(-)
S. crassifolium	(+)	(-)	(++)	(+++)	(+++)	(-)

*(+) – Presence in lower amounts; (-) – absence of component,
(++) – Presence in moderate amounts, (+++) – presence in higher amounts.

Phytochemical screening of macroalgae

Table 2 shows the summary of the phytochemical screening of the secondary metabolites found in *Ulva intestinalis* and *Sargassum crassifolium*. Alkaloids and flavonoids were present in low amounts (+) for *U. intestinalis* while saponins and steroids were present in high amounts (+++). For *S. crassifolium*, alkaloids were present in low amounts (+), flavonoids were present in moderate amounts (++) while saponins and steroids were present in high amount. Noteworthy, the presence of flavonoids and alkaloids are interesting because they have a huge potential in replacing synthetic antioxidant and antimicrobial (Abdel-Khaliq *et al.,* 2014). The phytochemical components such as alkaloids, glycosides, flavonoids, saponins, steroids, and tannins are secondary metabolites of the seaweeds that serve as defense mechanism against different infectious diseases (Baleta *et al.,* 2017).

Antibacterial activity of Algal extracts against Halomonas species using Kirby-Bauer disc diffusion assay

Ethanolic extracts of *Ulva intestinalis* and *Sargassum crassifolium* demonstrated antibacterial activity. The extracts were tested against the isolated Halomonas species using 30 µL of the algal extract. Fig. 6 and 7 show the zones of inhibition of the algal extracts of *U. intestinalis* and *S. crassifolium* against

the three Halomonas species.

The different concentrations of the algal extracts of *U. intestinalis* and *S. crassifolium* show inhibition of the bacterial strains of Halomonas albeit not comparable to the positive control. This finding is quite significant because it showed potential. Noteworthy, inhibition zones may increase if obtained pure extract concentration is also increased. In this case only 19.6% concentration was set to be the pure ethanolic extract for *S. crassifolium* and 9.6% concentration was set to be the pure ethanolic extract for *U. intestinalis*.

Table 3. Mean zones of inhibition produced by *Ulva intestinales* and *Sargassum crassifolium* using Kirby-Bauer Disc diffusion assay against three Halomonas species.

	Mean Zone of Inhibition (mm)							
	U. intestinalis				*S. crassifolium*			
	25%	50%	100%	Positive Control	25%	50%	100%	Positive Control
HSp.1	8.10	8.76	7.68	38.39	8.58	7.49	8.63	37.20
HSp.2	8.76	8.21	8.04	43.25	8.44	6.57	8.34	40.11
HSp.3	8.42	8.94	9.37	35.68	9.35	7.38	8.86	38.97
Overall Mean	8.427	8.637	8.363	39.107	8.79	7.147	8.610	38.760

Table 4. Analysis of Variance (ANOVA) for the zones of inhibition exhibited by different concentration of *Ulva intestinalis* extracts: 25%, 50%, 100%, and tetracycline on Halomonas species.

	Sum of sqrs	df	Mean square	F	p
Between groups:	2111.32	3	703.774	178.6	1.146E-06
Within groups:	31.5225	8	3.94031		
Total:	2142.84	11			

*p < 0.05 level of significance.

The mean zones of inhibition exhibited by the two seaweed extracts are shown in Table 3. The maximum activity of *U. intestinalis* was observed against Halomonas species 3 (9.37 mm) in 100% concentration of the algal extract, and the minimum inhibitory activity was observed against Halomonas species 1 (7.68 mm) in 100% concentration. The 25% concentration of the *S. crassifolium* extract yielded the maximum inhibitory activity (9.35 mm) against Halomonas species 3, while the 50% concentration of the brown seaweed yielded the smallest zone of inhibition (6.57 mm) against Halomonas species 2.

Table 5. Analysis of Variance (ANOVA) for the zones of inhibition exhibited by different concentration of *Sargassum crassifolium* extracts: 25%, 50%, 100%, and tetracycline on Halomonas species.

	Sum of sqrs	df	Mean square	F	p
Between groups:	2108.56	3	702.852	1033	1.079E-10
Within groups:	5.44086	8	0.680107		
Total:	2114	11			

*p < 0.05 level of significance

One-way Analysis of Variance (ANOVA) and Tukey's pairwise comparison

Results show significant difference between group means of the different concentrations of the *Ulva intestinalis* and *Sargassum crassifolium* extracts that yield positive for producing the zones of inhibition as determined by one-way ANOVA against the three Halomonas species with F= 178.6; p= 1.146 E-06 and F= 1033; p= 1.079E-10 as shown in Table 4 and 5 respectively.

Table 6. Tukey's pairwise comparison between the different *Ulva intestinalis* algal extracts and tetracycline that yield zones of inhibition against three Halomonas species.

	A	B	C	D
A	-			
B	0.9992	-		
C	1	0.9982	-	
D	0.0002306	0.0002306	0.0002306	-

*p<0.05 is significant; A.100%; B. 50%; C. 25%; D. Tetracycline.

Table 7. Tukey's pairwise comparison between the different *Sargassum crassifolium* algal extracts and tetracycline that yield zones of inhibition against the three Halomonas species.

	A	B	C	D
A	-			
B	0.1471	-		
C	0.993	0.2112	-	
D	0.0002306	0.0002306	0.0002306	-

*p<0.05 is significant; A.100%; B. 50%; C. 25%; D. Tetracycline.

Tukey's pairwise comparison further confirmed the differences between the zones of inhibition of the different extracts of *U. intestinalis* and *S. crassifolium* and the positive control tetracycline with p<0.05 as significant (Table 6 and Table 7).

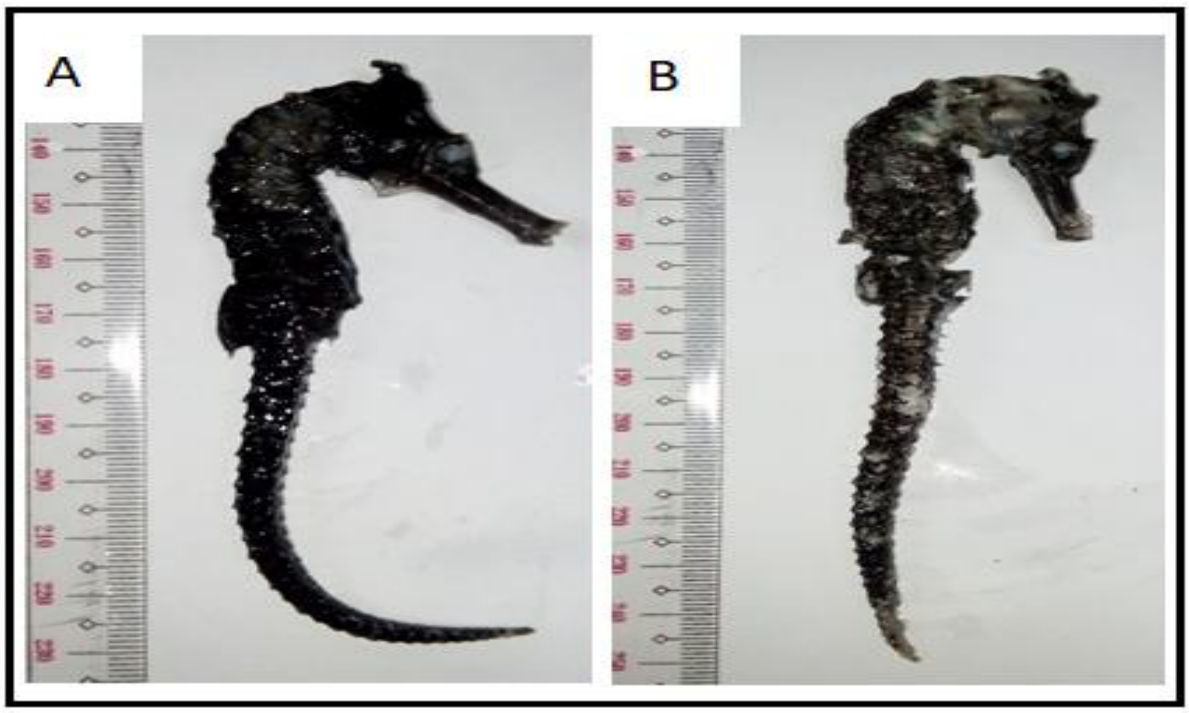

Fig. 1. (A-B) Infected *Hippocampus kuda* showing ulcerative dermatitis.

Results show that the algal extracts were able to produce zones of inhibition against the Halomonas species albeit zones of inhibition were not comparable to the commercial antibiotic, tetracycline. Given that the extracts yielded zones of inhibition despite the percent concentration of the extracts is very low, then

if the weight of dried seaweeds will be increased to specific amounts, from 200 grams to 1000 grams it might affect the zones of inhibition of the extracts. The results gathered were found to be important since, the algal extracts showed antibacterial potential against marine flesh-eating bacteria.

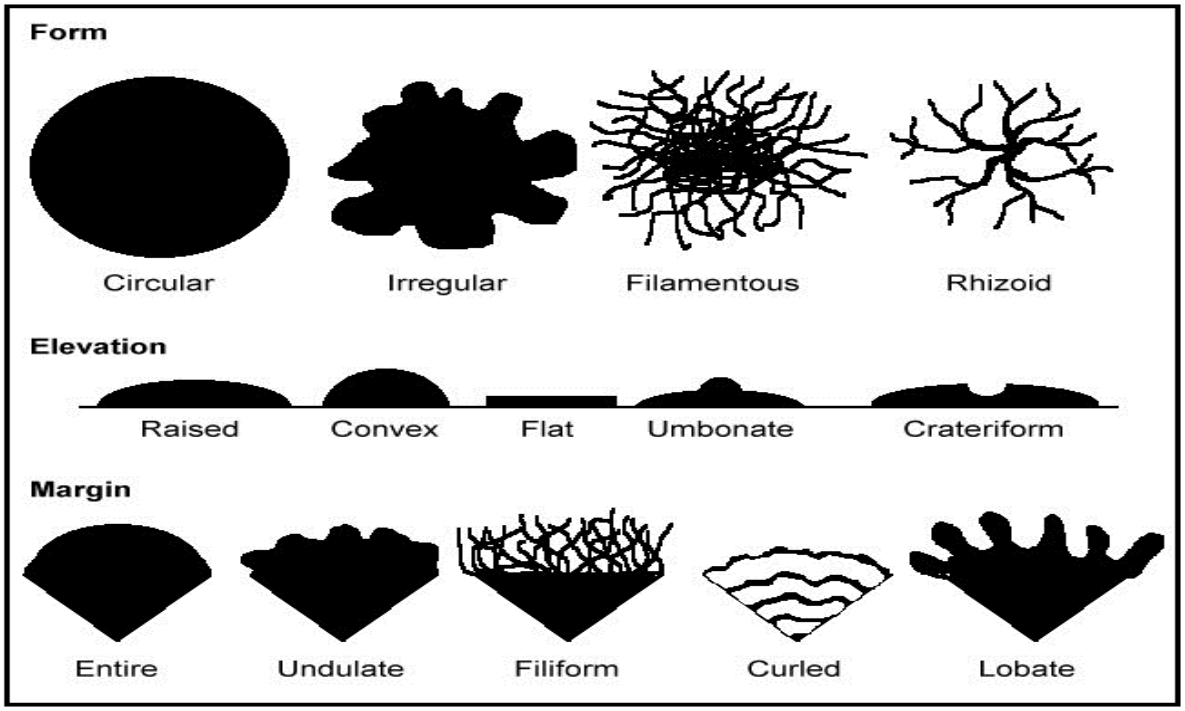

Fig. 2. Colony Morphology characteristics (microbeonline.com).

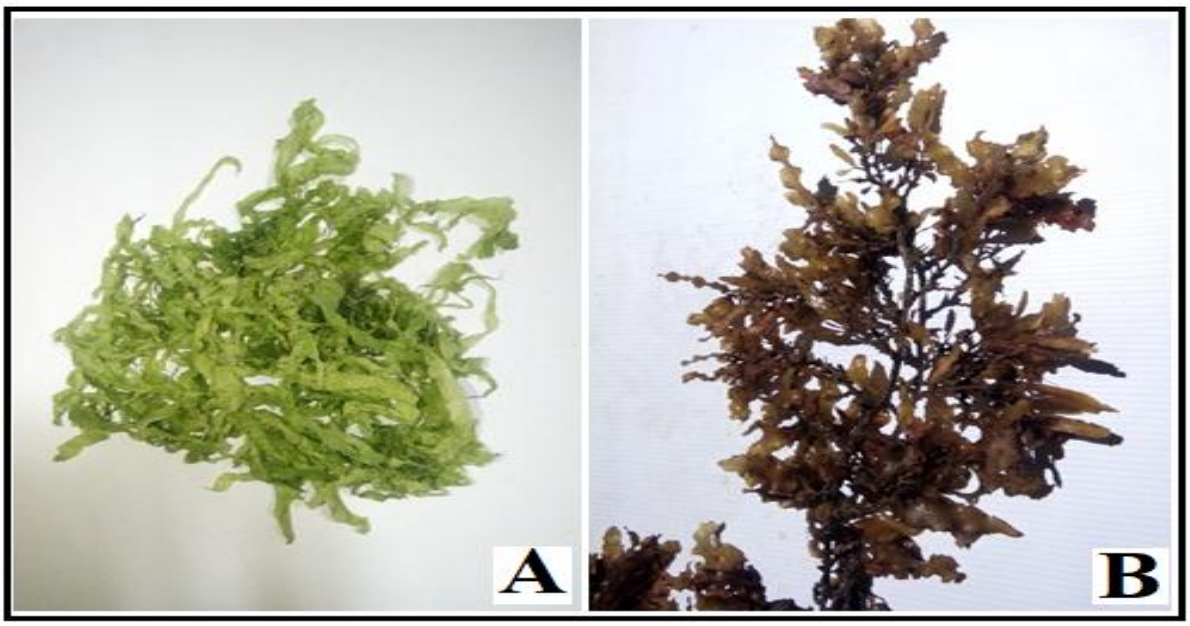

Fig. 3. Macroalgae used in this study: A. *Ulva intestinalis* (tubular thallus range from 80-340mm in height); (B) *Sargassum crassifolium* (up to 550 mm in height).

In the study of Rojas *et al.* (2009), pathogenicity of Halomonas species has been first associated with larval scallop mortality which causes larvae agglutination which blocks ciliary movements and

distends velum. They have high ability to adhere to the surface of the shell in order to rapidly increase in number by using the nutrients found in the shell. Halomonas species are very difficult to control because of their biofilms that could cause resistance to antibiotics (Olson *et al.,* 2002*;* Rojas *et al.,* 2009). Also, Halomonas species could still exist in the tanks after a long period of time (Rojas *et al.,* 2009). The antibacterial activity of green and brown macroalgae against the gram-negative and gram-positive bacteria has been published by many scientists but there are certain discrepancies on their inhibitory activity (Salem *et al.,* 2011).

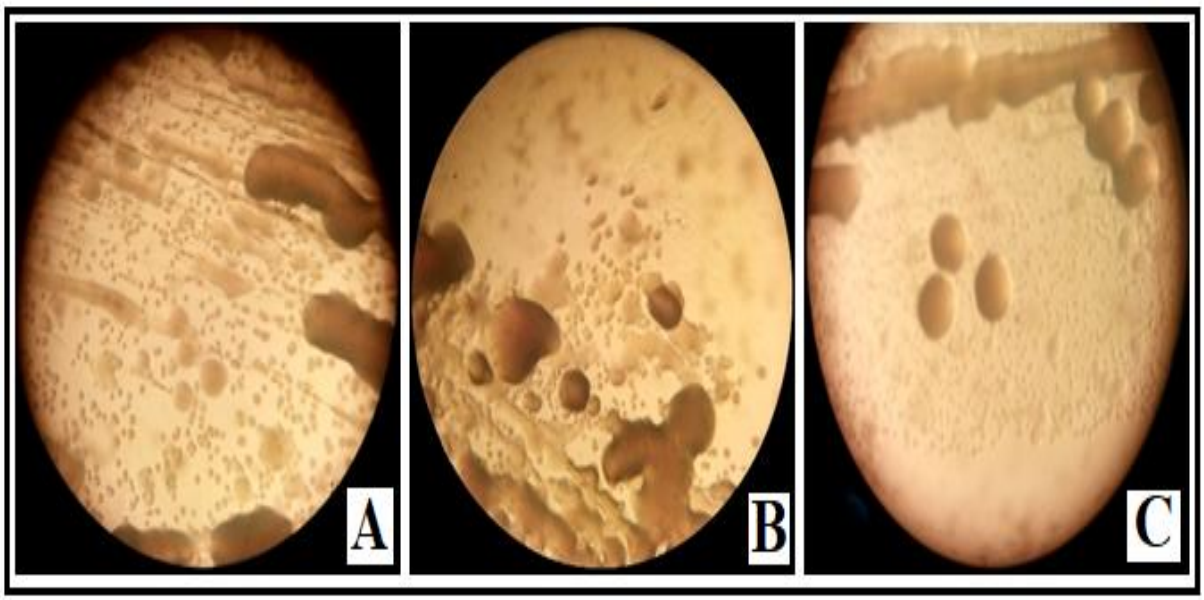

Fig. 4. Morphological characteristics of colonies: A.) *Halomonas* sp. 1; B.) *Halomonas* sp. 2; C.) *Halomonas* sp. 3 observed under the stereomicroscope.

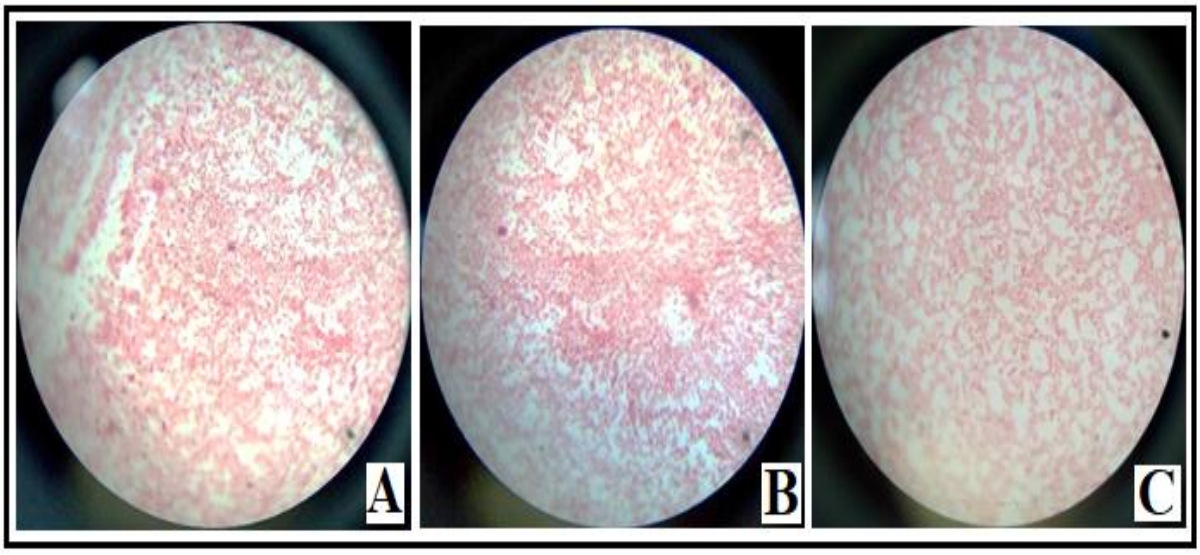

Fig. 5. Gram-negative, rod-shaped cells: A.) *Halomonas* sp. 1; B.) *Halomonas* sp. 2; C.) *Halomonas* sp. 3 observed under the microscope.

The results in the antimicrobial activity might be due to the presence of different antibacterial substances among the selected seaweeds (Lustigman and Brown, 1991), the time and place of sampling collection, and the capability of the extraction protocol being followed to screen the active metabolites (El-deen,

2011). The different concentrations of the algal extracts of *Ulva intestinalis* and *Sargassum crassifolium* showed inhibition on the bacterial strains of Halomonas albeit not comparable to commercial antibiotic tetracycline. Despite the small zones of inhibition, the findings were important since these seaweeds show potential source of antibacterial components (Abdel-Khaliq *et al.,* 2014). The presence of moderate amounts of flavonoids in the Sargassaceae and Ulvaceae are responsible for the antimicrobial activity of the algal extracts (Baleta *et al.,* 2017).

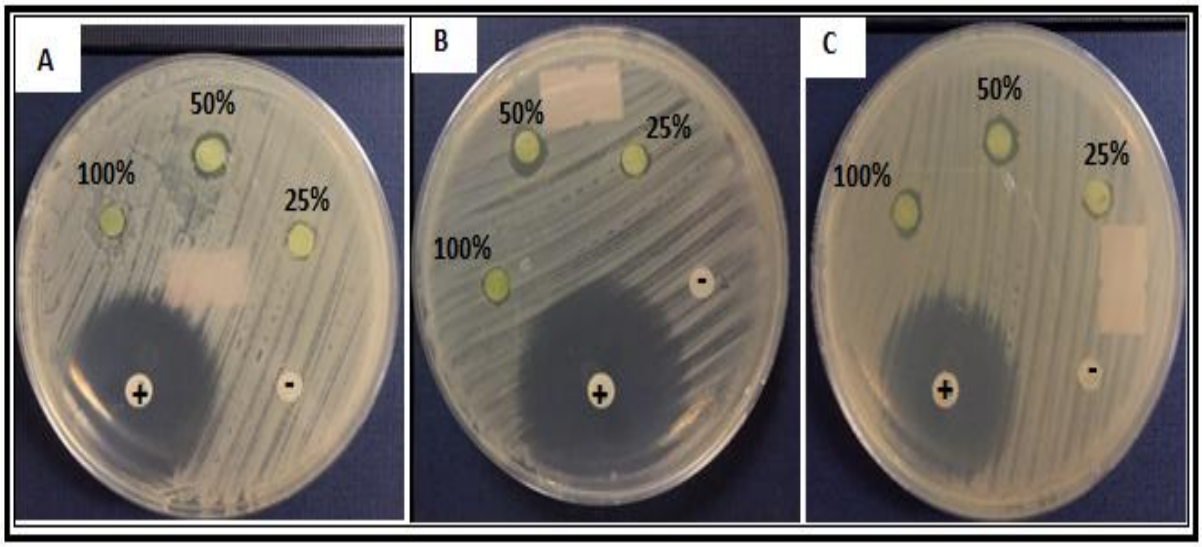

Fig. 6. Zones of inhibition of different concentrations of *Ulva intestinalis* against: A.) *Halomonas* sp. 1; B.) *Halomonas* sp. 2; C.) *Halomonas* sp. 3.

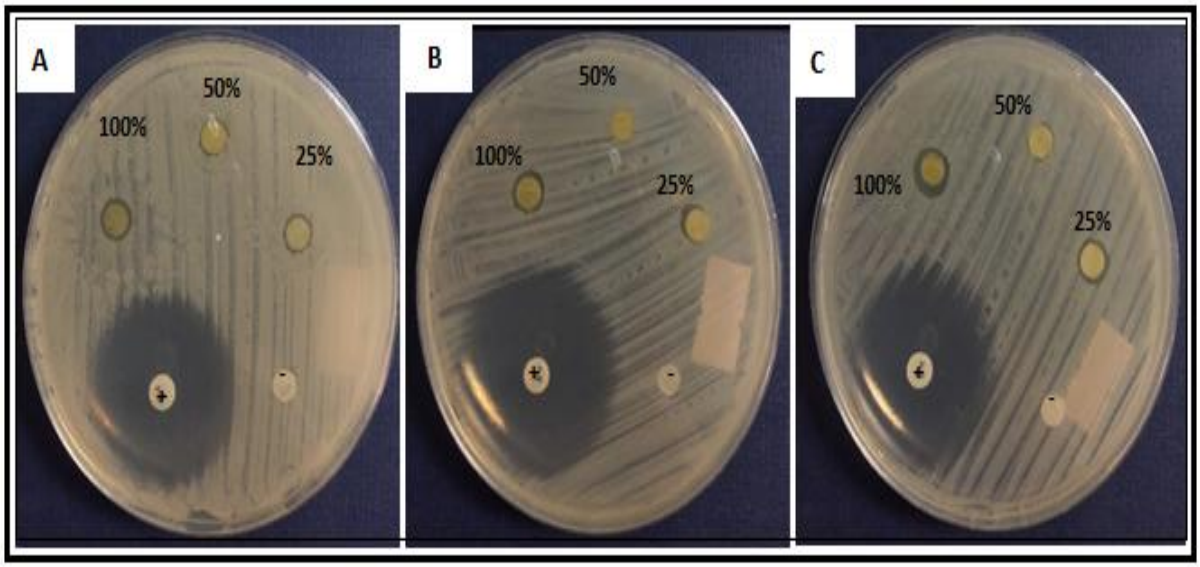

Fig. 7. Zones of inhibition of different concentrations of *Sargassum crassifolium* against: A.) *Halomonas* sp. 1; B.) *Halomonas* sp. 2; C.) *Halomonas* sp. 3.

Conclusion

Three different species of Halomonas were isolated from the epithelial surface of the infected dead seahorse and successfully identified by colony morphology and DNA barcoding via 16s rDNA bidirectional gene sequencing. Moreover, the antibacterial activity of secondary metabolites of seaweed extracts: *Ulva intestinalis* and *Sargassum crassifolium* was tested against isolates of marine flesh-eating pathogen, Halomonas species by Kirby-

Bauer disc diffusion assay. The extracts displayed an inhibitory activity but incomparable to commercial antibiotic used which was tetracycline. But such findings were significant, given that the extracts yield zones of inhibition despite the percent concentration of the extracts is very low, then if the weight of dried seaweeds will be increased to specific amounts, from 200 grams to 1000 grams, it might affect the zones of inhibition of the extracts. It is perceived that the presence of phytochemical components such as alkaloids, glycosides, flavonoids, saponins, steroids, tannins exhibit different biological activities and are important indicators of the microbial properties of seaweeds. Herewith, macroalgae can be used as an alternative source of antibiotics that could treat diseases that are caused by marine flesh-eating bacteria in seahorses hence, can be valuable in aquaculture.

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