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Epiphytic Bacteria Associated with the Green Algal *Halimeda Opuntia* as a Source of Antibacterial Agent

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Abstract: *Marine bacteria have great potential in developing pharmaceutical industry because they are able to produce many bioactive secondary metabolites. This study aimed to isolate, identify and evaluate the antibacterial activity of some bacteria associated with Halimeda opuntia, isolated from Red Sea, Jeddah. Four epiphytic bacteria were identified by morphological and biochemicals characters as well as 16S rRNA gene sequencing. Bacterial strains were found to belong to two major families, Bacillaceae and Vibrionaceae. The identified bacterial isolates were of genera Bacillus (two isolates), Vibrio (one isolate) and Lysinibacillus (one isolate). Primary screening results using well diffusion technique shown 75% of epibiotic bacteria exhibited antibacterial activity. Among them, Bacillus niacini extract showed significant antibacterial activity against all the tested Gram-negative E. coli, K. pneumonia and S. typhi with MICs 123.965, 389.164 and 268.54 mg⁄ml, respectively. Moreover, Vibrio parahaemolyticus extract showed the highest activity against E. coli, exclusively with MIC 121.51 mg⁄ml. These findings revealed that the Red Sea associated bacteria have antibacterial activity that could be incorporated in the pharmaceutical industries.*

Key words: *Red Sea, Halimeda opuntia, Epiphytic bacteria, Antibacterial activity, MIC, Ethyl acetate extract.*

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I. Introduction

The infectious diseases caused by bacteria, virus, fungi, and parasites are still a major risk to communities and universal economies although the significant progress in the field of chemical synthesis and engineered biosynthesis of antimicrobial compounds [1]. Indiscriminate use of antibiotics may lead to heavy infections and the pathogenic bacteria becoming resistant to drugs. Moreover, a high cost of chemical synthetic antibiotic and their adverse effect including hypersensitivity and reduction of normal flora in the gut [2]. As a result, production and development of antibiotics from natural sources has become an urgent need which may be efficient to combat a range of resistant bacteria [3].

The oceans occupy about 70% of earth planet as well as a rich resource of ecological, biological and chemical diversity [4]. Marine algae are one of the large and various ecosystems, it plays an important role in marine environment [5]. Around 70 algal species are utilized as nutriment, food additives, fertilizers and in cosmetic [6]. Additionally, several bioactive secondary metabolite have been isolated from marine algae and have been shown many interesting activities, such as antioxidant [7], cytotoxic [8], antibacterial [9], antiviral[10] and anti-inflammatory [11]. Furthermore, the surfaces of marine algae are colonized by microorganisms that play a vital role in morphogenesis of their host [12]. During recent decades, the attention to bacterial populations living in association with marine algae has increased because bacteria specially cooperate with their host in multiple and complex ways, they represent rich source of new bioactive compounds [13]. These bacteria may defend their host from foreign microbes existing in marine environment[14] but can also produce compounds may contribute to the pharmaceutical industry [15]. Several studies have shown that algal associated bacteria able to produce antimicrobial compounds [16]–[19].

Halimeda opuntia is a chlorophyte alga grow in lower intertidal in the zone of coral reefs or on dead corals at depth ranging from 0-25 meters. Earlier studies stated a considerable antimicrobial and antioxidant activity in *Halimeda opuntia* [20], [21]. But, the biological diversity of its epiphytic bacteria and their antimicrobial activity are still unknown. So, the aim of current study was isolating and identifying of the epiphytic bacteria of *Halimeda opuntia* surface and then estimating the antibacterial activity of isolated bacteria extracts.

II. Materials and Method

1. Algal Sample Collection and Associated Bacterial Isolation:

Halimeda opuntia belong to chlorophyta, green algae, was collected in Spring season of 2017 from North Obhur region in Jeddah, Saudi Arabia (N 21^0 42' 36.4 E 039^0 05' 45.9) at depth 5 meters , pH 8.5 and 28.5ºC (Figure 1). The algal sample was transported in sterile plastic zip bag into King Fahad Medical Research Center (KFMRC) under cooling conditions. Later, *Halimeda opuntia* sample (Figure 2) was washed serially by sea water followed by distilled water to remove sands and other adhering substances, dried and identified at Biology Department, Faculty of Science, KAU. Voucher samples were deposed at Biology Dep. Faculty of Science, KAU.

Algal associated bacteria were isolated by taking multiple swabs from different areas of alga. Swabs of isolation were spread directly on marine agar medium plates (medium 2216, Fisher Scientific Company, USA) and incubated at 27°C for 48 hours. The growing bacterial colonies on the plates were carefully chosen according to their morphological features such as shape, color and surface characteristics [22]. Purification of bacteria was carried out by sub-culturing single colonies by streaking technique and they were encoded with numbers according to their alga name. Purified bacterial isolates were preserved for further investigation in glycerol stock.

3. Morphological and Biochemical Characterization:

Pure bacterial isolates were grown on marine agar plate. Gram staining, spore formation and motility as well as biochemical tests for all bacterial isolates was performed as demonstrated by [23], [24], [25].

4. Genetic Characterization:

All bacterial isolates were subjected to DNA extraction with QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacture's instruction, followed by amplification of the extracted DNAs.

Figure. 1: Sampling Site of the used green algal species, *Halimeda opuntia*, obtained from North Obhur region in Jeddah, SA.

Figure 2: The dried chlorophyte algal sample, *Halmida opuntia* collected from North Obhur region in Jeddah , Saudi Arabia.

as described by Susilowati *et al*. [22]. Then, they were identified in Macrogen Laboratory, Korea according to 16S rRNA Gene Sequencing.

5. Bioactive Compound Extraction:

The bacterial isolate was cultured in 1L of marine broth 2216 (Fisher Scientific Company, USA) for 7 day at 27°C. after fermentation, bacterial supernatant were mixed with equal amount of ethyl acetate (Sigma, USA).These mixtures were separated, and the ethyl acetate layers were evaporated using rotary evaporator at 40°C. The crude extract was weighted and dissolved in Dimethyl sulfoxide (Sigma, USA) with final concentration (1gm/ml) [26].

6. Antibacterial Activity Assay:

The antibacterial activity of extracts was evaluated on different clinical strains: *Escherichia coli* ATTC 5044552, *Klebsiella pneumonia* ATTC 5205773, *Salmonella typhi* ATTC 6704712 and *Staphylococcus aureus* ATCC 25923. They were provided by Microbiology Unit in King Abdulaziz University Hospital (KAUH), Jeddah, Saudi Arabia. The agar well diffusion method was conducted for screening antibacterial activity of isolated bacteria extracts [27].

The pathogenic bacteria were suspended in 0.9% sterile NaCl solution with turbidity equivalent to 0.5 McFarland. Bacterial suspensions were spread on Mueller Hinton agar plates (Himedia, India). About 40 µL of each extract was poured in 5 mm well. Dimethyl sulfoxide was used as a negative control while ampicillin (25 µg/ml) as a positive control. Finally, the plates were incubated for 24 hours at 37°C. The antibacterial activity was well-defined by inhibition zones measured in mm.

7. Determination of MIC:

Microdilution test was performed to provide quantitative measurement of antibacterial activity of ethyl acetate extracts. Consistent with the method described by Elshikh *et al*. (2016), MIC of extracts were determined by using Resazurin dye (Sigma, USA) as a redox indicator [29]. This was carried out using 96-well plates and a change in the color was determined using ELISA reader (Bio-Tek, USA).

8. Statistical Analysis:

All the values of antibacterial activity were estimated in triplicate and presented as mean ± SD. All statistical analyses were performed using SPSS version 22.0. In order to determine whether there any variance between the activity of extracts, One-Way ANOVA and Turkey post hoc test ($p<0.01$, $a= 0.01$) were applied to result.

III. Results

1. Isolation of Associated Bacteria:

In the current study, four bacterial isolates were picked up from the surface of Red Sea alga, *Halimeda opuntia*. They were encoded with numbers according to their algal name (SHO_1, SHO_2, SHO_3 and SHO_4).

2. Morphological and Biochemical Characterization:

All bacterial isolate were characterized morphologically, and all colonies of growing bacterial strains showed pale yellow colors, their colonies were gelatinous with a high rate of their growth. Three bacterial

strains were Gram-positive while one isolate Gram-negative. Bacterial isolates under optical microscope have rod or curved rod shapethat could be found in cluster or pair. Also, the oxidase, catalase, motility and spore formation tests were performed. All these characteristics were described in detail in Table 1.

Table 1:The Morphological and biochemical characterization of *Halimeda opuntia* associated bacteria.

	SHO_1	**SHO_2**	**SHO_3**	**SHO_3**
Shape	Curved-Rod shape	Rod shape	Filamentous	Rod shape
Color colony	Pale yellowish Gelatinous	Pale yellowish Gelatinous	Pale yellowish Gelatinous	Pale yellowish Gelatinous
Rate of Growth	High	High	High	High
Gram Stain	Gram-negative	Gram-positive	Gram-positive	Gram-positive
Spore Formation test	Non-spore forming	Spore-forming	Spore-forming	Spore-forming
Motility	motile	Non-motile	motile	motile
Oxidase test	+ve	+ve	-ve	+ve
Catalase test	+ve	+ve	+ve	+ve

+ve: Indicating the presence of the enzyme, -ve :Indicating absence of the enzyme

3. Genetic characterization:

Four bacterial strains were recognized by 16S rRNA gene sequencing in Macrogen laboratory, Korea with similarity 92-99%. All bacterial strains were recorded in Table 2, with their accession numbers and their similarities to other species. The isolates were found to belong to two major families, 75% of the isolates were found belong to the family of Bacillaceae while 25% belong to Vibrionaceae. Out of all bacterial strains, two strains belong to *Bacillus* genus, one strains belong to *Vibrio* and one strain to *Lysinibacillus* genus.

Table 2: Identification of *Halimeda opuntia* associated bacteria based on 16S rRNA gene sequence.

	SHO_1	**SHO_2**	**SHO_3**	**SHO_4**
Bacterial ID	*Vibrio parahaemolyticus*	*Lysinibacillus* sp.	*Bacillus Algicola*	*Bacillus niacini*
Accession No.	KT986101.1	KU159206.1	KX816439.1	KT350462.1
Similarity	92%	98%	99%	99%

4. Antibacterial Assay of Bacterial Extracts:

evaluation of antibacterial activity of four bacterial extracts was conducted against different clinical strains. Inhibitory activity against at least one clinical isolate was detected for three isolates (Table.3). The inhibition zones of bacterial extract ranged between 14.00 and 19.67 mm as listed in Table.3. The extract of *Vibrio parahaemolyticus* presented the highest inhibition zone (19.67 mm) against *E. coli* with MIC 121.51mg/ml (Figure. 3). Moreover, *Bacillus niacini* extract was inhibited the growth of *E. coli, S. typhi* and *K. pneumonia* with inhibition zone 17.67, 16.67 and 14.67 mm and MICs 123.965, 268.54 and 389.164 mg/ml, respectively (Figure. 3). However, the extract of *Bacillus algicola* was inhibited *S. typhi* with inhibition zone 14.00 mm and MIC 470.126 mg/ml (Figure 3). Among isolated bacterial strains, *Lysinibacillus* sp. had no antibacterial activity against all clinical strains. Additionally, all bacterial extracts had no inhibitory effect on Gram-positive *S. aureus.* As a result, *B. niacin* and *V. parahaemolyticus* showed a highly significant variances compared to other extracts (p<0.0).

Table 3: Inhibition zone (mm) and MICs (mg/ml) of four bacterial extract against different clinical strains, *E. coli, K. pneumonia, S. typhi* and *S. aureus*.

	SHO_1		**SHO_2**		**SHO_3**		**SHO_4**	
	IZ	**MIC**	**IZ**	**MIC**	**IZ**	**MIC**	**IZ**	**MIC**
E. coli	19.67±0.54**	121.51	ND	-	ND	-	17.67±0.54**	123.965
K. pneumonia	ND	-	ND	-	ND	-	14.67±0.54**	389.164
S. typhi	ND	-	ND	-	14.00±1.00	470.126	16.67±0.54**	268.54
S. aureus	ND	-	ND	-	ND	-	ND	-

The values of inhibition zone were described as mean ± SD.
No Antibacterial Activity Detected (ND), Inhibition Zone (IZ) and Minimum Inhibitory Concentration (MIC).
** highly significant variance against individual tested pathogenic bacteria at (p< 0.01) compared to other extract

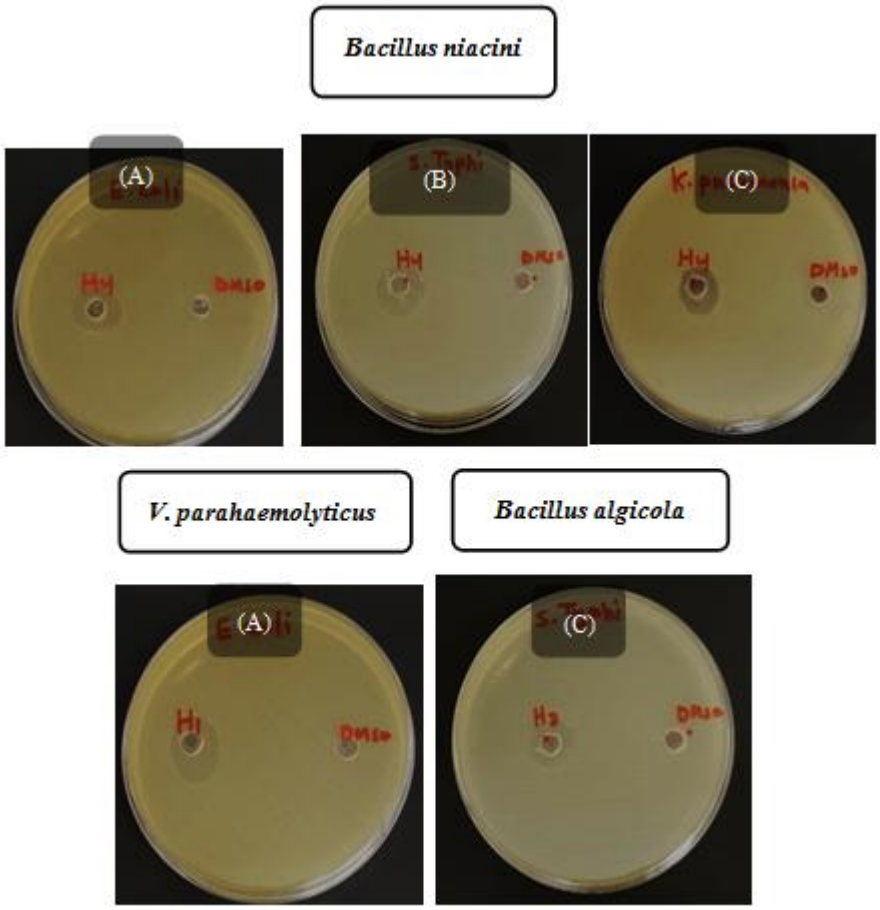

Figure 3: The Inhibition Zone of *Bacillus niacini*, *Vibrio parahaemolyticus* and *Bacillus algicola* extracts against *E. coli* (A), *K. pneumonia* (B) and *S. typhi* (C).

IV. Discussion

Antibiotics resistance has become one of the biggest threats to global health. Also, they have many side effects represented by harmful digestive upset and hypersensitivity [2]. The discovery of new antibiotic from natural source such as marine environment could be assist in reducing the side effect.

In the current study four bacterial strains were isolaled from Red Sea alga *Halimeda opuntia* and revealed antibacterial activity against different clinical strains. Most of the studies conducted in the Red Sea were linked to isolation of algae and testing their antimicrobial activity[30], [31]. The Red Sea bacteria did not receive attention as a source of medically effective compounds of medically effective compounds [28], [31]. Many studies around the world revealed the antimicrobial activity of associated bacteria which isolated from various places like Northern coast of Tunisia [34], Southeastern coast of India [35], [36], Eastren coast of Australia [37] and in the Maxico [19]. This tells the importance of marine bacteria as a source of natural antimicrobial agents regardless of its origin. Furthermore, the 16S rRNA gene sequencing of associated bacteria were phylogenetically examined. The isolated bacteria were found to be belonging to Bacillaceae and Vibrionaceae families. Our findings are agreeing with previous studies [22], [38].

In this study, the extract of *Bacillus niacini* was revealed significant activity against tested Gram-negative clinical strains.Moreover, the extract of *Bacillus algicola* in this study had exclusive inhibtory effect on *S. typhi*. This consistent withthe findings of bacteial strain associated with marine sponge *Xestospongia testudinaria* in Tanjung Kasuari, Papua. According to their studiy, the efficacy of ethyl acetat extract is due to the presence of phytochemical compounds such as alkolids, steroid and triterpenoid [39]. In contrast to our findings, a study was conducted on *Bacillus* sp. which were isolated from Tamilnadu Sea in India [28]. The antibacterial activity of *Bacillus* species could be returns to their production of secondary compounds such as exopolysaccharides or lipopeptides. This is compatible with the recent studies conducted on *Bacillus licheniformis* and *Bacillus methylotrophicus* and evidenced its inhibitory activity on *K. pneumoniae* [40], [41].

The extract of *Bacillus* sp. that were isolated from brown alga *Padina pavonica* gave inhibtion zone between 15-25 mm against different strains of *E. coli* [42]. This is correspond withfindings of *B. niacini* extracts in this study. The extract of *Lysibacillus* sp. had no antibacterial activity against tested clinical strains. A preveous study perfomed inon five endophytic *Lysinibacillus fusiformis* strains contrast the findings obtained by *Lysinibacillus* sp. in this study. *Lysinibacillus fusiformis* strains that were isolated from marine fish of *Triacanthus strigilifer* in Indian coast possesed antibacterial activity against *E. coli*, *K. pneumoniae*, *S. typhi* and *S. aureus* [38]. Another study indicated the antifungal activity of *Lysinibacillus* sp. that was isolated from forest soil specimens collected from different regions in China [43]. Moreover, the extract of *Vibrio parahemolyticus* had the highest antibacterial activity against *E. coli.* This contrary to a study conducted in Indonesia on brown algal associated bacteriathatwere inhibit the growth of *S. aureus* [22]. In earlier study, Al-Zereini *et al*. isolated seven novel maleimide compounds with antibacterial and cytotoxic activities from *Vibrio* sp. associated with soft coral *Sinularia polydactyla* [44]. This confirms the importance of *Vibrio* sp. in the production of pharmaceutical compounds.

The variation among our results and the ones of the literature may be attributed to many factors such as the environmental conditions, season of collection and extraction solvents. However, our findings still interesting specially when taking in consideration that the field of associated bacteria is new, easier and faster field compared to the host one, and it could be applied on a large scale. Further investigation is suggested to identify the isolated strains and determine the active metabolite behind the antibacterial activity which could be phytochemicals and/or secondary compounds as amino acids, carbohydrates, pigments, fatty acids and more [45].

V. Conclusion:

The epiphytic bacteria of the Red Sea alga, *Halimeda opuntia* could be a potential local cheap source of natural antibiotic that could be used against the pathogenic bacteria. The results of isolate *Bacillus niacini, Vibrio parahomolyticus* and *Bacillus algicola*that inhibited tested Gram-negative clinical strains were promising. More investigations are suggested to determine the responsible compounds of this activity. Moreover, identifying the effective strains by gene sequencing techniques will help to understand the results better. The antibacterial activity could be useful in food safety applications in Saudi Markets.

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