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Soft Coral Epibiotic Bacteria *Vibrio alginolyticus* from Sarangani Bay, Mindanao, the Philippines as Potent Source of Antibacterial Agent

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ABSTRACT

This study aims to explore the antibacterial properties of the different epibiotic bacterial species thriving in the surface tissues of the soft corals, and their potential application in developing novel antibacterial agents. There were ten soft coral species randomly collected from Sarangani Bay in Mindanao, Philippines: *3 sarcophyton sp., two sinularia sp., 1 nepthia sp., 1 stenonephthya sp., 1 anthelia sp., 1 lithophyton sp. and 1 asteropicularia sp*. All soft coral species collected harbored epibiotic bacteria. Fifteen epibiotic bacterial species were isolated and tested for antibacterial properties against *Staphylococcus aureus* (gram-positive) and *Escherichia coli* (gram-negative) as test organisms. Their respective zones of inhibition were compared to that of the commercial antibiotics Penicillin, Chloramphenicol, and Gentamycin (as positive controls). Results indicated varied antibacterial effects, with one isolate, Vibrio alginolyticus (EB6), showing significant activity, with the zone of inhibition of 9.33 mm against E. coli, comparable to that of Chloramphenicol and Gentamycin, with the zone of inhibition of 10 and 9 mm, respectively. These findings highlight the potential of marine-derived bacteria in developing new antibacterial agents.

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1. INTRODUCTION

For centuries, higher plants have been made as major sources of drugs used in many civilizations, although the nature of the compounds in the drug is not exactly known. Yan (2004) stated that after the discovery of penicillin, attention has been focused on searching for terrestrial microorganisms as new sources of drugs, and many new families of antibiotics are found from these microorganisms. As humans face ever-increasing population size and longevity. The demand for novel drugs to treat existing and newly emerged diseases is becoming an ever-pressing issue.

The growing number of drug-resistant infectious diseases point to the necessity that a major investment in finding new drugs is needed to confront the ever-pressing need for new drugs. Nature has continuously provided mankind with a broad and structurally diverse arsenal of pharmacologically active compounds that continue to be utilized as highly effective drugs to combat a multitude of deadly diseases or as lead structures for the development of novel synthetically derived drugs that mirror their models from nature (Prokcsh, *et al*, 2002). The last frontier that remains to be explored for possible sources of drugs lies within the so-called “inner space” or the oceans (Yan, 2004). According to Jha and Zi-Rong (2004), the marine environment contains several plants, animals, and microorganisms that due to the unique adaptations to their habitat elaborate a wide diversity of natural products that are mainly accumulated in living organisms with specific bioactivities.

To evaluate the biomedical potential of any plant or animal, one must consider both the chemical ecology of the organism and its evolutionary history. It is probable that chemical defense mechanisms evolved with the most primitive microorganisms but have been replaced in many more advanced organisms by physical defenses and/or the ability to run or swim away and hide. Sessile soft-bodied marine invertebrates that lack obvious physical defenses are therefore prime candidates to possess bioactive metabolites. They have a very long evolutionary history and have had ample opportunity to perfect their chemical defenses (Faulkner, 2000). Further, organisms that thrive despite pronounced biotic pressures can to some degree be expected to contain metabolites that are also of interest to drug prospectors searching the ocean.

Soft corals are colonial marine invertebrates that belong to the Phylum Cnidaria/Coelenterata: Class Anthozoa: Order Octocoralla: Family Alcyonacea. They lack a rigid skeleton (calcareous or proteinaceous) but are made up of either encrusting or erect colonies, mostly fleshy and flexible with a bizarre assortment of internal structural elements called sclerites that are embedded in the tissue rendering shape and structure. Species occur in all oceans, more in the tropics than in temperate areas, mainly in mid-depths of 5-10 meters. Their distinguishing characteristic is that their polyps always bear eight tentacles (hence octocoral), which are on both edges fringed by rows of pinnules. The popular term soft-coral points to the fact that most soft coral, in contrast to the related hard coral, has no massive external skeleton.

Epibiotic bacteria are bacterial species that inhabit the surface of the host. Armstrong *et al* (2001) studied the symbiotic role of living microbes on living surfaces and described the ecological role of epibiotic bacteria from seaweed surfaces. These epibionts may play a protective role, releasing compounds into the surrounding seawater that help prevent extensive fouling of the surface. It is also proposed that epibiotic bacteria on microalgae can also produce antifouling compounds that work in concert with the seaweed-derived compounds to protect the seaweed surface. Gil-Turness *et al* (1989) identified a beneficial association between the surface bacteria and their host. Epibiotic bacteria on the larvae of

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some crustaceans protect them from fungal infection by the production of simple antimicrobial compounds.

Many bottom-dwelling invertebrates such as sponges, tunicates, and soft corals have no value as food and hence, have not been considered economically important resources. However, chemical and pharmaceutical studies in the past three decades have revealed that these unpalatable organisms are important sources of biologically active (bioactive) compounds that have the potential for the development of new drugs and other useful products and are helpful for the invention and discovery of bioactive compounds primarily for deadly diseases (Dobresov, 2004; Dobresov, 2002).

Prokcsh (2002) stated that the numerous natural products from marine invertebrates show striking structural similarities to known metabolites of microbial origin, suggesting that microorganisms are at least involved in their biosynthesis or are the true sources of these respective metabolites however, this remains to be demonstrated. Bacteria associated with soft-bodied organisms are suggested to produce bioactive compounds against the attachment of invertebrate larvae and bacteria into the surface of these organisms are responsible for the antibiosis as displayed in certain species of soft corals. This motivates the researcher to conduct a study on the antibacterial activity of some marine epibiotic bacteria.

For centuries, higher plants have been primary sources of drugs, but recent focus has shifted to terrestrial microorganisms. Despite extensive research, marine environments, especially soft corals, remain underexplored. This study addresses this gap by uniquely investigating the antibacterial properties of epibiotic bacteria from soft corals in Sarangani Bay, an area previously underexplored for such purposes. The objective is to identify bacterial isolates with significant antibacterial activity against E. coli and/or S. aureus, contributing to the development of new antibacterial agents.

2. METHODS

2.1. The Collection Site

Collection of the soft coral samples was done in the coastal town of Glan, the largest municipality of Sarangani Province, Mindanao, Philippines. It is bounded on the east by the province of Davao del Sur, on the north by the municipality of Malapatan, Sarangani Province, on the west by Sarangani Bay, and the south by Celebes Sea. The location of collection site was in Barangay Kapatan (05° 54’ 32” N, 125° 14” 15.6” E) which is located in the southern portion of the municipality of Glan, facing Sarangani Bay (**Figure 1**). The area is a fishing ground for municipal and subsistence fishing by the residents.

2.2. Experimental Design

The study was laid out in Complete Randomized Design (CRD). Metabolites of fifteen (15) epibiotic bacterial isolates were tested against *S. aureus* and *E.coli*. Chloramphenicol (commercial broad-spectrum antibiotic for both gram-positive and gram-negative bacteria), Penicillin (commercial antibiotic for gram-positive bacteria), and Gentamycin (commercial antibiotic for gram-negative bacteria) were used as positive controls. Each treatment was replicated three times. Each replication contained 3 discs (**Figure 2**).

2.3. Collection of Soft Coral Samples

The collection of soft coral was done through the Self-Contained Underwater Breathing Apparatus (SCUBA). Ten (10) soft coral species were randomly picked and were assigned codes that were tagged on every species. The tags were written on pieces of yellow laminated cards. Two samples of each coral species were taken, one for the isolation of epibiotic bacteria

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and the other one for taxonomic purposes. From the site, the samples were first placed separately in resealable cellophane bags with seawater and each was assigned a code number similar to the original code number assigned to the stock where the sample was taken. Photographs of the coral species in their natural habitat were made during the sample collection. Specimens were identified using the taxonomic description by Fabricius and Alderslade (2001) and were validated by local experts on corals.

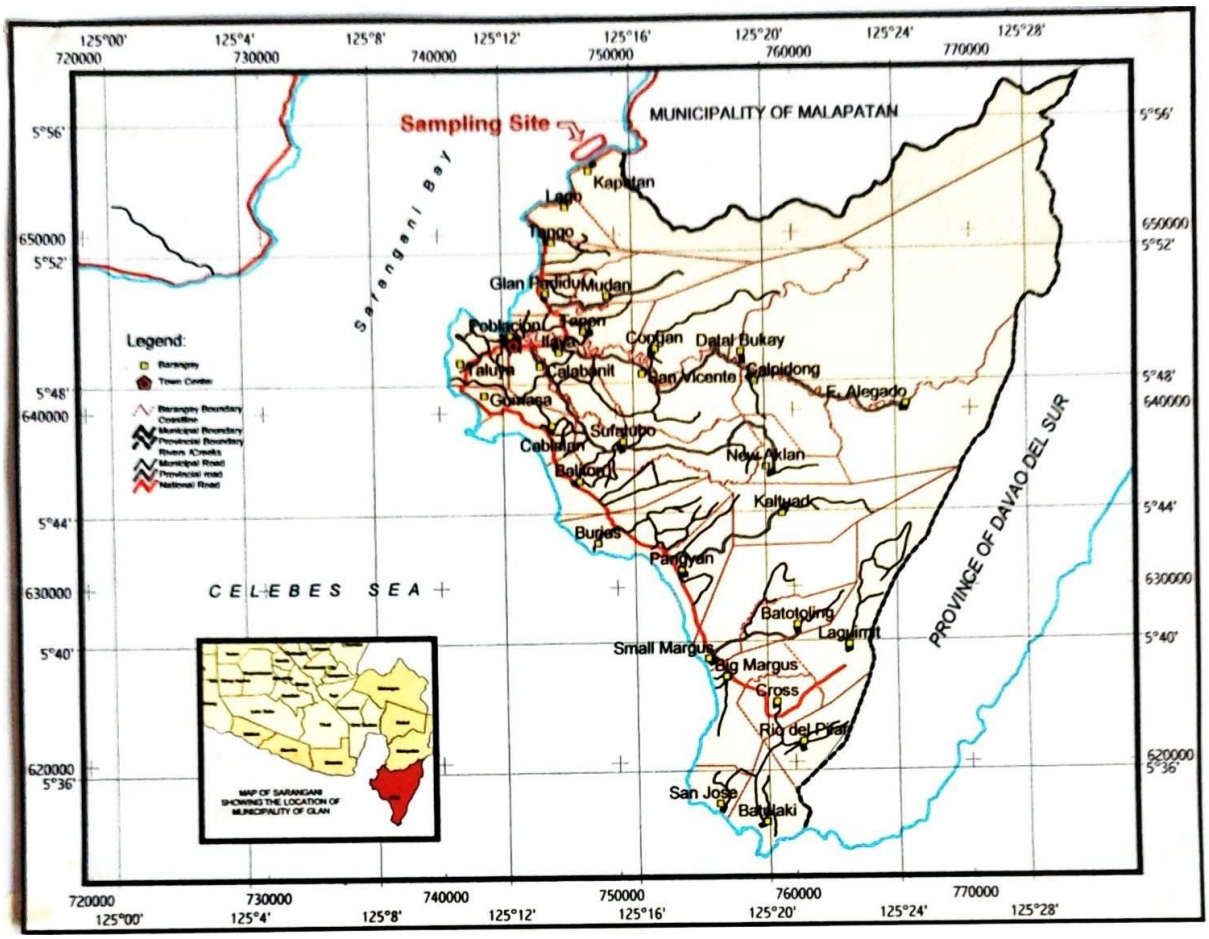

Figure 1. Site of soft coral collection.

2.4. Isolation of epibiotic bacteria

Soft coral samples were taken out from the water while on board the sampling vessel. The coral sample was washed with sterile seawater to remove extraneous particles adhering to the surface. Individually-wrapped pre-sterilized cotton swab, (PS-Swab Tube, Greiner bio-one, Maybachstr.2, D,72636 Frieckenhausen, Germany), was used to swab the surface of soft coral in an area of about 18 cm2. The cotton swab was immediately suspended in 10 ml sterile, filtered seawater in a screw-capped test tube and was shaken until the swab disentangled. One ml was transferred to sterile marine broth (0.5% peptone and 3% yeast extract in 0.22 µm-filtered seawater) instead of inoculating the bacteria directly from sterile seawater to marine agar. Samples in marine broth were incubated at 30°C for 24 hours, followed by serial dilution up to 1:1,000,000, inoculation in marine agar (0.5% peptone, 3% yeast extract, 1.5% agar in 0.22µm-filtered seawater) and incubation of two replicates at 30°C for 24 hours. Isolation of epibiotic bacteria was conducted using standard microbiological techniques. Fifteen (15) epibiotic bacteria were isolated. Samples were incubated at 30°C for 24 hours,

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followed by serial dilution and inoculation in marine agar. All bacterial isolates obtained were maintained in a marine agar slant for the antibacterial assay.

2.5. Antibacterial Assay

The metabolites of the fifteen (15) epibiotic bacterial isolates were tested against pure cultures of Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* ATCC 8739. Penicillin for gram-positive bacteria, Gentamycin for gram-negative bacteria and Chloramphenicol for broad spectrum (Mast Diagnostics, Ltd., UK) served as positive controls. For the epibiotic bacteria, circular discs (Whatman No. 1) were cut using a puncher before application on respective test organisms. Each plate received three discs that were placed equidistantly from each other to avoid overlapping the zone of inhibition (**Figure 2**). The antibacterial activity of marine bacterial isolates was determined based on the size of the zone of inhibition around each disc measured in millimeters. Statistical analysis was performed using ANOVA and Scheffe's Pairwise Comparisons test."

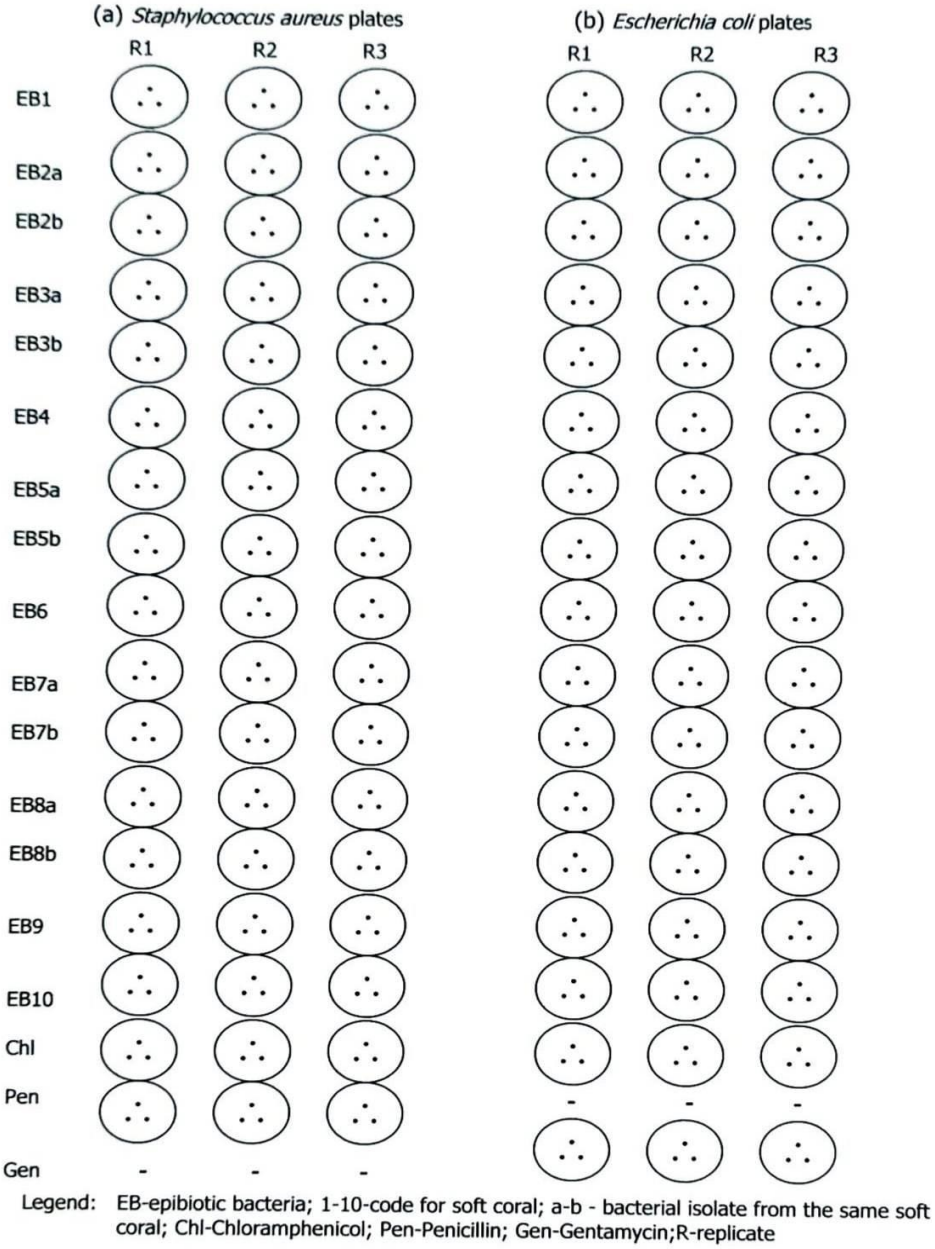

Figure 2. Experimental layout of the *S. aureus* and *E.coli* plates.

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2.6. Identification of Soft Coral Samples

Photographs of soft coral species in their natural habitat were made during the sample collection. Specimens were identified using the taxonomic descriptions by Fabricius and Alderslade (2001) and were validated by local experts. Identification was done down to the genus level. Specimens that belonged to the same genus were designated with a number next to the genus name (eg. *Sarcophyton 1, Sarcophyton 2*, and so on). Preserved specimens were properly labeled.

3. RESULTS AND DISCUSSION

Soft coral species were randomly collected from the sampling area located at 05° 54' 32" N, 125° 14' 15.6" E. There were ten (10) soft coral species collected and identified. All soft coral species collected harbored epibiotic bacteria. A total of fifteen epibiotic bacterial isolates with distinct colony morphotypes were isolated from the ten soft coral samples as shown in **Table 1**. Kirchman *et al* (1982) stated that marine biofilms in the natural environment are composed of species of bacteria that serve as an effective protection of soft-bodied marine invertebrates such as the soft coral and sponges against macrofouling.

Table 1. Soft coral species and associated epibiotic bacteria were isolated. (a,b – different epibiotic bacteria isolated from the same soft coral source; eg. 2a and 2b are two epibiotic bacterial isolates both from Soft coral 2).

Soft coral sample	Soft coral species	Epibiotic bacteria isolated
1	*Sarcophyton spp. 1*	EB1
2	*Sinularia spp. 1*	EB2a
		EB2b
3	*Sarcophyton sp. 2*	EB3a
		EB3b
4	*Nephthea spp.*	EB4
5	*Sinularia spp. 2*	EB5a
		EB5b
6	*Stereonephthya spp.*	EB6
7	*Anthela spp.*	EB7a
		EB7b
8	*Sarcophyton spp. 3*	EB8a
		EB8b
9	*Litophyton spp.*	EB9
10	*Asterospicularia spp.*	EB10

3.1. Antibacterial Activity Against *S. aureus*

The result of the antibacterial activities of the metabolites produced by the different epibiotic bacterial isolates against *S. aureus* is presented in **Table 2**, which shows that penicillin and chloramphenicol outperformed all the epibiotic bacterial isolates. This is confirmed by the Analysis of Variance (ANOVA), shown in **Table 3** that there is a significant difference in the average zones of inhibitions of the isolates and positive controls.

Scheffe's Pair-wise Comparisons test (**Table 4**) shows that there are five (5) groupings concerning the zones of inhibitions produced by epibiotic bacterial isolates and the positive controls against S. aureus. Members of the same group are not significantly different from each other. Group-a includes only Penicillin and Group-b includes only Chloramphenicol (with inhibition zones of 14.33 mm and 9.33 mm respectively) which significantly outperformed all

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epibiotic bacterial isolates in inhibiting S. aureus indicating their superior efficacy. All epibiotic bacterial isolates are not as effective as penicillin and chloramphenicol against S. aureus. The comparatively lower efficacy of some epibiotic bacterial isolates may be due to the non-concentrated nature of the metabolites used as compared to the positive controls.

Table 2. Mean zone of inhibition of different soft coral epibiotic bacterial isolates and commercial antibiotics against *S. aureus*.

Treatment (epibiotic bacterial isolates and positive control)	Mean zone of inhibition against *S. aureus* (mm)
1. EB1	0.67
2. EB2	1.00
3. EB2a	1.00
4. EB3a	0.00
5. EB3b	0.00
6. EB4	2.00
7. EB5a	0.00
8. EB5b	0.00
9. EB6	2.00
10. EB7a	0.00
11. EB7b	0.00
12. EB8a	0.00
13. EB8b	0.00
14. EB9	2.00
15. EB10	1.33
16. Chloramphenicol	9.33
17. Penicillin	14.33

Table 3. ANOVA of the zones of inhibitions of the different epibiotic bacterial isolates and commercial antibiotics against *S. aureus*.

SV	DF	SS	MS	F-value	Probability
Treatment	16	726.310	45.395	578**	0
Error	34	2.667	0.078431		
Total	50	728.977			

** significant @1% level of significance

Table 4. Scheffe's Pairwise comparisons test of the zones of inhibition of the different epibiotic bacterial isolates and commercial antibiotics against *S. aureus*.

Treatment	Mean zone of inhibition (mm) [1]	Homogenous group
Penicillin	14.33^{a}	a
Chloramphenicol	9.33^{b}	b
EB4	2.00^{c}	C
EB6	2.00^{c}	C
EB9	2.00^{c}	C
EB10	1.33^{cd}	cd
EB2a	1.00^{cd}	cd
EB2b	1.00^{cd}	cd
EB1	0.67^{cd}	cd
EB3a	0.00^{d}	d
EB3b	0.00^{d}	d
EB5a	0.00^{d}	d
EB5b	0.00^{d}	d
EB7a	0.00^{d}	d

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Table 4 (continue). Scheffe's Pairwise comparisons test of the zones of inhibition of the different epibiotic bacterial isolates and commercial antibiotics against *S. aureus*.

Treatment	Mean zone of inhibition (mm) [1]	Homogenous group
EB7b	0.00^{d}	d
EB8a	0.00^{d}	d
EB8b	0.00^{d}	d
F-value		578.78**
Probability		0.000

[1] Means with common letter superscript do not differ significantly by Scheffe's Pairwise comparisons test.

[a, b, c, cd, d] Group of epibiotic bacterial isolates and commercial antibiotic based on the zones of inhibition they produced against S. aureus.

** Significant @ 1% level

3.2. Antibacterial Activity Against *E. coli*

The result of the antibacterial activities of the metabolites of the different epibiotic bacterial isolates against *E. coli* is presented in **Table 5**, which shows that positive controls Chloramphenicol, Gentamycin, and EB6 have the highest zones of inhibition of 10 mm, 9.33mm, and 9 mm, respectively. The positive controls and EB6 outperformed the rest of the isolates. This significant superior effect is confirmed by the ANOVA in **Table 6** and Scheffe's Pairwise Comparisons Test in **Table 7**. The identification of *Vibrio alginolyticus* (EB6) with a zone of inhibition of 9.33 mm against *E. coli*, comparable to commercial antibiotics, underscores its potential in developing new antibacterial agents. These findings align with Dobretsov *et al*. (2003), who reported similar antibacterial activities in marine bacteria.

Table 5. Mean zones of inhibition of different soft coral epibiotic bacterial isolates and commercial antibiotics against *E. coli*.

	Treatment	Mean Zone of Inhibition against *E. coli* (mm)
1.	EB1	2.00
2.	EB2	1.67
3.	EB2a	1.33
4.	EB3a	2.00
5.	EB3b	2.00
6.	EB4	0.00
7.	EB5a	3.00
8.	EB5b	3.00
9.	**EB6**	**9.33**
10.	EB7a	2.00
11.	EB7b	2.33
12.	EB8a	2.00
13.	EB8b	2.00
14.	EB9	0.00
15.	EB10	2.00
16.	Chloramphenicol	10.00
17.	Gentamycin	9.00

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Table 6. Analysis of Variance of the zones of inhibition of the different epibiotic bacterial isolates and commercial antibiotics (positive control) against *E. coli*.

SV	DF	SS	MS	F-value	Probability
Treatment	16	464.08	29.005	369.81	0.000
Error	34	2.667	0.078		
Total	50	466.75			

** Significant @ 1% level of significance

Table 7. Scheffe's Pairwise comparisons test of the zones of inhibition of the different epibiotic bacterial isolates and commercial antibiotics against *E. coli*.

Treatment	Mean zone of inhibition (mm) [1]	Homogenous group
Chloramphenicol	10.00[a]	a
EB6	9.33[a]	a
Gentamycin	9.00[a]	a
EB5a	3.00[b]	b
EB5b	3.00[b]	b
EB7b	2.33[bc]	bc
EB7a	2.00[bc]	bc
EB1	2.00[bc]	bc
EB3a	2.00[bc]	bc
EB3b	2.00[bc]	bc
EB8a	2.00[bc]	bc
EB8b	2.00[bc]	bc
EB10	2.00[bc]	bc
EB2a	1.67[bc]	bc
EB2b	1.33[cd]	cd
EB4	0.00[d]	d
EB9	0.00[d]	d
F-value		369.81**
Probability		0.000

[1] Means with common letter superscript do not differ significantly by Scheffe's Pairwise comparisons test.

[a, b, bc, cd, d] Group of epibiotic bacterial isolates and commercial antibiotic based on the zones of inhibition they produced against S. aureus.

** Significant @ 1% level

3.3. Species Identification of EB6

The antibacterial assay shows that EB6 is the only epibiotic bacterial isolate having a zone of inhibition of 9.33 mm against *E. coli* which is statistically comparable to the positive control Chloramphenicol with a zone of inhibition of 10 mm and Gentamycin with 9.33 mm, as shown in the result of the Scheffe's Pairwise comparisons test depicted in **Table 7**.

The isolated bacteria EB6 was cultured in Marine Agar and Theosulfate Citrate Bile Salts Sucrose (TCBS) agar and was incubated at 30°C, and the morphology was determined. The bacterial isolate EB6 was further tested using several biochemical tests. Morphology of EB6 (**Table 8**) and the results of various biochemical tests (**Table 9**) revealed that EB6 was identified as *Vibrio alginolyticus*.

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Table 8. Morphology of bacterial Isolate on Marine Agar (MA) and Thiosulfate Citrate Bile Salts Sucrose (TCBS) Agar incubated at 30°C.

Isolate	Colony Morphology		Cell Morphology
	MA	TCBS	
EB6	White, creamy, smooth, convex colonies with entire margin	Yellow, shiny, smooth, circular, convex colonies with entire margin	Gram genitive, regular rods, arranged in singles, rarely in clusters, 0.80 – 1.25 µm

Table 9. Biochemical tests conducted.

Tests Conducted	Media and Reagents used	EB6
Oxygen requirement	Thioglycollate broth	Facultative Anaerobic
Indole Production	SIM Medium	-
Methyl Red test	MRVP broth/ Methyl Red Reagent	-
Voges Proskauer Test	MRVP broth/ VP Reagent	+
Citrate Utilization	Simmon's Citrate Agar	+
H_2S Production	SIM Medium	-
Motility	SIM Medium	+
Gelatin Liquefaction	Nutrient gelatin	+
Lysine Decarboxylase test	Lysine decarboxylase broth	-
Arginine Dehydrolase test	Arginine dehydrolase broth	-
Nitrate Reduction	Nitrate broth	+
Oxidase test	Oxidase reagent	+
Catalase Production	Hydrogen peroxide	+
Starch utilization	Starch agar/ Lugol's iodine	+
Urea Hydrolysis	Urea broth	-
Casein Hydrolysis	Casein agar	-
Tyrosine Utilization	Tyrosine agar	+
Lipid Utilization	Sieera's medium with Tween 80	+
Fermentation of:		
Glucose	Phenol red dextrose broth	+ acid, - gas
Sucrose	Phenol red sucrose broth	+ acid, - gas
Galactose	Phenol red galactose broth	- acid, - gas
Lactose	Phenol red lactose broth	- acid, - gas
Maltose	Phenol red maltose broth	+ acid, - gas
Dulcitol	Phenol red dulcitol broth	- acid, - gas
Assimilation:		
Arabinose	API 20 NE kit	-
Mannose	API 20 NE kit	+
Mannitol	API 20 NE kit	+
N-acetyl-glucosamine	API 20 NE kit	+
Gluconate	API 20 NE kit	+
Caprate	API 20 NE kit	-
Adipate	API 20 NE kit	-
Malate	API 20 NE kit	+

+ positive reaction

- negative reaction

Identification - *Vibrio alginolyticus*

- Very good identification – 99.3%

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4. CONCLUSION

There were ten (10) soft-coral species randomly collected which harbor epibiotic bacteria. A total of fifteen (15) epibiotic bacteria with distinct colony morphotypes were isolated from these soft coral species. Different epibiotic bacterial isolates reacted differently on various biochemical tests suggesting that each isolate belonged to a different species. Different epibiotic bacterial isolates show varied effects against S. aureus and E. coli. Commercial antibiotics (positive control) outperformed all the epibiotic bacterial isolates except for EB6, which demonstrated significant antibacterial activity against E. coli, comparable to commercial antibiotics. The identification of EB6 as Vibrio alginolyticus (shown in Tables 8 and 9) as a potent source of antibacterial agent against E. coli suggests its potential for developing new antibacterial drugs, which is crucial in the fight against antibiotic-resistant pathogens. These findings suggest that marine-derived bacteria could be valuable sources of new antibacterial agents, addressing the urgent need for novel antibiotics in combating resistant pathogens. The finding implies its potential applications in the field of biotechnology and medicine.

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6. AUTHORS' NOTE

The authors declare that there is no conflict of interest regarding the publication of this article. The authors confirmed that the paper was free of plagiarism.

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