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Characterization and Potentiating Effects of the Ethanolic Extracts of the Red Seaweed *Gracillaria* sp. on the Activity of Carbenicillin against *Vibrios*

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Cite This: *ACS Omega* 2022, 7, 46486−46493

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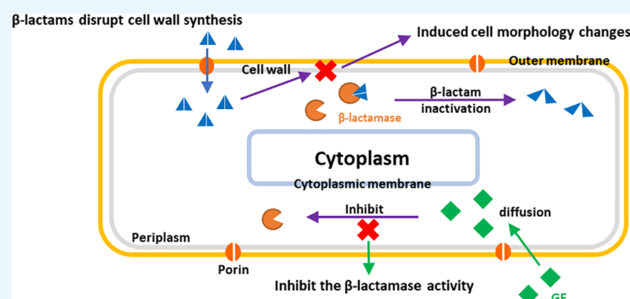

ABSTRACT: *β*-lactam-resistant *Vibrio* strains are a significant clinical problem, and *β*-lactamase inhibitors are generally coadministered with *β*-lactam drugs to control drug-resistant bacteria. Seaweed is a rich source of natural bioactive compounds; however, their potential as *β*-lactamase inhibitors against bacterial pathogens remains unknown. Herein, we evaluated the potential *β*-lactamase inhibitory effect of the ethanolic extracts of the red seaweed *Gracilaria* sp. (GE) against four *Vibrio* strains. The minimum inhibitory concentration, half-maximal inhibitory concentration, checkerboard assay results, and time-kill study results indicate that GE has limited antibacterial activity but can potentiate the activity of the *β*-lactam antibiotic carbenicillin against *Vibrio parahemolyticus* and *V. cholerae*. We overexpressed and purified recombinant metallo-*β*-lactamase, VarG, from *V. cholerae* for *in vitro* studies and observed that adding GE reduced the carbenicillin and nitrocefin degradation by VarG by 20% and 60%, respectively. Angiotensin I-converting enzyme inhibition studies demonstrated that GE did not inhibit VarG via metal chelation. Toxicity assays indicated that GE exhibited mild toxicity against human cells. Through gas chromatography and mass spectrometry, we showed that GE comprises alkaloids, phenolic compounds, terpenoids, terpenes, and halogenated aromatic compounds. This study revealed that extracts of the red seaweed *Gracillaria* sp. can potentially inhibit *β*-lactamase activity.

1. INTRODUCTION

Vibrio strains are ubiquitous in aquatic environments and pose a severe health threat to humans. To date, 70 *Vibrio* species have been identified. Of these, 13 are pathogenic, including *Vibrio parahemolyticus*, *V. vulnificus*, and *V. cholerae*.[1−3] Patients with vibriosis are clinically treated using antibiotics, including quinolones, cephalosporins, tetracyclines, and penicillins, with penicillins having the weakest effect.[4] Penicillin-resistant *V. parahemolyticus*[5] reportedly resists *β*-lactam antibiotics through the action of certain genes, including *acrE, crp, hns*, $bla_{CARB-17}$,[6] and bla_{CYM-2},[7] that encode efflux pumps or *β*-lactamase. The metallo-*β*-lactamase Vmh, identified in *Vibrio vulnificus*, exhibited broad degradation activity against *β*-lactam antibiotics, including penicillins, cephalosporins, and imipenems.[8] Moreover, reports show that *V. cholerae* can produce AmpC *β*-lactamase, carbapenemase,[8] and metallo-*β*-lactamases.[9] *β*-lactamases can hydrolyze the amide bond of the *β*-lactam ring, making the *β*-lactam antibiotics ineffective.[10] The Ambler classification, based on the amino acid sequence similarity, divides *β*-lactamases into four classes:[11] A, B, C, and D. Class A *β*-lactamases commonly hydrolyze penicillins, whereas class B *β*-lactamases, also known as metallo-*β*-lactamases, hydrolyze a broad spectrum of substrates, especially carbapenems.[12,13] Class C *β*-lactamases, known as cephalosporinases, catalyze cephalosporins better than penicillin. Class D *β*-lactamases, known as oxacillinases, hydrolyze oxacillin more efficiently than class A and C *β*-lactamases.[11]

Inhibitors with chemical structures based on the *β*-lactam ring, such as clavulanic acid (CLA), sulbactam, and tazobactam, are clinically used to inhibit class A *β*-lactamases. However, these inhibitors are ineffective against class B *β*-lactamases owing to the difference in catalytic mechanism.[14,15] Moreover, a few *β*-lactamase inhibitors have also been synthesized based on the structure of diazabicyclooctanes.[16] Recent studies indicate that inhibitors containing *β*-lactam ring result in the overexpression of *β*-lactamases.[17] Therefore, *β*-lactamase inhibitors with non-*β*-lactam ring structures can be

Received: August 17, 2022
Accepted: November 29, 2022
Published: December 9, 2022

ACS Publications

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Table 1. MIC and IC_{50} of *Gracilaria* sp. extracts and *β*-Lactam Antibiotics against *Vibrios*[a]

strains	CAR (μg/mL)		IMI (μg/mL)		CEP (μg/mL)		ATZ (μg/mL)		GE (μg/mL)		CLA (μg/mL)	
	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}
V. parahemolyticus 0577	256	128	<2	<2	8	4	<2	<2	>1000	>1000	>1024	>1024
V. parahemolyticus 1109O101	256	256	<2	<2	32	8	<2	<2	>1000	>1000	>1024	1024
V. parahemolyticus 1109O202	256	128	<2	<2	8	8	<2	<2	>1000	>1000	>1024	1024
V. cholerae CVD101	8	<2	<2	<2	<2	<2	128	<2	>1000	>1000	>1024	1024

[a]CAR = carbenicillin; IMI = imipenem; CEP = cephalothin; ATZ = aztreonam; GE = *Gracilaria* sp. extracts; CLA = potassium clavulanate.

Table 2. MIC and FICI of *Gracilaria* sp. Extract, Carbenicillin against *Vibrios*[a]

strains	MIC (μg/mL)			FIC (μg/mL)		FICI	
	CAR	GE	CLA	CAR + GE	CAR + CLA	CAR + GE	CAR + CLA
V. parahemolyticus 0577	256	>1000	>1024	64 + 7.8	64 + 8	0.26	0.26
V. parahemolyticus 1109O101	256	>1000	>1024	64 + 7.8	32 + 8	0.26	0.13
V. parahemolyticus 1109O202	256	>1000	>1024	16 + 7.8	16 + 8	0.07	0.07
V. cholerae CVD101	8	>1000	>1024	4 + 7.8	2 + 8	0.51	0.26

[a]CAR = carbenicillin; GE, *Gracilaria* sp. extract; CLA, potassium clavulanate.

used as alternatives.[18] Several natural compounds can reinforce the activity of antibiotics against resistant bacteria instead of exhibiting direct antibacterial activity. Studies have shown that plant extracts, such as *Cyperus rotundus*,[18] *Peganum harmala*,[16] *Berberis intergerrima*,[19,20] and *Stephania suberosa*,[21] could synergistically act with *β*-lactam antibiotics to inhibit *β*-lactamases.

Seaweed exhibits various beneficial biological activities, such as antioxidant, anticancer,[22] anti-inflammatory,[23] and antimicrobial[19,20] activities. Previous studies have reported that the methanol extracts from the seaweeds *Ulva intestinalis*, *Codium tomentosum*, *Dictyota dichotoma*, and *Halopteris scoparia* suppressed the activity of the *β*-lactamase GES-22 from *Acinetobacter baumannii*.[24] However, the mechanism underlying the synergistic effects of the combination of seaweed extracts and *β*-lactam antibiotics against bacteria remains unexplored. Therefore, we aimed to investigate the potential activity of the red seaweed *Gracilaria* sp. extract (GE) on the activity of *β*-lactam antibiotics against *Vibrio* strains and their possible inhibitory effects on the Ambler B *β*-lactamase.

2. RESULTS AND DISCUSSION

2.1. Minimum Inhibitory Concentration (MIC) and IC_{50} of the Ethanolic Extract of *Gracilaria* sp. and *β*-Lactam Antibiotics against *Vibrios*. *β*-lactams are one of the oldest classes of antibiotics for treating pathogen infections. They inhibit bacterial enzymes of the penicillin-binding proteins family, which play an essential role in synthesizing the bacterial cell wall. *β*-lactams inhibit cell wall synthesis and cause changes in bacterial morphology, such as elongation, bulge stagnation, and cell lysis. The antimicrobial activities of GE and *β*-lactam antibiotics against four *Vibrio* strains were evaluated by using the microdilution method. Table 1 presents the minimum inhibitory concentration (MIC) and IC_{50} values for *V. parahemolyticus* and *V. cholerae*. The MIC of carbenicillin (CAR), imipenem (IMI), cephalothin (CEP), and aztreonam (ATZ) for the three clinically isolated *V. parahemolyticus* strains were 256, <2, 8−32, and <2 μg/mL, respectively. The IC_{50} values of these three strains against CAR, IMI, CEP, and ATZ were 128−256, <2, 4−8, and <2 μg/mL, respectively. The MICs of CAR, IMI, CEP, and ATZ against *V. cholerae* were 8, < 2, < 2, and 128 μg/mL, respectively, whereas IC_{50} was <2 μg/mL against all the selected *β*-lactam antibiotics. Tan et al.[25] reported that 82.1% and 31.3% of 76 *V. parahemolyticus* strains were resistant to ampicillin and CEP, respectively, and all strains were sensitive to IMI. Ceccarelli et al.[26] indicated that 2% of the 178 clinically isolated *V. cholerae* strains were ampicillin-resistant, and 0%−2% were resistant to different generations of cephalosporins.

GE and CLA showed no antimicrobial activity against *V. parahemolyticus* and *V. cholerae* with MICs of >1,000 and >1,024 μg/mL, respectively. A previous study reported that the red seaweed *Gracilaria changii* shows no antimicrobial activity against Gram-positive and Gram-negative bacteria with a MIC of 3.13−6.25 mg/mL.[27] The ethanolic extracts of the brown seaweeds *Laminaria japonica* and *Sargassum horneri* and red seaweeds *Gracilaria* sp. and *Porphyra dentata* displayed mild antibacterial activities against the drug-susceptible *E. coli* strain Kam3.[28] However, GE did not exhibit similar inhibitory effects against the *V. parahemolyticus* and *V. cholerae* strains.

2.2. The Ethanolic Extract of *Gracilaria* sp. Potentiates Carbenicillin Activity against *Vibrios*. GE-mediated potentiation of CAR activity against *Vibrios* was evaluated by using checkerboard assays. The MIC of CAR against the *V. parahemolyticus* strain was decreased by 4- to 16-fold upon the addition of GE with a fractional inhibitory concentration (FIC) of 16−64 (CAR) + 7.8 (GE) μg/mL (Table 2).

This FIC result was similar to that of CLA, which has an FIC of 16−64 (CAR) + 8 (CLA) μg/mL. CLA alone exhibited no antibacterial effects and is known to inactivate *β*-lactamases through chemical reactions at their active sites.[29] CLA is a medication that is clinically used with *β*-lactam drugs to manage and treat bacterial infections, specifically for bacteria producing *β*-lactamases.[30] EUCAST[31] stated that synergistic, additive, indifferent, and antagonistic effects are observed when the fractional inhibitory concentration index (FICI) is ≤0.5, 0.5< FICI ≤ 1, 1< FICI < 2, and FICI ≥ 2, respectively. The combined use of CAR + GE and CAR + CLA demonstrated a FICI between 0.07−0.26, indicating the synergistic effects of both against *V. parahemoylticus*. Additionally, the MIC of CAR against *V. cholera* decreased 2-fold when GE was added with an FIC of 4 (CAR) + 7.8 (GE) μg/mL. The resultant FICI of CAR + GE was 0.51, indicating an additive effect against *V. cholerae*. Sasidharan et al. (2010) reported that *Pseudomonas*

https://doi.org/10.1021/acsomega.2c05288
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aeruginosa exposed to a methanol extract of marine algae *Gracilaria changii* with a MIC value of 6,250 μg/mL for 12 h exhibited a slightly rough cell appearance. However, the GE concentration used in this study (7.8 μg/mL) for the checkerboard and time-kill assay was markedly lower than the MIC (>1,000 μg/mL), suggesting that the effects of GE on bacterial morphology are limited.

Teethaisong et al.[32] used 95% ethanol to isolate the extract from the roots of *Stephania suberosa*. The extract showed a synergistic effect (FIC < 0.5) with ampicillin against ampicillin-resistant *Staphylococcus aureus* in the checkerboard assay. The ethanolic extract from *S. suberosa* also inhibited the degradation ability of class D β-lactamase from *Enterobacter cloacaei* by showing the presence of residual benzylpenicillin after the degradation reaction. Our data suggested that GE exhibited potentiating activity with CAR against *V. parahemolyticus* and *V. cholerae*. We further investigated this potentiating activity using time-kill and drug degradation assays.

2.3. Evaluation of the Effect of GE Using a Time-Kill Assay.

To determine the synergistic effect of GE with CAR over time, we monitored the dynamic changes in the viable counts of *V. parahemolyticus* 0577 and *V. cholerae* in CAR and CAR + GE using a time-kill assay. As shown in Figure 1A, *V. parahemolyticus* was allowed to grow until the log phase and then exposed to CAR, GE, or CAR + GE. The control group (untreated) reached the stationary stage at 9 h with a cell count of 8.8 Log CFU/mL, whereas the GE-treated group initially showed slow growth and reached the stationary stage at 9 h with a similar cell count (8.8 Log CFU/mL). In the CAR-treated group, the cell count decreased from 5.5 to 3.7 Log CFU/mL in the first 6 h and sharply rose to 8.4 Log CFU/mL at 12 h. In contrast, the number of bacteria in the CAR + GE-treated group gradually decreased and reached 4.1 Log CFU/mL after 12 h of incubation.

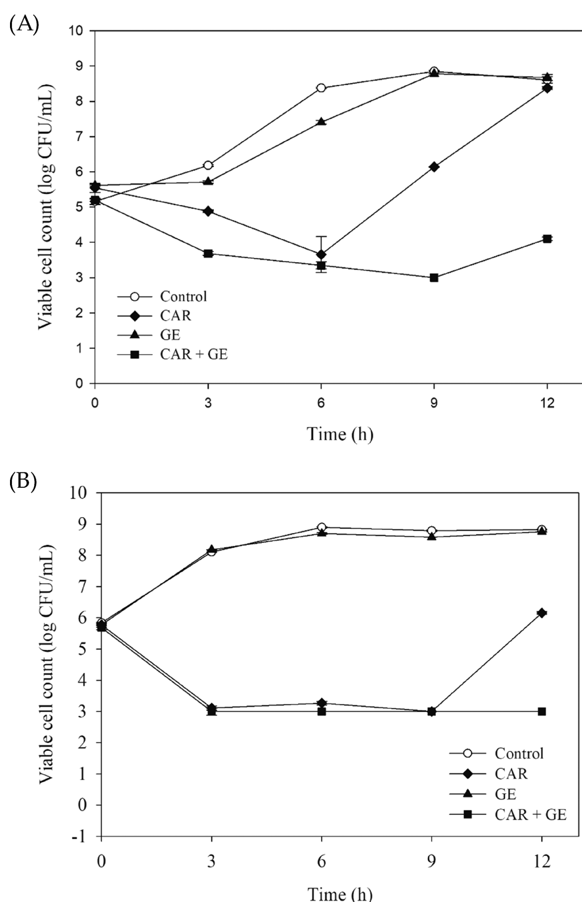

Figure 1. Time-kill curve of *Vibrios* with CAR, GE alone, or in combination. (A) *V. parahemolyticus* (B) *V. cholerae*. Data are expressed as mean ± SD ($n = 3$). CAR, carbenicillin; GE, *Gracillaria* sp. extract. For *V. parahemolyticus*, CAR (64 μg/mL) and GE (7.81 μg/mL); for *V. cholerae*, CAR (4 μg/mL) and GE (7.81 μg/mL).

Figure 1B presents the synergistic effect of GE with CAR over time on *V. cholerae*. *V. cholerae* cultures were allowed to reach the log phase and then exposed to CAR, GE, or CAR + GE. The growth pattern of the bacteria incubated with GE alone was similar to that of the control group, which reached the stationary stage at 6 h, with a cell count of 8.9 Log CFU/mL. The cell counts of the bacteria in the CAR-treated group decreased from 5.8 to approximately 3.1 Log CFU/mL in the first 3 h but increased to 6.16 Log CFU/mL at 12 h. Intriguingly, the cell counts of the CAR + GE-treated group decreased from 5.8 to approximately 3 log CFU/mL at 3 h and remained constant until 12 h.

Regrowth of bacteria cultured with antibiotics might occur because of the degradation of the antibiotics or the expression of proteins conferring drug resistance.[33] Using a time-kill assay, Siqueira et al. measured the translation levels of various genes in multidrug-resistant *Pseudomonas aeruginosa*. They reported that the regrowth of *P. aeruginosa* treated with 1/2 MIC Meropenem at 6 h increased with an increase in the expression of the β-lactamase gene, *ampC*. Keepers et al.[34] observed bacterial regrowth in *E. coli* and *Pseudomonas aeruginosa* cultures incubated with penicillin and cephalosporin at multiple times their MIC using a time-kill assay. Although adding β-lactamase inhibitors can retard bacterial regrowth, the inhibitory effect depends on the antibiotic-inhibitor combination and bacterial isolates. This suggests that the bacterial regrowth might be attributed to the β-lactamase-mediated degradation of the β-lactam antibiotics.

In this study, the CAR-treated group showed regrowth of *V. parahemolyticus* and *V. cholerae* at 6 and 9 h, respectively, similar to Keepers et al.[34] et al. The addition of GE and CAR deterred the regrowth of *V. parahemolyticus* and *V. cholerae*, possibly suppressing CAR degradation during cultivation.

2.4. Overexpression and Purification of the Recombinant β-Lactamases and Inhibitory Assay.

Previous studies have reported that metallo-β-lactamases VarG from *V. cholerae* confers resistance against penicillins, cephalosporins, and carbapenems.[35] To determine the possible inhibitory effect of GE against *V. cholerae*, a known β-lactamase, VarG, was overexpressed to determine its degradation activity on CAR and nitrocefin in the presence of GE. *E. coli* cells harboring the *varG* genes were induced with IPTG, and the crude VarG enzyme extracts were obtained through osmotic shock. VarG was further purified using nickel affinity chromatography and examined through SDS−PAGE. As shown in Figure S1, the prominent bands in lanes 3 and 4 represent VarG proteins with an approximate molecular weight of 43 kDa. We monitored the hydrolysis efficiency of VarG on CAR (1000 μg/mL) combined with various concentrations of GE (0.05−10 μg/mL) and observed that it decreased with an increase in GE concentration. The final relative activity at 10 μg/mL GE was 78% compared with that of VarG alone (Figure 2). This

https://doi.org/10.1021/acsomega.2c05288
ACS Omega 2022, 7, 46486−46493

indicated that GE mildly inhibited VarG-mediated CAR hydrolysis.

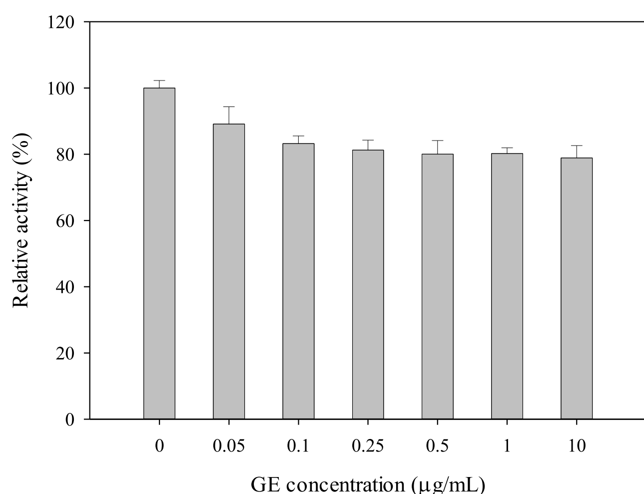

Figure 2. Effect of *Gracilaria* sp. extracts on VarG for carbenicillin degradation.

We further investigated the possible inhibitory effects of GE on VarG using the nitrocefin test, wherein β-lactamase-sensitive nitrocefin is rapidly degraded and observed as a visible color change.[36] EDTA, a known metal chelator, was used as the positive control. We monitored the degradation of nitrocefin by VarG for 25 min in the presence and absence of EDTA and GE. As shown in Figure 3, the hydrolysis efficiency

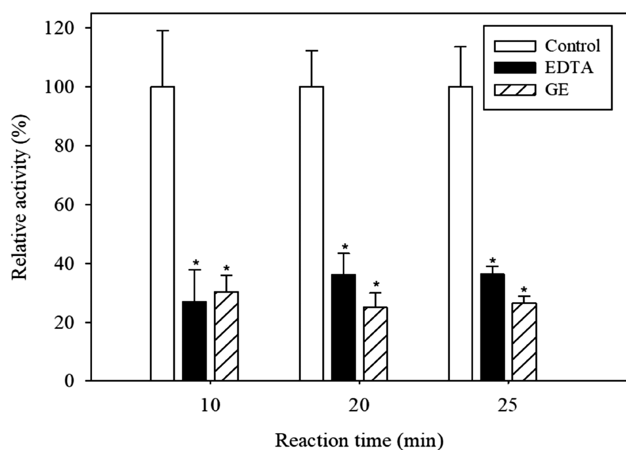

Figure 3. Effect of *Gracilaria* sp. extracts on VarG. Using nitrocefin as a substrate, and the variety of OD486 was measured. EDTA was used as positive control with the concentration of 1 mM. GE was used with the concentration of 1 μg/mL. All treatments were performed in triplicate and expressed as mean ± SD (n = 3). Significantly different between different groups and control are showed with *.

of VarG on nitrocefin significantly decreased in the presence of GE to 26%−40% relative activity compared with the control. Moreover, EDTA demonstrated higher inhibitory effect than the control.

Our data showed that VarG degraded nitrocefin and carbenicillin at different rates, suggesting a substrate-dependent inhibition mechanism. The proposed mode of action for the GE potentiating the activity of carbenicillin against *Vibrios* have been illustrated (Figure S2). β-lactam antibiotics can pass through the porins on the outer membrane of bacteria and interfere with cell wall synthesis, therefore inducing cell morphology changes and causing bactericidal activity against susceptible microorganisms. Some bacteria are capable of producing β-lactamases to inactivate β-lactam antibiotics and become resistant. The addition of GE could possibly retard the activity of bacterial β-lactamases and therefore potentiate the bactericidal activity of β-lactam antibiotics.

A previous study reported that 70% ethanolic extracts of *Hibiscus cannabinus* inhibited the metallo-β-lactamase NDM-1 with an IC_{50} of 0.5 μg/mL.[37] Yang et al.[38] identified the methanolic extracts of *Fissistigma cavaleriei* as salicylsalicylic acid, which inhibited β-lactamase from *Pseudomonas aeruginosa* with an IC_{50} of 18.5 μg/mL. Ju et al.[39] classified the mechanism of the metallo-β-lactamase inhibitors into four groups, including the binding with metals, forming covalent bonds with β-lactamase, allosteric inhibition, and others. Of these, metal binding is the most common inhibition strategy. These inhibitors either remove metal ions from the active sites or combine with the metal ions and surrounding residues to prevent the entry of antibiotics. Based on this, we investigated whether the inhibitory effect of GE on VarG was mediated via metal chelation.

2.5. ACE Inhibition Assay and Cytotoxicity Test of *Gracilaria* sp. Extracts. As zinc ions are required for metallo-β-lactamase activity, we investigated whether GE reduced VarG's activity by chelating the zinc ions. Angiotensin I-converting enzyme (ACE) is a zinc-dependent protease that catalyzes the conversion of angiotensin I to a potent vasoconstrictor angiotensin II. As this metalloenzyme requires zinc ions for its activity, we evaluated its activity in the presence of GE to investigate whether GE exerted its inhibitory effect via metal chelation.

Using captopril, a known competitive inhibitor of ACE, as the positive control, we measured the activity of ACE in the presence of GE (1 and 10 μg/mL) and ACE inhibitor captopril (10 μg/mL) (Figure 4). In the ACE assays, captopril significantly decreased the relative activity compared to the control. At the same time, GE showed no inhibitory effect on

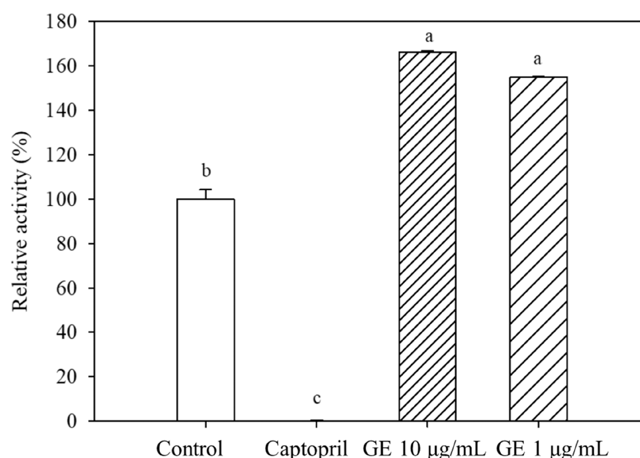

Figure 4. Effect of *Gracilaria* sp. extracts on ACE. Hippuryl-histidyl-leucine (HHL) was used as a substrate, and hippuric acid (HA) was measured at OD228. Captopril was used as positive control with the concentration of 10 μg/mL. All treatments were performed in triplicate and expressed as mean ± SD (n = 3). Different letters are show significantly different between groups.

https://doi.org/10.1021/acsomega.2c05288
ACS Omega 2022, 7, 46486−46493

ACE (Figure 4), suggesting that GE is not a metal chelator. Hence, we conclude that GE does not inhibit the activity of metallo-β-lactamase VarG via metal chelation.

The *in vitro* cytotoxicity of GE was evaluated by monitoring the cell viability of the human liver cells, HepG2, at various concentrations of GE. Each GE sample was dissolved in ethanol, and ethanol alone was used as the vehicle control (0 μg/mL of GE). Figure 5 shows that the cell viability at 1.95, 3.91, 7.81, 15.63, and 31.25 μg/mL of GE was 85.2% ± 6.3%, 72.9% ± 5.5%, 63.4% ± 5.0%, 51.3% ± 4.5%, and 45.5% ± 6.3%, respectively, compared with that of the control group. The IC_{50} of GE in HepG2 cells was >15.63 μg/mL.

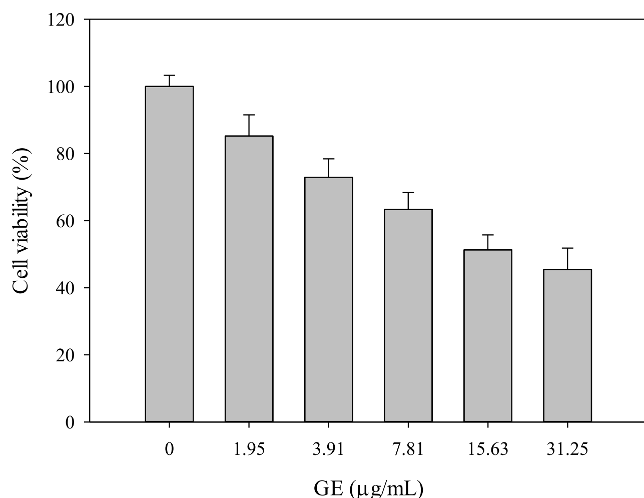

Figure 5. Cytotoxicity test of *Gracilaria* sp. extracts in human hepatic HepG2 cells. Data are expressed as mean ± SD (n = 3).

Cotas et al.[40] demonstrated that tetrasporophyte and female gametophyte carrageenan extracts from the red seaweed *Gigartina pistillata* markedly reduced the cellular viability of human colorectal cell lines, SW620 and SW480 to under 50% at a concentration of 33 μg/mL and 100 μg/mL, respectively. Yerlikaya et al.[41] observed no significant change in the viability of human Hela cells when incubated with three *Ononis natrix* extracts (water, methanol, and ethyl acetate) at concentrations ranging from 0.1 μg to 10 mg. However, cytotoxic activity in PC3 cells was observed with 2.5 mg water and 1 mg methanol extracts. Our cytotoxicity data showed that the IC_{50} of GE was >15.63 μg/mL in human HepG2 cells. Our checkerboard data indicated that the GE could potentiate the activity of carbenicillin against *V. parahemolyticus* and *V. cholera* at a concentration of 7.8 μg/mL, suggesting that GE reinforces the activities of the β-lactam antibiotics at its subhalf inhibitory concentration.

2.6. Compositions Analysis of the *Gracilaria* sp. Extracts. As shown in Table 3, the GC-MS analysis of GE revealed the abundance of alkaloids, phenolic compounds, terpenoids, terpenes, and halogenated aromatic compounds at 92.87%, 5.44%, 1.05%, 0.37%, and 0.26%, respectively.

Several plant-derived β-lactamase inhibitors have been characterized. Yang et al.[38] identified that the root extracts of *Fissistigma cavaleriei* contained salicylsalicylic acid, a type of phenolic acid, which inhibited the β-lactamase activities of a clinical *Pseudomonas aeruginosa* strain G19 in a dose-dependent manner. Chandar et al.[37] identified six ethanol extracts from medicinal plants that reduced the enzymatic activity of NDM-1 from 40% to 77% when degrading nitrocefin and showed a synergistic effect when combined with colistin, Meropenem, and tetracycline. Additionally, phytochemical analysis revealed the presence of several active compounds, including phenolic compounds, terpenoids, alkaloids, glycosides, steroids, flavonoids, and saponins, in these extracts. Our MIC and checkerboard assay results (Table 2) indicate that certain antibacterial substances from the seaweed extracts restored the activity of carbenicillin and exerted an inhibitory effect on metallo-β-lactamase VarG. This inhibitory effect might be due to a single compound in GE or the combined effect of the various GE components. Further chromatographic purification of GE and evaluation of the inhibitory effects of the purified compounds are required for further clarification.

3. CONCLUSIONS

β-lactamase inhibitors are increasingly used as part of novel drug combinations to treat resistant β lactamase-resistant

Table 3. Classification of GC-MS Analysis of the Composition in *Gracilaria* sp. Extracts

classification	percentage (%)	subclasses	compositions of *Gracilaria* sp. extracts	retention time (min)
alkaloids	92.87			
			3′-methylphthalanilic acid	5.80
			7H-dibenzo (a,g) carbazole, 12,13-dihydro-	7.46
		phenylethylamine alkaloids	acetamide, N-(2-hydroxyphenyl)-	5.34
		alkaloids	colchicine	12.37
		pyrimidines	sulfameter	4.95
phenolic compounds	5.44			
		simple phenol	phosphoramidic acid, phenyl-, diphenyl ester	8.96
		simple phenol	p-pentylacetophenone	9.10
		simple phenol	2,4-di-*tert*-butylphenol	12.71
		simple phenol	fenipentol	8.50
terpenoids	1.05			
		monoterpenoids	2,6-di-*tert*-butyl-1,4-benzoquinone	11.92
		monoterpenoids	benzaldehyde, 3,4,5-trimethoxy-	13.01
	0.37			
		terpenes	cyclohexene, 1-methyl-4-(1-methylethylidene)-	6.55
halogenated aromatic compounds	0.26			
		benzenes	benzene, (1-bromoethyl)-	5.83

https://doi.org/10.1021/acsomega.2c05288
ACS Omega 2022, 7, 46486−46493

bacteria. The ethanolic *Gracilaria* sp. extracts could potentiate the activity of carbenicillin against *Vibrio* strains. It also inhibited the activity of metallo-β-lactamase VarG from *V. cholerae*. Additionally, we found that GE did not inhibit the metallo-β-lactamase activity via metal chelation and exhibited limited cell toxicity. In conclusion, further investigations are warranted that focus on GE purification, identification of purified fractions/compounds for β-lactamase inhibition, constant inhibitor determination, and cytotoxicity tests to harness the ability of GE to block β-lactamases and β-lactamase-producing pathogens.

4. MATERIALS AND METHODS

4.1. Bacterial Strains, Media, and Antibiotics. *V. parahemolyticus* 0577 was isolated from food poisoning patients by Dr. Hin-Chung Wong's laboratory, Department of Microbiology of Soochow University, Taiwan. *V. parahemolyticus* 1109O101 and 1109O202 were isolated from oyster by Dr. Hsin-I Hsiao's laboratory, Department of Food science of National Taiwan Ocean University, Taiwan. *V. cholerae* CVD101 was obtain from Dr. Adrian Robert Walmsley's laboratory, Department of Biosciences of Durham University, United Kingdom. Carbenicillin (CAR), cephalothin (CEP), imipenem (IMI), potassium clavulanate (CLA), hipury-histidyl-leucine (HHL), and angiotensin-converting enzyme (ACE) were obtained from Sigma-Aldrich, USA. Aztreonam (ATZ) was obtained from Bionovas, Canada. Nitrocefin was obtained from Toronto Research Chemicals, Canada. The antibiotics were purchased with purity levels exceeding 98%, and other compounds were 95%. Mueller-Hinton (MH) broth was obtained from Formedium, U.K.

4.2. Preparation of Ethanolic Extracts from Seaweed. The fresh *Gracilaria* sp. was bought in Taiwan. The seaweed was washed, air-dried and ground into a powder with a superfine pulverizer. The powder was extracted by 95% ethanol at room temperature for 24 h two times before lyophilization. The solid ethanolic *Gracilaria* sp. extracts (GE) were obtained by rotary evaporation in lyophilization and redissolved in 95% (v/v) ethanol to a final concentration of 50 mg/mL. The GE stock was stored in the dark at −20 °C before use.

4.3. Minimal Inhibitory Concentration (MIC) and Half-Maximal Inhibitory Concentration (IC_{50}) Determinations. MIC and IC_{50} of CAR, IMI, CEP, ATZ, CLA, and GE against *Vibrios* were performed according to Lu et al.[42] In brief, a 96-well microplate containing two-folds serial dilution of antibiotics, CLA or GE with cultures that were adjusted with MH broth to obtain a final concentration of 5.5×10^5 CFU/mL. A serial dilution of the tested antibiotics and GE in MH medium was performed from column 1 to 12 in a 96 well plate. The microplates were incubated at an appropriate temperature for 12 h. The bacterial growth is determined at OD600, and defined MIC as the lowest concentration of antibiotic that no visible bacterial growth while IC_{50} as the lowest concentration of antibiotic that inhibits 50% the bacterial growth.

4.4. Checkerboard Assays. The synergistic effect between antibiotics and CLA or GE was determined using checkerboard assay following the method described by Eumkeb et al.[43] In brief, a 96-well microplate containing a two-fold serial dilution of antibiotic combined with CLA or GE from MIC, respectively. A serial dilution of the tested antibiotics in MH medium was performed from row A to H in a 96-well plate, and a serial dilution of GE was performed from column 1 to 12. The MIC determination was described in section 4.3. The fractional inhibitory concentration index (FICI) was calculated for the interaction between two compounds as the following equation:

$$\mathrm{FICI} = \mathrm{FIC_A} + \mathrm{FIC_B}$$

$$\mathrm{FIC_A} = (\mathrm{MIC_A}\ \text{in combination})/(\mathrm{MIC_A}\ \text{alone})$$

$$\mathrm{FIC_B} = (\mathrm{MIC_B}\ \text{in combination})/(\mathrm{MIC_B}\ \text{alone})$$

When FICI ≤ 0.5 represents as synergistic; $0.5 < \mathrm{FICI} \leq 1.0$ represents as additive; $1.0 < \mathrm{FICI} < 2.0$ represents as indifference; FICI ≥ 2.0 represents antagonism.[31]

4.5. Time-Kill Assay. A time-kill assay was performed to observe the variation in the bacterial count of GE when used alone and in combination with CAR following the method of Michael et al.[44] In brief, the cultures were adjusted with MH broth to obtain a final concentration of 10^5 CFU/mL before exposure to CAR, GE, or combination. All testing groups were incubated in MH broth with different agents and diluted in peptone after removing 100 μL for plate counting at five different time points (0, 3, 6, 9, 12 h). The colonies were counted after incubating the plates at an appropriate temperature for 12 h. The adequate number of colonies was 25−250; the lowest detectable counting was 10^3 CFU/mL. For *V. parahemolyticus*, the concentration of CAR and GE was 64 μg/mL and 7.81 μg/mL; for *V. cholerae*, the concentration of CAR and GE was 4 μg/mL and 7.81 μg/mL.

4.6. β-Lactamase Purification. The gene of *varG* was cloned into pET-26b(+) (Novagen) as in the previous study by Lin et al.[35] before transforming into *E. coli* C43 (DE3). The *E. coli* C43 (DE3) harboring VarG cells was induced by IPTG at 24 °C for 12 h. The cells were collected by centrifugation (5000 × g, 4 °C, 10 min) and disrupted cells by osmotic shock. The cultures of C43/pET26b-*varG* were resuspended with ice-cold resuspension buffer containing 30 mM Tris base (pH 8), 1 mM EDTA, and 20% sucrose, kept on ice for 20 min, then harvested by centrifugation (5000 × g, 4 °C, 10 min). As described above, the cell pellet was resuspended with ice-cold 5 mM $MgSO_4$, kept on ice for 20 min, then harvested by centrifugation (5000 × g, 4 °C, 10 min), and the supernatant was collected. The buffer solubilized VarG were purified by using nickel affinity column (HiTrap chelating column, GE Healthcare). The purified VarG were stored at −80 °C for further experiments.

4.7. β-Lactamases Inhibitory Assay. The inhibitory effect on β-lactamases was carried out following the method of Houchi et al.[24] and Liu et al.[45] Using nitrocefin as a substrate of the hydrolysis reaction and detected the increasing absorbance at OD486. β-lactamases were preincubated with inhibitors or GE at room temperature for 5 min before adding 50 μM nitrocefin. The relative activity of control was defined as 100%, which reacted without inhibitors. The β-lactamase inhibitory activity of purified VarG was monitored by the decrease in β-lactam absorbance that results from the opening of the β-lactam ring during hydrolysis. The reactions were performed at 25 °C in a mixture containing purified VarG, 50 mM MOPS (pH 7.5), 50 mM $ZnSO_4$, and GE (0.05−10 μg/mL), and the decrease in absorbance was monitored. The extinction coefficients and measured wavelength were 400 M^{-1} cm^{-1} and 240 nm for carbenicillin.

4.8. ACE Inhibition. The inhibitory effect on ACE was carried out following the method of Jimsheena and Gowda.[46] Using HHL as a substrate, and measured the absorbance at

https://doi.org/10.1021/acsomega.2c05288
ACS Omega 2022, 7, 46486−46493

OD228 after the products of hippuric acid (HA) and (HL) were separated with HPLC. The relative activity of control was defined as 100%, which reacted without inhibitors.

4.9. GC-MS. The compositions of GE were analyzed using gas chromatography−mass spectrometry (GC-MS) with PolarisQ Ion Trap GC-MS/MS system (Thermo Scientific, Waltham, Massachusetts, U.S.A.), and Equity-5 column with 30 m × 0.25 mm i.d. × 0.25 μm film thickness was used. Helium was used as mobile phase, with the splitting ratio of 10:1, while the temperature of sampling was set at 280 °C. The oven was kept at 50 °C for 2 min, before increasing to 250 °C in a speed of 15 °C/min. The data were identified using the software AMDIS of National Institute of Standards and Technology (NIST).

4.10. Statistical Analysis. All data were statistically analyzed using software of IBM SPSS Statistics (IBM, Armonk, New York, U.S.A.) and expressed as mean ± SD. One-way analysis of variance (ANOVA) was used to determine the statistical differences between groups and conducted using the Tukey's HSD test with the level of significance set at $p < 0.05$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c05288.

SDS-PAGE of VarG; the proposed mode of action of GE (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Center of Excellence for the Oceans, National Taiwan Ocean University from The Featured Areas Research Center Program within the framework of the Higher Education Sprout Project by the Ministry of Education (MOE) in Taiwan (NTOU-RD-AA-2021-1-02018).

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ACS Omega 2022, 7, 46486−46493