HOSTED BY
ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Short communication http://dx.doi.org/10.1016/j.apjtb.2015.07.012

In vitro antimicrobial activities of methanolic extract from marine alga *Enteromorpha intestinalis*

Darah Ibrahim, Sheh-Hong Lim*

Industrial Biotechnology Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia

ARTICLE INFO

Article history:
Received 31 Mar 2015
Received in revised form 1 Jun 2015
Accepted 15 Jun 2015
Available online 5 Aug 2015

Keywords:
Antibacterial activity
Enteromorpha intestinalis
Marine algae
Methicillin-resistant
Staphylococcus aureus

ABSTRACT

Objective: To extract the bioactive compound from *Enteromorpha intestinalis* (*E. intestinalis*) and determine its *in vitro* antimicrobial activity.
Methods: *E. intestinalis* was extracted by methanol and subjected to antimicrobial screening. The antimicrobial activity was studied by using disc diffusion and broth dilution method. The effect of the extract on the growth profile of the bacterial was also examined via time-kill assay. Microscopy observations using SEM was done to determine the major alterations in the microstructure of methicillin-resistant *Staphylococcus aureus* (MRSA).
Results: The results showed methanolic extract of *E. intestinalis* exhibited a favourable antimicrobial activity against tested bacteria with produced inhibition zone ranging from 8.0 to 19.0 mm. However, all the tested fungi and yeast were resistant to the extract treatment. Time kill assay suggested that methanolic extract of *E. intestinalis* had completely inhibited MRSA growth and also exhibited prolonged antibacterial activity. The main abnormalities noted from the microscopic observations were the structural deterioration in the normal morphology and complete collapsed of the bacteria cells after 36 h of treatment.
Conclusions: The significant antibacterial activity shown by crude extract suggested its potential against MRSA infection. The extract may have potential to develop as anti-bacterial agent in pharmaceutical use.

1. Introduction

Various therapeutic benefits available in natural products interest researchers to search for alternatives to combat the rising prevalence of global antimicrobial resistance problems. Moreover, concerns on the safety of some chemical in drugs have prompted an increased great interest in natural additives.

Marine organisms such as algae offer unlimited source of new agents for pharmaceutical industries. Recently, the ability of marine algae to produce secondary metabolite has been extensively documented [1,2]. Today, marine algae have been shown to possess substances with therapeutic values [3,4]. Algae extracts have been investigated, which contains antimicrobial, cytotoxic activity, antitumour and anti-inflammatory properties [5,6]. The antimicrobial agents obtained from marine algae such as chlorellin derivatives, acrylin acid, halogenated aliphatic compounds, phenolic inhibitors were observed to inhibit the growth of microorganisms [7]. Therefore, enhanced screening method for detection of antimicrobial activity and advanced technology in oceanography is necessary to help in promoting the studies in this area. The continuous research for new antimicrobial agent from marine algae is considered an alternative to overcome the emergence of multi-drug resistance organisms and the higher risk of infectious diseases caused by these organisms.

Enteromorpha intestinalis (*E. intestinalis*) is a marine alga and has a wide distribution around Peninsular Malaysia. This alga belongs to the family of Ulvaceae. *E. intestinalis* is often one of the first macroalgae to colonize newly cleared surface on rocky shores, tide pools, in estuaries, and on the hauls of ships passing from salt water to freshwater [8]. In this communication, we report the antibacterial activity of the preparations of methanolic crude extract of *E. intestinalis* and the effect of this extract on the alteration of MRSA cells by microscopy study.

*Corresponding author: Lim Sheh-Hong, Industrial Biotechnology Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia.
Tel: +60 12 4629487
Fax: +60 4 6565125
E-mail: limshehhong77@gmail.com
Peer review under responsibility of Hainan Medical University.

2. Materials and methods

2.1. Preparation of the extract

E. intestinalis was collected from the dock at Gelugor fishing village, Penang, Malaysia, during low tide period. The algal samples were washed thoroughly with running tap water and left to dry for 4–7 days at ambient temperature. The whole parts of the algae were cut into small pieces approximately 1.0 cm and use for extraction. Extraction was carried out according to the method described by Lim and Darah [9]. The 100 g of small pieces of algae were soaked in 1 000 mL of methanol for 3 days at room temperature (30 ± 2) °C. Stirring the mixture to ensure homogeneity during the extraction process. The mixture was filtered by using filtered paper (Whatman No. 1, United Kingdom). The filtrate was obtained then evaporated to obtain a dark oily paste of concentrated extracts.

2.2. Microorganisms and cultural maintenance

All the microorganisms used in this study were obtained from the stock cultures of Industrial Biotechnology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia. The bacterial strains were grown and maintained on nutrient agar slants (Oxoid, United Kingdom) at 37 °C. However, fungi and yeast were maintained on potato dextrose slants (Oxoid, United Kingdom) at 30 °C. All the stock cultures were stored at 4 °C for further use.

2.3. Antimicrobial activity test

Disc diffusion method was applied in this test. Inoculum containing 1×10^8 cells/mL were added to molten nutrient agar and distributed uniformly. When the agar was solidified, sterilized antibiotic disc (Whatman, United Kingdom) were placed on the agar and 20 μL of each extract at concentration of 50 mg/mL were pipetted onto it. Similarly, each plate was placed with sterile antibiotic disc by using chloramphenicol (30 μg/mL) or ketoconazole (30 μg/mL) as positive control and solvent as negative control. The inoculated plates were then incubated at 37 °C for bacteria and 30 °C for fungi and yeast. Clear zones of growth inhibition indicated antimicrobial activities were present.

2.4. Determination of minimal inhibitory concentration (MIC)

The MIC values were studied for the microorganisms that were susceptible in the antimicrobial activity test. MIC value of the methanolic *E. intestinalis* extract was determined by broth dilution assay method [10]. Briefly, extracts were subjected to a serial dilution to give final concentrations between 0.39 and 50.00 mg/mL. Extracts with different concentration were added aseptically into different labelled test tubes containing 1.5 mL sterile nutrient broth. Then, 0.5 mL of the bacteria suspension with 1×10^8 cells/mL were inoculated into the respective test tubes. The inoculated test tubes were incubated at 37 °C overnight. The lowest dilution of the tube that showed no visual turbidity was determined as the MIC value.

2.5. Time kill assay

The effect of *E. intestinalis* methanolic extract was observed on MRSA. This experiment was performed using the modified method of Lim and Darah [9]. A total of 250 mL Elenmeyer flasks containing 50 mL of extracts and nutrient broth were prepared with final concentration of half MIC, MIC and two times MIC. One millilitre of inoculums containing 1×10^8 cells/mL was added, and incubated at 37 °C, with shaking at 150 r/min for 48 h. Every 4 h intervals, about 4 mL of the mixture was withdrawn and the optical density (OD) was read at 540 nm.

2.6. Morphological study of the bacteria cell after exposure to the extract

Scanning electron microscope observations were carried out on MRSA cells. One millilitre of the MRSA cell suspension at a concentration of 1×10^8 cells/mL was inoculated on a nutrient agar plates and then incubated at 37 °C for 4 h. The extract (2 mL), at a concentration of 6.25 mg/mL (MIC), was then dropped onto the inoculated agar and was further incubated for another 36 h at the same incubation temperature. A 1% dimethyl sulfoxide (v/v) treated culture was used as control. A small block of bacteria containing agar was withdrawn from the inoculated plates at various time intervals (0, 12, 24 and 36 h) and were fixed for scanning electron microscopy (FESEM LEO Supra 50 VP, Carl Zeiss, Germany) study [11].

2.7. Statistical analysis

The triplicate data obtained were subjected to an analysis of a completely random design using SPSS (version 12.0) software (SPSS, Chicago, IL, USA). Statistical significance was assumed at the 0.05 levels ($P < 0.05$).

3. Results

The antimicrobial activity of *E. intestinalis* methanolic extract was shown in Table 1. Among the tested microorganisms only *Bacillus cereus* (*B. cereus*), *Bacillus subtilis* (*B. subtilis*) and methicillin-resistant *Staphylococcus aureus* (*S. aureus*)

Table 1
Antimicrobial activity of methanolic extract of *E. intestinalis*.

Microorganisms		Antimicrobial activity		
		ME	PC	NC
Bacteria	*B. cereus*	+	+++	-
	B. subtilis	+	+++	-
	Escherichia coli	-	++	-
	Klebsiella pneumoniae	-	+++	-
	Pseudomonas aeruginosa	-	+++	-
	S. aureus	-	+++	-
	Salmonella sp.	-	-	-
	MRSA	++	+++	-
	Citrobacter freundii	-	+++	-
Yeast	*Candida albican*	-	++	-
	Candida utilis	-	++	-
Fungi	*Penicillium* sp.	-	+	-
	Microsporum gypseum	-	++	-
	Rhizopus sp.	-	+	-
	Trichoderma viride	-	+	-

ME: Methanolic extract; PC: Positive control; NC: Negative control; Activity was classified according to the diameter of the inhibition around the point of application of the disc (+++: ≥15.0 mm; ++: >9.0 mm – 15.0 mm; +: ≤9.0 mm; -: No activity).

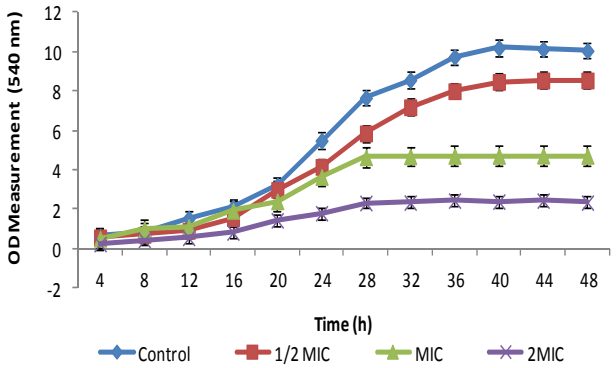

Figure 1. Growth profile of MRSA after exposure to the methanolic extract of *E. intestinalis*.

(MRSA) were susceptible to the extract. The inhibition zone produced by the commercial antibiotic disk was larger than that produced by the extract disk ($P < 0.05$).

The MIC values of *E. intestinalis* extract against three species of bacteria showed positive results in antimicrobial screening test. The results revealed that the lowest MIC value on MRSA was about 6.25 mg/mL. However, the MIC values on *B. cereus* and *B. subtilis* were 12.5 mg/mL. Thus, further study was concentrated on MRSA since it showed lower MIC value at 6.25 mg/mL and also known as a pathogen on humans, which causes urinary tract infections, open wounds infection, respiratory system infections and a variety of systemic infections.

The growth profile of the selected test microorganism, MRSA after treated with the methanolic extract of *E. intestinalis* at different concentrations, was shown in Figure 1. This study was carried-out over a period of 48 h with the bacteria cells exposed to MIC value, half of the MIC value and twice of the MIC value of the extract. At $^1/_2$MIC (3.13 mg/mL) value, the methanolic extract of *E. intestinalis* exhibited a drop in OD of the growth profile of MRSA after 36 h of exposure, which led to the stationary phase of the bacteria growth compared to the control. However, at the MIC (6.25 mg/mL) and 2MIC (12.5 mg/mL) values, the methanolic extract of *E. intestinalis* produced absolute bacteria eradication after 28 h of exposure. The time-kill curves described that the methanolic extract of *E. intestinalis* has potency to inhibit MRSA cells (bacteriostatic) at lower concentration with $^1/_2$MIC value of 3.13 mg/mL and to kill the cells (bacteriocidal) at concentrations of MIC (6.25 mg/mL) and above.

The effect of methanolic extract of *E. intestinalis* of 6.25 mg/mL (MIC) on MRSA cells were clearly observed under SEM. Figure 2A shows the control bacterial cells which were not exposed to the extract. The MRSA cells in the control were tightly packed smooth cocci undergoing cell division. A progressively increasing number of cells were seen to be degenerating over 12 h of exposure, progression of changes occurred was characterized by the appearance of discrete blebs on the cell surface (Figure 2B). After 24 h of treatment the formation of pores on the corrugated cells was seen (Figure 2C). As for the final incubation period (Figure 2D), dramatic lysis was apparent on the cell. Almost complete destructions of the cells were observed. At this point, the cells have lost its metabolic functions completely.

4. Discussion

In this study methanolic extract of *E. intestinalis* exhibited inhibition zones on some tested bacterial and none on tested fungi and yeast. These findings demonstrated that antibacterial compounds presence in that extract concentration when using methanol as an extraction solvent. The results were agreeable with the findings reported by Sasidharan *et al.* [12]. Moreover, Manivannan *et al.* [13] have reported solvents extraction systems were important in obtaining significant antibacterial

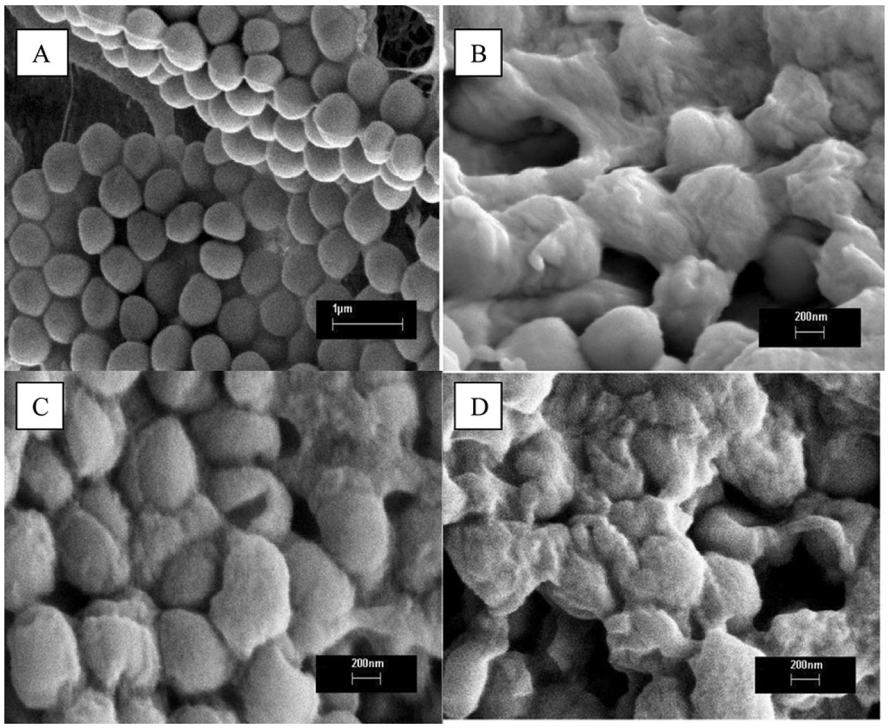

Figure 2. SEM micrographs of MRSA treated with crude methanolic extract of. *E. intestinalis*.
A: Control; B: Exposure for 12 h; C: Exposure for 24 h; D: Exposure for 36 h.

activity from plants including algae. According to Imbs *et al*. [14], antimicrobial activities of extracts that varied considerably between assay microorganisms also suggest that the microbial growth inhibition was mediated by a variety of antimicrobial compounds.

In present study, the MIC values ranged from 6.25 to 12.5 mg/mL. Sahgal *et al*. [15] attributed the differences of the MIC value could be due to the morphological structure of the bacterial cells and their composition in the cells. The results also showed that the higher concentrations of extract, the greater the effect on the bacterial growth inhibition occurred. The reduction in growth possibly occurred due to interference by active compounds in the extract [16]. Similarly, Lim *et al*. [10] and Darah *et al*. [17] reported that the higher concentration of the extract was needed to kill the microorganisms cells than to inhibit the growth of these cells on time-kill profile study.

According to Darah *et al*. [18], many studies were reported to investigate the mechanism of actions involved in bacterial killing process. Among them are the interactions of antimicrobial compound with the cell membrane. The ultrastructural changes in the bacterial cell produced by antibiotics are the expression of profound biochemical alterations [19]. According to Braga and Ricci [20], the integrity of cell wall and bacterial structure are imperative in maintaining the vitality and the virulence of bacteria. Based on SEM findings, it can be suggested that the cells had undergone some distinct morphological and cytological alterations. The same phenomenon was also observed on *S. aureus* after exposed to fosfomycin [21]. MRSA cells after treated with the extract for 36 h were seen to be a conglomerate of structurally collapsed and lysed. The cells were deformed evidently and lost their metabolic function would lose their virulence at this stage [21]. In fact with micrograph from SEM, crude extract of *E. intestinalis* were capable of collapsing the MRSA cells with the antimicrobial compounds it borne.

In this work, conclusively demonstrates the antibacterial potential of methanolic extract from *E. intestinalis*. This extract is proven to be bacteriostatic and bactericidal agent for MRSA. Furthermore, it may have potential for clinical treatment of MRSA infection disease although this use will require additional investigation. Further purification of active compound(s) and its individual antibacterial activity study can be suggested on the basis of the present study.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The authors acknowledge The Ministry of Science, Technology and Innovation (203/PBIOLOGI/6730001) and Universiti Sains Malaysia for awarding the RUI research grant (RUT1001/pkimia/855006) to support this study.

References

[1] Jeyaseelan EC, Kothai S, Kavitha R, Tharmila S, Thavaranjit AC. Antibacterial activity of some selected algae present in the costal lines of Jaffna Peninsula. *Int J Pharm Biol Arch* 2012; **3**: 352-6.

[2] Darah I, Tong WY, Nor-Afifah S, Nurul-Aili Z, Lim SH. Antimicrobial effects of *Caulerpa sertularioides* extract on foodborne diarrhea-caused bacteria. *Eur Rev Med Pharmacol Sci* 2014; **18**: 171-8.

[3] Christobel GJ, Lipton AP, Aishwarya MS, Sarika AR, Udayakumar A. Antibacterial activity of aqueous extract from selected macroalgae of southwest coast of India. *Seaweed Res Util* 2011; **33**: 67-75.

[4] Vijayabaskar P, Shiyamala V. Antibacterial activities of brown marine algae (*Sargassum wightii* and *Turbinaria ornata*) from the Gulf of Mannar biosphere reserve. *Adv Biol Res* 2011; **5**: 99-102.

[5] Sridharan MC, Dhamotharan R. Antibacterial activity of marine brown alga *Turbinaria conoides*. *J Chem Pharm Res* 2012; **4**: 2292-4.

[6] Al-Saif SS, Abdel-Raouf N, El-Wazanani HA, Aref IA. Antibacterial substances from marine algae isolated from Jeddah coast of Red sea, Saudi Arabia. *Saudi J Biol Sci* 2014; **21**: 57-64.

[7] Chakraborthy K, Lipton AP, Paulraj R, Chakraborthy RD. Guaiane sesquiterpenes from seaweed *Ulva fasciata* Delile and their antibacterial properties. *Eur J Med Chem* 2010; **45**: 2237-44.

[8] Sze P. *Biology of the algae*. New York: McGraw-Hill Company Inc; 1998.

[9] Lim SH, Darah I. Assessment of anticandidal activity and cytotoxicity of root extract from *Curculigo latifolia* on pathogenic *Candida albicans*. *J Med Sci* 2013; **13**: 193-200.

[10] Lim SH, Darah I, Jain K, Suraya S. Gallic acid: an anticandidal compound in hydrolysable tannin extracted from the barks of *Rhizophora apiculata* Blume. *J Appl Pharm Sci* 2011; **1**: 75-9.

[11] Darah I, Nisha M, Lim SH. Polygalacturonase production by calcium alginate immobilized *Enterobacter aerogenes* NBO2. *Appl Biochem Biotechnol* 2015; **175**(5): 2629-36.

[12] Sasidharan S, Darah I, Jain K. *In vitro* and *in vivo* antifungal activity of the methanol extract from *Gracilaria changii*. *Internet J Pharmacol* 2007; **6**: 12.

[13] Manivannan K, Karthikai devi G, Anantharaman P, Balasubramanian T. Antimicrobial potential of selected brown seaweeds from Vedalai coastal waters, Gulf of Mannar. *Asian Pac J Trop Biomed* 2011; **1**: 114-20.

[14] Imbs TI, Chaykina EL, Dega LA, Vashchenko AP, Anisimon MM. Comparative study of the chemical composition of ethanol extract from brown algae and their effects on seedling growth and productivity of soya *Clycine max* (L.) MERR. *Russ J Bioorg Chem* 2011; **37**(7): 871-6.

[15] Sahgal G, Ramanathan S, Sasidharan S, Mordi MN, Ismail S, Mansor SM. *In vitro* and *in vivo* anticandidal activity of *Swietenia mahogany* methanolic seed extract. *Trop Biomed* 2011; **28**(1): 132-7.

[16] Beatrice OTI, Darah I, Supayang PV. Antimicrobial activity of crude ethanolic extract from *Eleutherine americana*. *J Food Agric Environ* 2010; **8**: 1233-6.

[17] Darah I, Jain K, Lim SH, Wendy R. Efficacy of pyroligneous acid from *Rhizophora apiculata* on pathogenic *Candida albicans*. *J Appl Pharm Sci* 2013; **3**: 7-13.

[18] Darah I, Lim SH, Ninthianantham K. Effects of methanolic extract of *Wedelia chinensis* Osbeck (Asteraceae) leaves against pathogenic bacteria with emphasize on *Bacillus cereus*. *Indian J Pharm Sci* 2013; **75**: 533-9.

[19] Lorian V, Gemmell CG. Effect of low antibiotic concentration on bacteria: effect on ultrastructure, virulence, and susceptibility to immune defence. In: Lorian V, editor. *Antibiotic in laboratory medicine*. United Kingdom: Williams & Wilkins; 1991, p. 493-547.

[20] Braga PC, Ricci D. Imaging bacterial shape, surface and appendages before and after treatment with antibiotics. In: Braga PC, Ricci D, editors. *Methods in molecular biology, atomic force microscopy: biomedical methods and applications*. New York: Humana Press Inc.; 2002, p. 179-88.

[21] Lorian V. Effects of subminimum inhibitory concentrations of antibiotics on bacteria. In: Lorian V, editor. *Antibiotics in laboratory medicine*. Baltimore: Williams & Wilkins; 1981, p. 349-79.