Inhibition of *Klebsiella pneumoniae* ATCC 13883 Cells by Hexane Extract of *Halimeda discoidea* (Decaisne) and the Identification of Its Potential Bioactive Compounds

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The inhibitory effect of the *Klebsiella pneumoniae* ATCC 13883 strain caused by the hexane extract of *Halimeda discoidea* (Nor Afifah et al., 2010) was further evaluated by means of the microscopy view and its growth curves. The morphological changes of the *K. pneumoniae* ATCC 13883 cells were observed under the scanning electron microscope (SEM) and transmission electron microscope (TEM) after they were treated at minimum inhibitory concentration (MIC; 0.50 mg/ml) (Nor Afifah et al., 2010) for 12, 24, and 36 h. The results showed the severity of the morphological deteriorations experienced by the treated cells. The killing curve assay was performed for 48 h at three different extract concentrations (1/2 MIC, MIC, and 2 MIC). An increase in the extract concentration of up to 2 MIC value did significantly reduce the number of cells by approximately 1.9 log₁₀, as compared with the control. Identification of the potential compounds of the extract responsible for the antibacterial activity was carried out through the gas chromatography-mass spectrum (GC-MS) analysis of the active subfraction, and the compound E-15-heptadecenal was identified and suggested as the most potential antibacterial compound of this extract. The subsequent cellular degenerations showed by the data might well explain the inhibitory mechanisms of the suggested antibacterial compound. All of these inhibitory effects have further proven the presence of an antibacterial compound within *H. discoidea* that can inhibit the growth of *K. pneumoniae* ATCC 13883.

Keywords: *Klebsiella pneumoniae* ATCC 13883, green macroalgae, transmission electron microscope, *Halimeda discoidea*, gas chromatography-mass spectrum

*Klebsiella pneumoniae* used to be correlated with lung inflammation disease [33, 47] and meningitis [6, 29], but now the attention has been extended to another disease called pyogenic liver abscesses [26, 39]. The group of people that are in the high-risk category to be infected by these diseases includes immunocompromised patients, infants, and the elderly. This strain has become an important pathogen in nosocomial and community-acquired infections [29]. The virulence factor that led to the uncontrollable spread of this bacteria is the thick capsule that encases the entire cell [1, 7]. Many of the multiresistant *K. pneumoniae* strains managed to develop the resistant barrier towards the antibiotic treatments including the broad-spectrum antibiotics and the extended-spectrum antibiotics [4, 28, 30], thus causing serious outbreaks of *K. pneumoniae* infections throughout the world.

Owing to this challenging worldwide threat, new targets of useful natural products to combat against the pathogens were discovered. Because of the large area covered by the oceans on the Earth, marine life consists of a rich diversity of chemical classes. These chemical compounds have been positively turned into useful marine-derived drugs with promising bioactivities, including the antimicrobial activity [14, 42, 51]. Macroalgae are an example of a marine source with potential bioactive compounds [20, 24, 27, 52]. Macroalgae such as *nori* has been consumed by the Japanese a long time ago because of the high mineral and soluble dietary content that can lower the glycemic index in humans [18]. Malaysia’s waters, located at the equatorial line with a tropical climate, are the habitat of four types of marine macroalgae: Cyanophyta, Chlorophyta, Rhodophyta, and Phaeophyta [41]. Along with the tropical climate is the sandy and rocky coastal zone, which is presumed to become major factors for the growth of a more diverse macroalgae and their chemical compounds. In Malaysia, macroalgae such as *Gracilaria changii*, *Gracilaria tetrispitata*, *Gracilaria
Euchema spp., and two Caulerpa spp. are eaten as salads by some communities [41].

In this present study, a green macroalga called Halimeda discoidea (Decaisne) was selected as the experiment subject. This macroalga was found to grow abundantly on its own without any major competition by other types of macroalgae and sea macroorganisms. The fact that this macroalga is less favored by sea grazers is because of its chemical and physical defence features, which are the secondary metabolites and the calcification properties, respectively [40, 55]. Therefore, the aim of the present study was to evaluate the potential secondary metabolites extracted from the collected H. discoidea to inhibit an opportunistic pathogen, K. pneumoniae ATCC 13883. This study is a part of the isolation of the bioactive compound from the macroalgae with antimicrobial properties.

Briefly, an antimicrobial screening through disc diffusion method was performed earlier [37] in order to evaluate the antimicrobial activity of the hexane extract of H. discoidea. The positive results obtained showed that the hexane extract was capable to inhibit the growth of K. pneumoniae ATCC 13883. Relating to that, the minimum inhibitory concentration (MIC) was determined through the broth dilution method with the MIC value of 0.50 mg/ml. Hence, using this MIC, the structural degenerations and killing curves assays were carried out on the K. pneumoniae ATCC 13883.

**Materials and Methods**

**Plant Sample**
Macroalga H. discoidea (Decaisne) was collected during the low tide at the coastal zone of Kera Island, Penang, Malaysia in August and October 2008. The plant species was identified and authenticated by Professor Dr. Shaida Fariza Sulaiman, School of Biological Sciences, Universiti Sains Malaysia based on the morphological examination checked in the book, "Rumpai Laut Malaysia" [21].

**Extraction**
Prior to extraction, the macroalgal sample was cleaned thoroughly under flowing tap water and dried in an oven at 45°C for 4–7 days. The extraction was carried out using the Soxhlet apparatus. Twenty grams of dried powder formed algae was wrapped in 24.0 cm Whatman No. 1 filter paper and placed in the extraction chamber. Four hundred milliliters of hexane solvent was filled in the round flask and placed just underneath the extraction chamber. The flask was heated by a mantel to an appropriate temperature for 2–3

**Bacterial Culture and Inoculum Preparation**
Strain K. pneumoniae ATCC 13883 was obtained from the Phytochemistry Laboratory, School of Biological Sciences, Universiti Sains Malaysia. The bacteria was grown on Nutrient Agar (NA) slant, incubated at 37°C for 24 h, and kept at 4°C until further use. Bacterial suspension was prepared by inoculating one loopful of the 24-h-old bacterial colonies into 10.0 ml of sterilized distilled water.

The inoculums size was adjusted to match the turbidity of McFarland 0.5 scale (1 × 10^8 cells/ml) and diluted with sterilized distilled water to the inoculums size of 1 × 10^7 cells/ml.

**Scanning Electron Microscope (SEM)**
The bacterial suspension was prepared as described in the section above. For each sample, 1.0 ml of the 24-h-old bacterial suspension was inoculated in a 50.0 ml Erlenmeyer flask containing 30.0 ml of sterilized nutrient broth (NB) and incubated in a shaker (Infors HT Ecolotron) at 37°C, 150 rpm for 18 h. The bacterial suspension (inoculums) was then added to the extract stock solution (the final concentration within each flask was at the MIC value) and incubated at the required incubation time (12, 24, or 36 h). For the control, the Erlenmeyer flask containing inoculums was added with 1.0 ml of 100% methanol. Methanol was used as the negative control.

The SEM sample was prepared based on the HMDS (hexamethyldisilazane) method [10, 17, 36]. The pellet of the bacterial suspension was fixed with Mc Dowell–Trump fixative solution in 0.1 M phosphate buffer (pH 7.2) for at least 2 h to fix the cell's original condition [32]. For the post-fixation step, the sample was resuspended in 1% osmium tetroxide in phosphate buffer for 1 h, re-centrifuged, and the supernatant was discarded. Then, the pellet was dehydrated using 50% ethanol, 75% ethanol, 95% ethanol, and 100% ethanol and HMDS for 10 min consecutively. Centrifugation was performed each time the samples were resuspended and the supernatant was also discarded after each centrifugation. Finally, after the HMDS solution was decanted, the Eppendorf tube containing the cells was left to air-dry in a desiccator at room temperature. The dried cells then were mounted on the specimen holder with a double-sided tape. The sample was coated with 5–10 nm of gold palladium alloy and viewed under an SEM (Leica Cambridge, S-360, UK).

**Transmission Electron Microscope (TEM)**
The sample preparation and a part of the TEM preparation was done according to the steps described in the section above. For the HMDS method, the steps were the same until the post-fix wash stage. The steps were continued by embedding the pellet of fixed cells in the agar solution before it was left to solidify, and the agar containing the cells was cut into small cubes of about 1 mm × and placed in a vial containing 50% ethanol. The cubes were then dehydrated with 75% ethanol, 95% ethanol, and 100% ethanol for 15 min and lastly resuspended with 100% acetone for 10 min. The vial was then added with a mixture of acetone:Spurr’s resin [1:1 (v/v)] and rotated for 15–30 min for infiltration. The vial was then replaced with only Spurr’s resin and rotated overnight. A new change of Spurr’s resin was infiltrated for another 5 h in the rotator. The sample was embedded in the Spurr’s resin and cured at 60°C for 12–48 h. A cross-section method was conducted to cut the cells according to the required size using a microtome instrument (Sorvall Ultra Microtome MT500, USA). The sample was stained with uranyl acetate and lead citrate solutions, respectively. The sample was viewed under a TEM (LIBRA 120 EFTEM, Germany).

**Time-Killing Curves of Klebsiella pneumoniae ATCC 13883 in the Presence of Hexane Extract of H. discoidea**
Bacterial suspensions of K. pneumoniae ATCC 13883 were prepared as described above. Hexane extract stock was prepared at concentrations of 25.00, 12.50, and 6.25 mg/ml. One milliliter of each extract stock was added into the conical flask containing 23.0 ml of sterilized NB