CLEANING AND EXTRACTION PROTOCOL OF GRACILARIA CHANGII

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Abstract. Under examination by a compound light microscope and stereoscope, several epiphytic animals and algae were found on the unclean seaweed surface. These epiphytes were mainly filamentous red and green algae, diatoms (Cocconeis sp), tube-dwelling polychaetes, and small mussels. Two methods were used to clean the seaweed surface, which were sonication for 15 min and hand cleaning using a soft brush with three rinsing using filtered seawater. Examination by a compound microscope, stereoscope and scanning electron microscope (SEM) of the cleaned weeds surface showed removal of the majority of the epiphytes, however, some of the filamentous algae and Cocconeis sp still remained. A much more efficient cleaning method is necessary to remove the filamentous algae and Cocconeis sp from the Gracilaria changii surface. The extraction method of Gracilaria changii is also described.

Introduction

Interest in isolating bioactive compounds from marine algae is now increasing tremendously. This is because they play an important role in medication especially seaweed of the genera Macrocystis, Laminaria, Fucus, Sargassum, Turbinaria, Homorphysa and Hydroclutes that has proven effective on goiter, dysentery and diarrhea. Recently halogenated compound isolated from Malaysian Laurencia has been reported active against marine bacteria (1). Some Sargassum species contain antitumor compounds such as fucoidan from S. thunbergii and S. kjellmanianum (2,3). There are a few compounds from Sargassum sp. known for its cytotoxicity activities such as dihydroxysaragquinone and sargaxtriol from S. tortile (4) and recent study also showed that S. crispum contain bicyclic diterpene that exhibited substantial cytotoxic activities (5).

In case of Gracilaria, many previous studies concentrated on taxonomy and habitat characteristic, agar content, characteristic of shape and type of species and relationship of growth of alga with respect to the environment, and nutritional and biochemical composition (6). Beside that, some species of Gracilaria has been studied for its potential use as bioaccumulator of heavy metal (7). Several studies has been done on isolation of chemical compounds from other Gracilaria species such as Gracilaria coronopifolius (8), Gracilaria edulis, Gracilaria textori and Gracilaria verrucosa. A series of apleysiatoxins and malyngamide has been isolated from Gracilaria coronopifolius. Polycavernoside A2, Polycavernoside B2 and stigmasteryl derivatives are reported to be characteristic compounds from Gracilaria edulis. Polysaccaride with antiviral property was isolated from Gracilaria cornicata (9). Some malyngamide derivatives was reported to display
mild antimicrobial and cytotoxic activity (10). Since there is no report on phytochemical and biological activity studies on *Gracilaria changii*, there is a great opportunity to discover new bioactive compounds from these species. Because of the nature of this study, it is important that the seaweed samples used for extraction of bioactive compounds must be relatively free of any epiphytes and the extract should be standardised. From field experience, thalli of *Gracilaria changii* collected from natural populations are never free of epiphytic algae and animals. Therefore, an effective epiphyte removal technique was necessary. The TLC analysis of every cleaning and drying batch should be monitored to ensure there is no chemical compounds change during the cleaning and drying process. The TLC profiling of every batch of extract was important to standardize the extract that will be used later in bioassay studies.

**Material and Methods**

**Collection of Seaweed**

The *Gracilaria changii* were collected from Morib, Selangor once in two months. Every sampling will take only two days to maintain the freshness of *Gracilaria changii*.

**Cleaning Process**

**Field**

Samples were rinsed with seawater to remove debris and epiphyte. Samples were kept in polystyrene box, wrapped with wet cotton (the cotton was dipped in sea water).

**Laboratory**

Samples were immediately put in tank filled with seawater, which was aerated and the seawater was changed several times. The samples were sorted, to separate it from other marine alga species. The entire epiphyte was thoroughly removed using soft brush. The cleaned seaweed were rinsed with filtered sea water and further examined under dissecting and compound microscope to confirm it is free from epiphytes. The cleaned seaweeds were weighed to give the wet weight. The whole cleaning process must finish in the shortest period of time to ensure the freshness of *Gracilaria changii*. The morphology of the *Gracilaria changii* is always monitored to avoid damage of the sample. The damage sample will not be used in extraction process.

**SEM**

Two methods were used to clean the seaweed surface, sonicated for 15 min at maximum setting was done in filtered seawater. Seawater was filtered through 0.45μ cellulose membrane filter using a vacuum pump. Hand cleaning using a soft brush with three rinsings using filtered seawater. The cleaned seaweeds were then examined again under SEM for remaining epiphytes.

For SEM, seaweed samples were fixed in 4% Gluteraldehyde rinsed in 0.1M Cacodylate Buffer placed on Aluminium Stub and viewed under LEO SEM.

**Drying process**

Batches of *Gracilaria changii* were dried under the shade. The temperature and the moisture content of *Gracilaria changii* were determined. The fresh weight were reduced to 10%, or until the dry weight is constant.
Extraction process.

The dried *Gracilaria changii* were cut into smaller pieces. Soaked in methanol for three days. The extraction is repeated for three times. Then followed by solvent partitioning with diethyl ether, ethyl acetate and butanol. The extracts were evaporated under reduced pressure.

TLC Profiling

Any chemical components changed during soaking, cleaning, and drying process was monitored by TLC profiling. Several types of spray reagents were used for visualization of the spot on the TLC. The TLC profile of all batches was recorded for standardization and for future verification.

Results and Discussion

In the process of searching bioactive compounds from *Gracilaria changii*, we establish a standard protocol for sampling, cleaning, drying and extraction methods. For the preliminary step, the ephiphytic algae or animal that could contribute to the interference of *Gracilaria changii* extract was determined. We found the only ephiphyte involved were filamentous algae (*Chaetomorpha* sp.) and diatom (*Cocconeis* sp.).

Several ephiphytic animals and algae were found on the uncleaned seaweed surface by examination under a compound light microscope and stereoscope. These ephiphytes were mainly filamentous red and green algae (Figure 1), diatoms, tube-dwelling polychaetes, and small muselles. SEM examination of the same seaweeds showed the diatom, *Cocconeis* sp as the dominant diatom ephiphyte (Figure 2). These ephiphytes are typical of those found on other *Gracilaria* species (11). Examination of the cleaned seaweeds showed removal of the majority of the ephiphytes such as small muselles and polychaete tubes. However, examination by a compound microscope, stereoscope, and SEM of the cleaned seaweeds still showed some of the filamentous algae (*Chaetomorpha* sp.) (Figure 3) and *Cocconeis* sp remaining on the cleaned seaweed surface (Figure 4). Based on the above results, a much more efficient cleaning method is necessary to remove the filamentous algae and *Cocconeis* sp from the *Gracilaria changii* surface. It appears that the remaining ephiphytes are well attached to the seaweed surface either by penetrating into the thallus, as in the filamentous algae, or by mucilage produced through the raphe on the valve attached to the seaweed surface as in *Cocconeis* sp (12). Based on the SEM analysis, we can conclude that the diatom *Cocconeis* sp was a major ephiphyte that could interfere with the extract of *Gracilaria changii*.

The importance of using a standardized extract for bioassays must not be overlooked. As of this, we did the TLC profiling to monitor if any chemical change during the cleaning and the drying process. The cleaning process of the seaweed was a very tedious and a time consuming process. During the cleaning process the sample must be kept fresh until the drying process. According to our experience on *Gracilaria changii* collected from Morib, it takes four weeks to clean the whole 30 kg of sample. The TLC profile of every batch was as in Figure 5 and the Rf values is given in Table 1. From the TLC profiling (Figure 5), we can conclude that there is no obvious chemical change observed during the four weeks time, with continuous aeration and frequent change of the seawater during the cleaning process. This standard protocol will be followed in whole process of investigating bioactive compound from *Gracilaria changii*.

Acknowledgement

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References


**Figure 1:** Uncleaned *Gracilaria changii* showing the filamentous algae (*Chaetomorpha* sp.)

**Figure 2:** SEM of uncleaned *Gracilaria changii* showing the Cocconies sp as major ephyphite

**Figure 3:** SEM of cleaned *Gracilaria changii* showing the filamentous algae (*Chaetomorpha* sp.)

**Figure 4:** SEM of cleaned *Gracilaria changii* showing majority of the Cocconies sp is removed from the thallus

Figure 5. TLC profiling of several batches of MeOH extract from Gracilaria changii to monitored whether there is a chemical change during the cleaning and the drying process.

Table 1. Rf values of TLC profile of several batches of MeOH extract using various solvet system

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<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
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<tr>
<td>Solvent System: CHCl₃</td>
<td>Solvent System: CHCl₃; Ethyl acetate (9:1)</td>
<td>Solvent System: CHCl₃; Ethyl acetate (1:1)</td>
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<tr>
<td>Rf = 0.90, UV 254 nm (Brown), I₂</td>
<td>Rf = 0.82, UV 254 nm (Brown), UV 366 nm (Red), I₂</td>
<td>Rf = 0.80, UV 366 nm (Red), I₂</td>
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<tr>
<td>Rf = 0.44, UV 254 nm (Brown), I₂</td>
<td>Rf = 0.71, UV 254 nm (Brown), Spray Vanilin (pink)</td>
<td>Rf = 0.74, Spray Vanilin (pink)</td>
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<tr>
<td>Rf = 0.52, UV 366 nm (Red)</td>
<td>Rf = 0.60, UV 366 nm (Red), UV 254 nm (Brown)</td>
<td>Rf = 0.62, UV 366 nm (Red)</td>
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<td>Rf = 0.26, UV 254 nm (Brown), UV 366 nm (Red), I₂</td>
<td>Rf = 0.51, UV 254 nm (Brown), UV 366 nm (Red), I₂</td>
<td>Rf = 0.4, Spray Vanilin (pink)</td>
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<td>Rf = 0.14, UV 366 nm (Red), Spray Vanilin (pink)</td>
<td>Rf = 0.36, UV 366 nm (Red)</td>
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