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ANTIFUNGAL ACTIVITY OF DICHLOROMETHANE AND HEXANE EXTRACTS OF FOUR MALAYSIAN SEAWEED SPECIES AGAINST *Ganoderma boninense*

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ABSTRACT

Ganoderma boninense is a major oil palm fungal pathogen that caused basal stem rot disease and serious efforts are required to identify alternative methods to control this disease. To date, little attempt was done to explore the antifungal potential of bioactive compounds in seaweeds. This study reported the antifungal activity of seaweed extracts against *G. boninense*. Seaweeds $_{cf}$*Sargassum oligocystum*, *Caulerpa racemosa* var. *lamourouxii*, *Caulerpa racemosa* and $_{cf}$*Halimeda macrophysa* were collected from Port Dickson, Malaysia and extracted using dichloromethane and hexane. The antifungal activity assay towards *G. boninense* was carried out using the poisoned food technique followed by gas chromatography-mass spectrometry (GC-MS) to screen the compounds in the seaweed extracts. Our findings revealed that *C. racemosa* var. *lamourouxii*-dichloromethane extract exhibited the highest antifungal activity at a concentration of 0.25 mg/mL with 46.82% inhibition of *G. boninense*'s growth followed by *C. racemosa* var. *lamourouxii*-hexane extract with 36.43% inhibition. Phytol and tetradecanoic acid were found to be the dominant compounds in the extracts and further analysis of phytol standard proved its antifungal activity. This study highlights the potential of local Malaysian seaweed species as a source of natural and powerful antifungal compounds which could be useful for alternative oil palm disease control in Malaysia.

Key words: seaweeds, antifungal, *Ganoderma boninense*, GC-MS

INTRODUCTION

Oil palm (*Elaeis guineensis*) is a major source of edible oil, industrial products and recently as biofuel (Barriuso *et al*., 2013). However, oil palm production is affected by a fungal disease caused by *Ganoderma boninense*. *G. boninense* causes basal stem rot (BSR) disease which has been known as a major threat to the oil palm industry of Malaysia (Hasan & Turner, 1998; Mercière *et al*., 2017). BSR disease not only results in the changes of the physical appearance of the palm but also lowers the yield of the fresh fruit bunch (Flood *et al*., 2002; Ferdous Alam *et al*., 2015). Basal stem rot disease is usually been controlled by commercial fungicides such as hexaconazole (Chung, 2011), triadimefon (Jayaratne *et al*., 2001), benomyl, cycloheximide and drazoxolone (Ramasamy, 2000; Chung, 2011) but the subsistence of some of these chemicals both in soil and water result in serious environmental problems. Therefore, attempts for alternative control for this disease have been suggested including the utilization of natural sources for antifungal compounds. However, not many studies on the utilization of seaweed as a source of antifungal compounds specifically against *G. boninense* have been reported. The bioactive compounds from

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natural sources could be an environmentally friendly mean for plant disease control hence the application of synthetic fungicides and harmful chemicals can be reduced (Hernández *et al*., 2007). A study proved the potential of papaya leaf extract for their antifungal activity against *G. boninense* where nine major compounds were identified in the extract namely decanoic acid, 2-methyl-, Z, Z-10-12-hexadecadien-1-ol acetate, dinonanoin monocaprylin, 2-chloroethyl oleate, phenol,4-(1-phenylethyl)-, phenol,2,4-bis(1-phenylethyl)-, phenol-2-(1-phenylethyl)-, ethyl iso-allocholate and 1- monolinoleoylglycerol trimethylsilyl ether (Tay & Chong, 2016). Another study has shown that aloe vera gel showed significant antifungal activity against four common postharvest pathogens, *Penicillium digitatum*, *P. expansum*, *Botrytis cinerea* and *Alternaria alternata* (Barkai-Golan, 2001).

Seaweeds are rich with bioactive compounds such as carotenoids (Yip *et al*., 2014), phenolics (Matanjun *et al*., 2008; Airanthi *et al*., 2011), alkaloids (Dheeb, 2015; Pawar & Nasreen, 2016), sterols (Abdel-Aal *et al*., 2015), essential fatty acids (Rajasulochana *et al*., 2013), vitamins (Hamid *et al*., 2015) polyphenols, dietary fiber, polysaccharides and proteins (Ibañez *et al*., 2012). Seaweeds have unique properties based on their photosynthetic pigments and have been utilised for various purposes. Malaysia is abundant with seaweeds, but they are mostly underutilized. Nermal *et al*. (2014) demonstrated the significant antifungal activity of brown algae, *Sargassum tenerrimum* J.G. Agardh and *Turbinaria ornate* J. Agardh extracts against *Aspergillus niger* and *Penicillium janthinellum*. Another study showed five brown algae extracts namely *Sargassum vulgare*, *Cystoseira barbata*, *Dictyopteris membranacea*, *Dictyota dichotoma*, and *Colpomenia sinuosa*, have high antifungal activities against eight fungal species (Am *et al*., 2015). Some algal extracts exhibited antifungal activity that is relative to commercial antifungal medicine (Am *et al*., 2015). This study aims to test further the antifungal potential of Malaysian seaweed extracts against the oil palm pathogen, *G. boninense* as how it was described previously (Abdul Aziz *et al.,* 2019). This project is hoping to reveal the antifungal potential of bioactive compounds from dichloromethane and hexane seaweed extracts and screen for the compounds responsible for the antifungal activity. These findings are crucial in the search for natural and environmental control for fungal infection in oil palm and the sustainability of palm oil production.

MATERIALS AND METHODS

Collection of seaweeds

Seaweeds were collected from Teluk Kemang, Port Dickson, Malaysia (2° 26' N latitude; 101° 51' E longitude). A total of four seaweeds were sampled: $_{cf}$*S. oligocystum*, *C. racemosa*, *C. racemosa* var. *lamourouxii* and $_{cf}$*H. macrophysa*. They were washed thoroughly with seawater and rinsed with tap water to remove all attached materials. The samples were freeze-dried for seven days to remove the moisture content. The seaweeds were then ground into fine powder and stored at 4°C until use.

Preparation of extracts

Portions of the seaweed powder (10 g) were packed in a Soxhlet extractor and extracted with 250 mL of dichloromethane and hexane separately at 40-70°C temperature. The liquid extract was concentrated using rotary evaporator at 50ºC. The concentrated extracts were weighed and kept at 4°C until further use. The extraction yield can be calculated using the following formula:

$$\text{Yield (\%)} = \frac{\text{Dry weight of extract}}{\text{Dry weight of plant powder}} \times 100$$

Fungal strain

G. boninense used in this study was kindly obtained from the Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia. The fungal strain cultures were maintained on a potato dextrose agar (PDA) medium at 27°C.

Antifungal assay

Antifungal activity against *G. boninense* was determined by poisoned food technique (Schmitz, 1930). The seaweed extracts were prepared using dimethyl-sulfoxide (DMSO, 1.0% v/v) as the initial solvent carrier followed by dilution with PDA (at about 50°C) containing a final concentration of 100 µg/mL antibiotics ampicillin and penicillin to give the desired concentrations of 0.25, 0.50 and 1.0 mg/mL. The agar was left to solidify and a 6 mm mycelial disk was cut from the periphery of one-week-old *G. boninense* cultures, placed in the center of each PDA plate, and then incubated at 27°C for seven days. PDA plates treated with an equal quantity of DMSO were used as negative control while PDA plates treated with triadimefon were used as a positive control. Each treatment which consisted of duplicates was repeated for three times. The mycelial growth (mm) in both treated and control petri dishes was measured diametrically in two different directions.

Identification of potential antifungal compounds using GC-MS

GC-MS technique was used in this study to identify the compounds in the seaweed extracts using Thermo Scientific TSQ Quantum XLS Gas Chromatography (country of origin: USA) interfaced with Mass Spectrometer equipped with GC capillary column with HP-5MS stationary phase (30 m × 0.25 mm × 0.25 μm) and composed of (5%-phenyl)-methylpolysiloxane. For GC-MS detection, the emission current used was 70 μA. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1.0 mL min^{-1} and an injection volume of 1 μL (split ratio 10:1). The injection temperature was set at 250°C and ion source temperature at 280°C, the oven temperature was programmed from 110°C (isothermal for 2 min) with an increase of 10°C min^{-1}, to 200°C, then 5°C min^{-1} to 280°C (isothermal for 9 min). Mass spectra were taken at 70 eV (a scanning interval of 0.5 seconds and fragments from 40 to 550 Da). Total GC running time was 36 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The software used to handle mass spectra and chromatograms was Xcalibur (Upgade & Bhaskar, 2013).

Data analysis

The percentage inhibition of fungal growth was calculated at day 7, using the following equation: Inhibition (%) = [(colony diameter of control – colony diameter of treatment) / (colony diameter of control – mycelial disks diameter)] × 100. Each treatment was replicated three times and the results were expressed as median ± standard deviation. The median values were subjected to Kruskal-Walls H tests (SPSS statistical package, version 22) was used to determine the significant differences ($p < 0.05$) between treatments.

RESULTS AND DISCUSSION

Extraction yield

The utilization of hexane as the extraction solvent gave higher extract yield for most types of seaweed species compared to dichloromethane extract (Figure 1). The extraction yield for $_{cf}$*S. oligocystum*-dichloromethane extract is 1.19% while $_{cf}$*S. oligocystum*-hexane extract is 5.07%. Dichloromethane and hexane extracts of *C. racemosa* showed slight differences with the extraction yield of 1.57% and 1.88% respectively. For *C. racemosa* var. *lamourouxii* species, hexane extract produced higher yield with 5.09% compared to dichloromethane extract with 4.22%. In contrast with $_{cf}$*H. macrophysa* species, the dichloromethane extract produced slightly higher extraction yield with 7.84% compared to hexane extract with 7.47%.

Extraction yield of seaweed extracts was obtained by measuring the weight of crude extracts from soxhlet extraction. Soxhlet extraction is a traditional method that is still used due to its efficiency in extracting marine bioactive compounds compared to alternative methods such as microwave-assisted extraction (MAE) and supercritical fluid extraction (SFE). Although this method is time and solvent consuming, soxhlet extraction is cheaper and allowing the recovery of higher amounts of bioactive compounds relative to other alternative techniques (Grosso *et al*., 2015). Besides, the selection of extraction solvent also crucial for extraction of the marine compound of interest (Misra *et al*., 2015). In this research, higher extraction yield was obtained in hexane extracts of most seaweed species compared to dichloromethane extracts. The polarities of different compounds in the seaweed might contribute to the variations in extraction yield of different seaweed species.

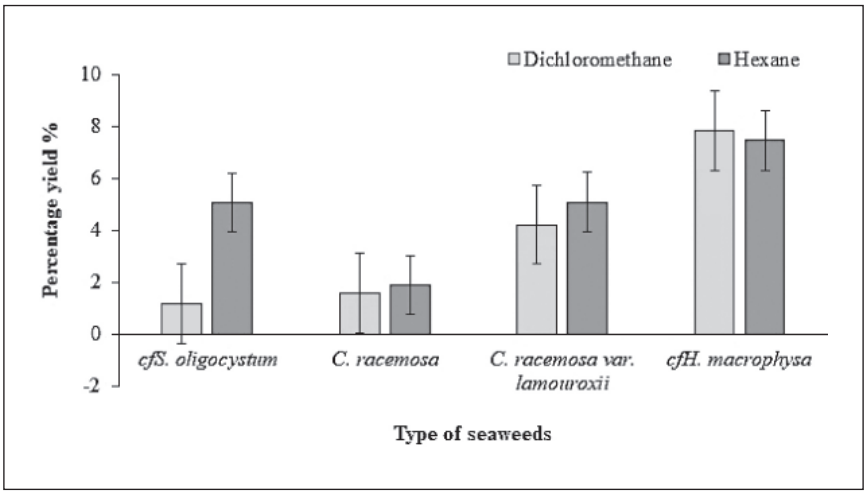

Fig. 1. Variation in percentage yield of seaweed extracts with different organic solvents.

Antifungal activity assay

The antifungal activity of the seaweed crude extracts from the two solvents was studied via poisoned food technique against *G. boninense* (Figure 2). The growth of *G. boninense* should be at the maximum at day seven where the mycelia should have covered the entire diameter of the 9 cm petri dish. The plates without any addition of seaweed extracts acted as control (Figure 3C). It was observed that there was a significant reduction of *G. boninense* growth rates in plates containing both dichloromethane and hexane seaweed crude extracts. At the lowest tested concentration (0.25 mg/mL), *C. racemosa* var. *lamourouxii*-dichloromethane extract showed a great inhibition activity of 46.82% but somehow showed a decrease in the activity as the concentration increased. In the case of the other seaweed extracts, the pattern of inhibition activity is fluctuated for the three concentrations tested. *C. racemosa* and $_{cf}$*S. oligocystum* extracts showed the highest inhibition at concentration 0.5 mg/mL while $_{cf}$*H. macrophysa* highest inhibition activity was shown at the highest concentration, 1.0 mg/mL (Figure 2a). The morphology of *G. boninense* was observed on plates incorporated with dichloromethane extracts (Figure 3a). The mycelial growth zone was unclear, and the structure was not white and cotton-like compared to the negative control which indicates the mycelial growth was stunted and malformed (Figure 3c).

For hexane extract, *C. racemosa* var. *lamourouxii* extract appeared as the extract with the highest inhibition activity with 40.62% at concentration 0.5 mg/mL. *C. racemosa* portrayed the second highest inhibition activity with 33.33% at concentration 1.0 mg/mL while $_{cf}$*S. oligocystum* and $_{cf}$*H. macrophysa* extract showed fluctuating inhibition pattern. $_{cf}$*S. oligocystum* extract showed highest inhibition at concentration 0.5 mg/mL while $_{cf}$*H. macrophysa* extract at concentration 1.0 mg/mL. Mycelial growth of *G. boninense* in hexane extracts assay had white cotton-like mycelia but the growth was also malformed and stunted (Figure 3b). This might be because of antifungal compounds in the seaweed extract that damages the cell membrane and inhibit the development of mycelia (Inoue *et al*., 2005). However, all the tested concentrations of dichloromethane and hexane seaweed crude extracts failed to completely inhibit the growth of the pathogen. Statistical analysis indicated that fungal growth treated with both the dichloromethane and hexane crude extracts were significantly lower ($p< 0.05$) as compared to the negative control.

Marine algae have been reported for various important biological activities. Seaweed extracts have shown to have antifungal activity against some fungi. In this research, the antifungal activity of seaweed extracts of two different solvents was determined by poisoned food technique (Schmitz, 1930). The results showed that the growth of *G.*

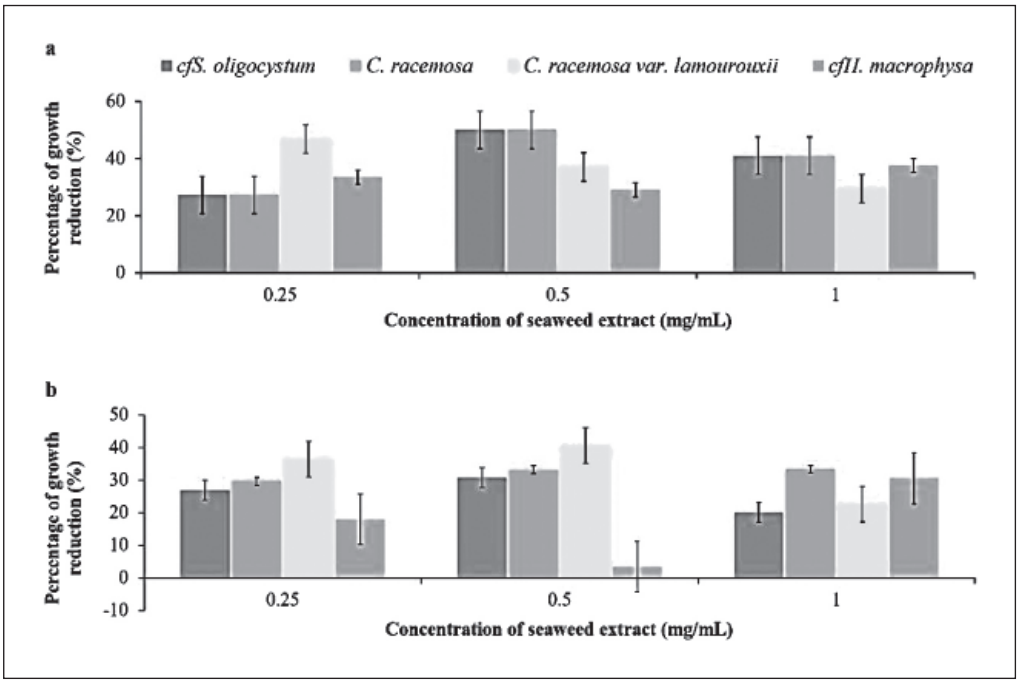

Fig. 2. *G. boninense* mycelial growth on PDA incorporated with three different concentrations of (a) dichloromethane and (b) hexane extract of seaweeds. Average was obtained from three replicates of each test.

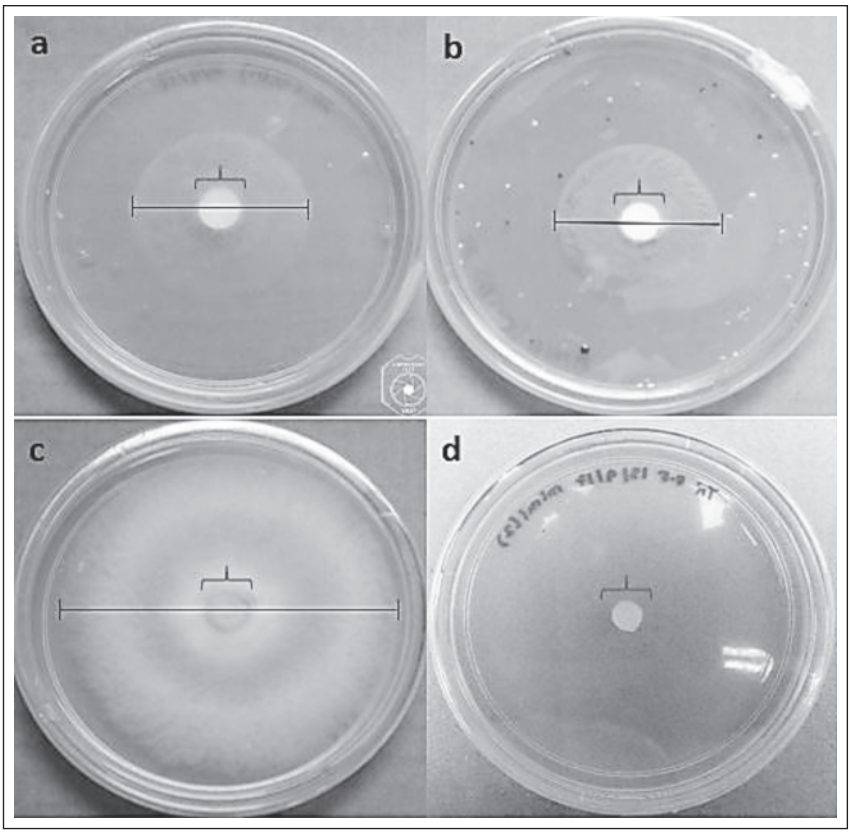

Fig. 3. Antifungal Assay of *G. boninense* on extract-incorporated PDA after 7 days. (a) Dichloromethane extract of *C. racemosa* var. *lamourouxii*, (b) Hexane extract of *C. racemosa* var. *lamourouxii* (c), Negative control, (d) Positive control (Triadimefon), Red bracket: 6mm fungus inoculum, Black line: Diameter of fungal growth.

boninense was positively inhibited at a certain level in the presence of *C. racemosa* var. *lamourouxii* extracts compared to the other tested extracts. Antimicrobial studies on *C. racemosa* var. *lamourouxii* extracts have been proven to have potential against various pathogenic organisms (Jebasingh & Rosemary, 2011; Etcherla & Narasimha Rao, 2014; Nagaraj & Osborne, 2014). The antifungal activity of hexane extract of $_{cf}$*H. macrophysa* showed less significant antifungal activity. However, there are many factors involved in the observation obtained. There are factors that affect the biological activity of seaweeds namely their habitat, season, and also their growth stage (Karthikaidevi *et al*., 2009). Selection of solvents to extract compounds from seaweed sources is also crucial (Thiripurasundari *et al*., 2008). Apart from that, the understanding of the capability of fungi is also important as some fungi are resistant to these metabolites due to their ability to conquer or divert the metabolic pathway and result in less inhibitory effect (Clarke, 1972).

A Kruskal-Wallis H test was conducted to explore the effect of solvents and species of extracts towards growth reduction percentage of *G. boninense*. There is a statistically significant difference, r=6.06, p=0.01 in growth reduction percentage between the different solvent with a mean rank growth reduction percentage of 30.43 for solvent dichloromethane and 42.57 for solvent hexane. The effect of different seaweed species tested towards growth reduction percentage showed no statistically significant difference, r=5.2, p=0.16 with a mean rank growth reduction percentage of 38.64 for species $_{cf}$*S. oligocystum*, 32.81 for species *C. racemosa*, 29.97 for species *C. racemosa* var. *lamourouxii* and 44.58 for species $_{cf}$*H. macrophysa*.

GC-MS analysis of the most effective seaweed extracts

GC-MS analysis of *C. racemosa* var. *lamourouxii*-dichloromethane and hexane extracts identified a mixture of various compounds. Results obtained indicated that seven active phytocompounds were characterized and identified in the *C. racemosa* var. *lamourouxii*- dichloromethane and hexane extracts as shown (Table 1 and 2).

Seaweeds are rich with valuable bioactive compounds for instance fatty acids (Agoramoorthy *et al*., 2007; Balamurugan & Selvam, 2013), phenols (Ibañez *et al*., 2012; Samarakoon & Jeon, 2012), proteins and amino acids (Abirami & Kowsalya, 2012) which may enhance the antimicrobial activity

Table 1. Chemical compounds of *C. racemosa* var. *lamourouxii*-dichloromethane extracts

RT (min)	Compounds	MW (m/z)	Area (%)
13.99	Spirost-8-en-11-one,3-hydroxy, (3á,5á,14á,20á,22á,25R)-	428	7.29
14.29	Isobutyl methylphosphonofluoridate	154	25.64
16.73	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	278	3.28
17.76	Benzenamine,2-[2-(4-pyridinyl) ethyl]-	198	2.87
21.05	Cyclopentane carboxylic acid, 4 neopentylidene-2-phenyl-, methylester	272	13.60
31.66	α-Tocopherol, O-methyl-	430	6.19
33.85	Furo[3',4':6,7] naphtho[2,3-d]-1,3-dioxol-6(5aH)-one, 5,8,8a,9-tetrahydro-5-(3,4,5-trimethoxyphenyl)-,[5R-(5à,5aá,8aà)]-	398	6.13

RT: Retention time; MW: Molecular weight; m/z: mass per charge number of ions.

Table 2. Chemical compounds of *C. racemosa* var. *lamourouxii*-hexane extracts

RT (min)	Compounds	MW (m/z)	Area (%)
11.71	Tetradecanoic acid	228	1.24
14.00	Benzenamine, 2-[2-(4-pyridinyl) ethyl]-	198	6.39
14.22	n-Hexadecanoic acid	256	4.07
14.29	Isobutyl methylphosphonofluoridate	154	18.27
16.30	Phytol	296	8.93
16.73	9,12,15-Octadecatrienoic acid, (Z, Z, Z)-	278	2.42
21.04	Hydrocortisone acetate	404	6.90

RT: Retention time; MW: Molecular weight; m/z: mass per charge number of ions.

Table 3. Mechanisms of identified compounds with potential biological activities

Compounds (Class)	Mechanisms	Reference
Phytol (Diterpene Alcohol)	Causing damage to cell membranes	(Inoue *et al.*, 2005)
9,12,15-octadecatrienoic acid, (Z,Z,Z)- (Fatty acid)	Signals jasmonates pathway to induce the synthesis of a family of wound-inducible defensive proteinase inhibitors	(Farmer & Ryan, 1992)
Tetradecanoic acid (Fatty acid)	Disturbance of protein function in fungus	(Carballeira *et al.*, 2005)
n-Hexadecanoic acid (Fatty acid)	Inhibit spore germination of *Colletotrichum lagenarium* and *Fusarium oxysporum f.* sp. *lycopersici*	(Liu *et al.*, 2008)

of seaweeds against fungi. GC-MS is one of the best techniques to study the constituents in seaweed extracts. Table 3 shows some of the compounds in the seaweed extracts identified through GC-MS analysis which possess biological activities proven by previous studies.

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