Antifungal activities against oil palm pathogen *Ganoderma boninense* from seaweed sources

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Abstract. Basal stem rot (BSR) disease is the most devastating disease in oil palm which is caused by a fungal pathogen, *Ganoderma boninense*. However, to date, there is no reliable control for this disease. This study investigated the antifungal potential of seaweed extracts against *G. boninense* and screening of the compounds possessing this antifungal activity. Four seaweed species namely *Sargassum oligocystum*, *Caulerpa racemosa*, *Caulerpa racemosa* var. *lamourexii* and *Halimeda macrophysa* were collected from Teluk Kemang, Port Dickson, Malaysia and their antifungal potential against *G. boninense* were evaluated. Two solvents with different polarities were used for crude extraction namely methanol and chloroform. Antifungal assay using crude methanolic and chloroform extracts from these seaweed species were carried out at various concentrations using the poisoned food technique. *Caulerpa racemosa* var. *lamourexii* chloroform extract showed strong antifungal activity against *G. boninense* with 27.44% inhibition of the fungus followed by *C. racemosa* methanolic extract with 26.92% inhibition of the fungus at the lowest extract concentration of 0.25 mg/mL. The extracts were subjected to Gas Chromatography-Mass Spectrometry analysis and the dominant bioactive compounds detected in both extracts were phytol and l-(+)-ascorbic acid 2,6-dihexadecanoate which were also found in plant extracts showing antimicrobial activities in previous studies. The findings suggested that local Malaysian seaweed species have high potential as a source of antifungal compounds which could be useful specifically for the application in the oil palm industry.

Keywords: antifungal activity, bioactive compounds, seaweeds, oil palm, basal stem rot, *Ganoderma boninense*

INTRODUCTION

Fungal infection is a serious case where it affects plants and interferes with the growth of plants mainly by utilizing the plants' nutrients. It is very severe and can even cause death to plants (Tucker and Talbot 2001). *Ganoderma boninense* is a soil borne pathogen which causes the basal stem rot

disease in oil palm (Hushiarian *et al.*, 2013). Neglecting the disease will affect the yield of fresh fruit bunches which will trim down the production of crude palm oil. It was estimated that in 2020, 400 thousand hectares of oil palm plantations could be affected by *G. boninense*

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(Roslan and Idris 2012). Fungal infection is not new in the oil palm industry of Malaysia. However, to date, no proper control measure is available, and this situation should be controlled urgently. Many approaches to prevent the fungal infection in oil palm have been done. The most common method is by the application of fungicide but these attempts require high cost and are not environmental friendly (Idris *et al.*, 2002). Therefore, a more effective and sustainable remedy to this disease would be worth exploring. Research suggested that the application of thiamine activated the host defense response and suppressed plant diseases (Rapala-Kozik *et al.*, 2012; Sylvander *et al.*, 2013). It was proven in a study by Monaim (2011), whereby thiamine treatment makes up hydrogen peroxide generation, callose disposition, and host resistance (HR) cell death in grapevine, hence, weakening the downy mildew disease. Likewise, thiamine was also able to induce defense-related enzymes in soybean plants resulting in success control of charcoal rot disease (Abdel-Monaim, 2011). Another study demonstrated the upregulation of thiamine biosynthesis as a result of the application of endophytes which also suggest the suppression of *G. boninense* (Kamarudin *et al.*, 2017).

On the other hand, a study by Tay and Chong (2016) on papaya leaf extract against *G. boninense* demonstrated a significant inhibition activity where at highest concentration tested (45 mg/mL) results in high inhibition activity. The fungicidal agent found in the extracts contained five classes of compounds: carboxylic acid, ester, fatty acid ester, phenol and steroid (Tay and Chong 2016). The potential of antimicrobial activities has also been discovered in certain seaweed extracts. Seaweeds are rich with useful bioactive substances that play important roles in biological activities such as antimicrobial (Demirel *et al.*, 2009), antiviral (Newman *et al.*, 2003), anti-inflammation (Ranganayaki *et al.*, 2014), anticoagulant (Chanda *et al.*, 2010), antifouling (Maréchal *et al.*, 2004) and antifungal activity (Ambika and Sujatha 2015; Andreea *et al.*, 2000; Pandian *et al.*, 2011; Saidani *et al.*, 2012). Seaweeds are productive sources of bioactive metabolites (De Almeida *et al.*, 2011; Hu *et al.*, 2011). Green, brown and red algae have been known to be a rich source of biologically active metabolites with

antifungal (Demirel *et al.*, 2009), cytostatic, antiviral, antihelmintic and antibacterial activities. For example, Rajasulochana and colleagues (2013) carried out a study on the potential of marine seaweed, *Kappaphycus alvarezii*. From the study, it was reported that *K. alvarezii* exhibited high antifungal activity against fungus *Aspergillus fumigates* and showed maximum antibacterial activity against bacteria *Staphylococcus aureus* (Rajasulochana *et al.*, 2013).

In this project, four common seaweeds in Malaysia namely *S. oligocystum*, *C. racemosa*, *C. racemosa* var. *lamouroxii* and *H. macrophysa* were tested for their antifungal activity specifically against a disease-causing fungus in oil palm, *G. boninense*.

MATERIALS AND METHODS

Collection of seaweeds. Brown seaweed (*S. oligocystum*) and green seaweeds (*C. racemosa*, *C. racemosa* var. *lamouroxii* and *H. macrophysa*) were collected from Teluk Kemang, Port Dickson, Malaysia (2° 26' N latitude; 101° 51' E longitude). The collected seaweeds were washed immediately with seawater and rinsed with tap water to remove any residual salt. The seaweeds were transported to the laboratory in liquid nitrogen and identified by Prof. Phang Siew Moi from Institute of Graduate Studies, Universiti Malaya. The samples were freeze-dried using a freeze-dryer for seven days, ground into fine powder using a mechanical blender and weighed (Abirami and Kowsalya, 2012).

Methanol and chloroform extract preparation. 250 mL of solvent methanol and chloroform were used to extract ten grams of seaweed powder via Soxhlet extraction, respectively. The liquid extract was then cooled down and concentrated using rotary evaporator at 50-60°C. The concentrated extracts were allowed to dry in a hot-air oven, weighed, and kept at 4°C until further use (Abirami, 2012). The percentage yield was calculated by using the following formula:

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

Preparation of fungus culture. *Ganoderma boninense* culture was provided by Dr. Nusaibah Syed Ali from Faculty of Agriculture, Universiti Putra Malaysia. The examination of the fungus structure was done by observing under light microscope at 40 x 10 magnification. The growth rate of *G. boninense* culture was measured from the colony diameter of the culture daily for 14 days by using slide caliper repeated for three times in triplicates.

Antifungal assay. The antifungal activity of seaweed extracts were tested using the poisoned food technique (Bussaman *et al.*, 2012; Schmitz 1930). One percent of dimethyl-sulfoxide (DMSO) was used as co-solvent to dissolve the seaweed extracts and were reconstituted to the concentration of 0.25, 0.5 and 1.0 mg/mL. The seaweed extracts were mixed with warm potato dextrose agar (PDA) containing 100 µg/mL ampicillin and 100 µg/mL penicillin before poured in a 9 cm sterile petri dish. The agar was left to solidify, and a 6 mm agar piece of one-week old fungus mycelia was inoculated to each petri dish. The cultured plates were incubated at 27°C and the diameters of fungal colonies were measured daily for seven days. PDA plates treated with an equal quantity of DMSO were used as a negative control while triadimefon (Sigma-Aldrich, USA) was chosen as positive control due to its ability in inhibiting fungal growth (Jayaratne *et al.*, 2001). All treatments consisted of duplicates repeated three times and the averages of the experimental results were determined. The percentage inhibition of fungal growth was obtained from the following equation:

$$\% \text{ Inhibition} = \frac{\text{ADC-ADT}}{\text{ADC}} \times 100$$

ADC: Average diameter of fungal culture on negative control plate

ADT: Average diameter of fungal culture on plates treated with seaweed extracts

Identification of compounds via GC-MS analysis. Methanol extracts were prepared by solid phase extraction (SPE) method (Abdullah *et al.*, 2004), while the chloroform extract were subjected to syringe filtration to remove impurities. Compounds from both extracts were identified with Thermo Scientific TSQ Quantum

XLS Gas Chromatography (USA) by referring to method by Upgade and Bhaskar (2013).

Data analysis. Each treatment was replicated three times and the results were expressed as mean $\pm$ standard deviation. The mean values were subjected to Kruskal-Wallis H tests (SPSS statistical package, version 22) was used to determine the significant differences ($p < 0.05$) between treatments.

RESULTS

Morphological identification of seaweeds.

The collected seaweeds were authenticated by Prof. Phang Siew Moi by comparing the morphology of the seaweeds from seaweeds library. Figure 1 shows the four Malaysian seaweeds collected.

Figure 1. Four Malaysian seaweed species collected. (a) *S. oligocystum* (b) *C. racemosa* (c) *C. racemosa* var. *lamourouxii* (d) *E. macrophylla*.

Microscopic identification and growth rate of *G. boninense*.

Figure 2 shows the identification of *G. boninense*. The formation of clamp connection (Figure 2a) is similar to identified *G. boninense* strain (Kandan *et al.*, 2010). The shape of *G. boninense* is filamentous while the elevation view is raised. *Ganoderma boninense* went through lag phase starting from day one until day four followed by exponential phase on day five until day eight. At day nine and ten, the fungus undergoes stationary phase and started to die on day twelve (Figure 2b).

Figure 2. (a) Microscopic identification of *G. boninense*. The black arrow showing clamp connection and the morphology similar to identified *G. boninense* strain. (b) Growth rate of *G. boninense* was observed within 14 days. The growth started to deteriorate at day 12.

Percentage yield of seaweed extracts. Two of the different extraction solvents were used per the order of their increasing polarity as different compound gets extracted in different solvents (Figure 3). From 10 g of seaweed powder, methanol extraction relatively produced higher yield than chloroform in all seaweed species. The extraction yield of methanol extracts ranged from 7.11% to 21.20% and chloroform extracts ranged from 1.98% to 7.11%.

Figure 3. Percentage yield of methanolic and chloroform extracts of seaweeds. Methanolic extracts portrayed higher yield in all seaweed species compared to chloroform extracts.

In vitro inhibitory effect of seaweed extracts against *G. boninense*. The results showed that most of the seaweed extracts affect the growth of *G. boninense* at all three concentrations tested and comparative effectiveness of *C. racemosa* var. *lamouroxii* extract is shown in (Figure 4). The highest antifungal activities for methanolic extract against *G. boninense* at concentration of 0.25 mg/mL were exhibited by *C. racemosa* and *C. racemosa* var. *lamouroxii* extracts, with inhibition percentage of 28.06% and 24.50% (Figure 4a), respectively. On the other hand, the highest antifungal activities for chloroform extract against *G. boninense* at concentration of 0.25 mg/mL were

exhibited by *C. racemosa* var. *lamouroxii* and cfH. *macrophysa* extracts, with inhibition percentage of 27.44% and 25.74%, respectively (Figure 4b). Lower antifungal activities were detected in cfS. *oligocystum*-chloroform extract in which the inhibition percentage was 6.82% while the methanolic extract of cfS. *oligocystum* enhanced the growth of *G. boninense*.

Figure 4. Susceptibility of *G. boninense* against seaweed-extracts. (a) Methanol extracts (b) chloroform extracts. Each measure represents average $\pm$ standard deviation from three replicates per treatment.

The morphology of *G. boninense* was observed on PDA plates incorporated with methanol and chloroform extracts. In the seaweed-methanol extract plates, the mycelia growth zone was unclear, and the structure was not white and cotton-like (Figure 5a) while the culture grew in the chloroform extracts had white cotton-like mycelia, but the growth was malformed and stunted (Figure 5b).

GC-MS analysis of seaweed extracts. Compounds from seaweed extracts were identified by comparing the output of the analysis to the National Institute of Standard and Technology (NIST) database library software. For methanolic extract, a total of six major compounds were identified in cfS. *oligocystum* extract, five major compounds in *C. racemosa* extract, seven major compounds in *C. racemosa* var. *lamouroxii* extract, and four major compounds were identified in cfH. *macrophysa* extract (Table 1). For chloroform extracts, seven major compounds detected in cfS. *oligocystum*, *C. racemosa* var.

lamouroxii and *H. macrophysa* extracts while five major compounds in *C. racemosa* extract (Table 2). Several compounds identified in methanol extracts were similar to that were found in chloroform extracts. The major classes of compounds found in all of the extracts were alkaloids, terpenes, fatty acids and sterols.

Figure 5. Growth of *G. boninense* on extract-incorporated PDA after 7 days. (a) Methanolic extract of *C. microphysa*, (b) chloroform extract of *C. racemosa*, (c) negative control, (d) positive control. Red bracket: 6 mm fungus inoculum, black line: diameter of fungal growth.

DISCUSSION

Morphological identification of *G. boninense* was carried out by using light microscopy under 40 x 10 magnification, and we observed clamp connection structure of *G. boninense*. The mycelia were white cotton-like whereas the mature sporophores on upper surface can be in light to dark brown color. These characteristics have been previously reported for *Ganoderma* species (Kandan *et al.*, 2010). The growth of *G. boninense* was observed until day fourteen by measuring the diameter of mycelia of the fungus. During the lag phase, *G. boninense* grew through spore germination and formed mycelia which then developed primordia. Commonly, at the exponential stage, *G. boninense* produces fruiting bodies known as young sporophores and grew rapidly to mature sporophores (Kandan *et al.*, 2010). The stationary phase was between day nine

to day ten in which the growth was stunted and the shrunk of mycelia indicates its death phase at day twelve.

From this study, methanol extracts displayed better extraction capability by showing high extraction yield than chloroform extracts. This might be due to the polarity of methanol which makes it able to extract not only the polar compounds but also some non-polar compounds that is miscible to methanol such as phenolics, fatty acids, sterols, alkaloids, hydrocarbons and more (Abdel-Aal *et al.*, 2015). Chloroform on the other hand is a non-polar solvent which can only solubilize non-polar compounds such as phenolics, diterpene alcohols, fatty acids and other non-polar compounds (Abdel-Aal *et al.*, 2015). The major classes of compounds found in both extracts were diterpene, alkaloid, vitamin, fatty acids and steroids (Table 1).

In this study, almost all the algal extracts tested showed antifungal activity against *G. boninense* proving that extractable materials from marine algae collected from Port Dickson, Malaysia contained bioactive compounds with potential remedial interest. The broad range of classes of bioactive compounds found in seaweeds are carotenoids (Yip *et al.*, 2014), phenolics (Airanthi *et al.*, 2011; Matanjun *et al.*, 2008), alkaloids (Dheeb, 2015; Pawar and Nasreen, 2016), sterols (Abdel-Aal *et al.*, 2015), essential fatty acids (Fayaz *et al.*, 2005; Rajasulochana *et al.*, 2013), vitamins (Hamid *et al.*, 2015), polyphenols, dietary fiber, polysaccharides and proteins (Ibañez *et al.*, 2012). A Kruskal-Wallis H test was conducted to know the effect of solvents and species of extracts towards growth reduction percentage of *G. boninense*. There is statistically significant difference, $r=9.6$, $p=.002$ in growth reduction percentage between the different solvent with a mean rank growth reduction percentage of 44.14 for solvent methanol and 28.86 for solvent chloroform. The effect of different seaweed species tested towards growth reduction percentage showed no significant difference, $r=5.3$, $p=0.15$ with a mean rank growth reduction percentage of 40.61 for species *S. oligocystum*, 31.94 for species *C. racemosa*, 30.00 for species *C. racemosa* var. *lamouroxii* and 43.44 for species *H. macrophysa*.

Table 1. Chemical constituents of seaweed-methanol extracts.

Type of seaweed	Retention time (min)	Component name	Molecular weight (m/z)	Area (%)
<i>S. oligocystum</i>	8.87	Phenol, 2,4-bis(1,1-dimethylethyl)-	206	10.48
	13.71	Hexadecanoic acid, methyl ester	270	3.01
	14.23	l-(+)-Ascorbic acid 2,6-dihexadecanoate	652	21.28
	16.66	cis-Vaccenic acid	282	4.46
	22.05	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	330	9.34
	33.70	Cholest-5-en-3-ol, 24-propylidene-, (3 α)-	426	4.37
<i>C. racemosa</i>	13.99	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	278	19.73
	14.21	Benzenamine, 2-[2-(4-pyridinyl) ethyl]-	198	11.59
	16.29	Phytol	296	2.40
	16.61	9,12-Octadecadienoic acid (Z,Z)-	280	7.91
	16.71	Isobutyl methylphosphonofluoridate	154	9.63
<i>C. racemosa</i> var. <i>lamouroxii</i>	14.19	l-(+)-Ascorbic acid 2,6-dihexadecanoate	652	11.59
	20.62	Ethyl iso-allocholate	436	3.57
	21.01	Hexacyclo [7.2.2.2(4,7).0(3,8).0(12,14).0(13,15)] pentadecan-1-ol-2-one	438	3.16
	32.70	Benzenamine, 2-[2-(4-pyridinyl)ethyl]-	198	7.94
	33.91	Spirost-8-en-11-one, 3-hydroxy, (3 α , 5 α , 14 α , 20 α , 22 α , 25R)-	428	3.97
	35.48	1-Monolinoleoylglycerol trimethylsilyl ether	498	5.45
	36.00	Isobutyl methylphosphonofluoridate	154	6.34
<i>H. macrophysa</i>	14.19	l-(+)-ascorbic acid 2,6-dihexadecanoate	652	3.93
	24.63	Isobutyl methylphosphonofluoridate	154	3.75
	26.49	1-Monolinoleoylglycerol trimethylsilyl ether	498	3.07
	33.22	Benzenamine, 2-[2-(4-pyridinyl) ethyl]-	198	4.02

m/z = mass per charge number of ions

All eight extracts were further analyzed for their compounds via GC-MS. Based on the results obtained (Tables 1 and 2), all eight extracts mainly consist of compounds from the classes of alkaloids, terpenes, fatty acids and sterols. Previous studies have shown that there are a few compounds that were identified in methanol and chloroform extracts such as phytol (Shobier *et al.*, 2016) and l-(+)-ascorbic acid 2,6-dihexadecanoate (Karthikeyan *et al.*, 2014) were correlated with strong biological activities. Phytol is a precursor of vitamin E and vitamin K and was known for their antimicrobial activity (Inoue *et al.*, 2005; Pejin *et al.*, 2014). Phytol is widely used in the fragrance industry, cosmetics, shampoos, toilet soaps, household cleaners, and detergents (Ghaneian *et al.*, 2015; McGinty *et al.*, 2010). While l-(+)-ascorbic acid 2,6-dihexadecanoate characterized from plants and marine sources was also demonstrated powerful antimicrobial activity

against *Staphylococcus aureus*, *S. faecalis*, *P. aeruginosa*, *Escherichia coli*, *Streptococcus pneumonia* and *Proteus mirabilis* (Okwu and Ighodaro, 2009). Therefore, these compounds may be associated with the observed inhibitory effect on the growth of *G. boninense* in this study.

To summarize, methanol produced higher yield than chloroform due to its ability to extract polar and non-polar compounds. Three different species of marine seaweeds collected, namely, *C. racemosa*, *C. racemosa* var. *lamouroxii* and *H. macrophysa* are potential species as they exhibited antifungal activities at the lowest concentration tested while *S. oligocystum* extract slightly inhibited the growth of *G. boninense*. Further studies to identify the antifungal compounds and their synergism should be carried out as they could be utilized as the source of antifungal compounds which could be useful to the Malaysian oil palm industry.

Table 2. Chemical constituents of seaweed-chloroform extracts.

Type of seaweed	Retention time (min)	Component name	Molecular weight (m/z)	Area (%)
<i>S. oligocystum</i>	11.72	Tetradecanoic acid	228	2.29
	12.65	Phytol	296	13.02
	14.28	l-(+)-Ascorbic acid 2,6-dihexadecanoate	652	12.88
	22.60	Diisooctyl phthalate	390	4.42
	26.03	Tetratriacontane	478	4.98
	28.26	2H-1-Benzopyran-6-ol,3,4-dihydro-2,8-dimethyl-2-(4,8,12-trimethyl tridecyl)-, [2R-[2R*(4R*,8R*)]]-	402	3.94
	31.68	α-Tocopherol, O-methyl-	430	5.61
<i>C. racemosa</i>	11.75	Tetradecanoic acid	228	2.87
	14.21	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	278	17.89
	14.57	Benzenamine, 2-[2-(4-pyridinyl) ethyl]-	198	32.41
	16.34	Phytol	296	1.72
	17.01	Isobutyl methylphosphonofluoridate	154	5.19
	31.72	α-Tocopherol, O-methyl-	430	1.72
<i>C. racemosa</i> var. <i>lamouroxii</i>	14.19	l-(+)-Ascorbic acid 2,6-dihexadecanoate	652	2.69
	16.29	Phytol	296	6.02
	17.25	Hexadecanamide	255	3.15
	20.26	Cholesta-5,7,9(11)-trien-3-ol acetate	424	4.50
	21.03	Isobutyl methylphosphonofluoridate	154	21.57
	31.61	(+)-α-Tocopherol, O-methyl-	430	6.80
	33.50	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-(5a,5a,8a)]-	398	9.51
<i>H. macrophysa</i>	13.49	Benzenamine,2-[2-(4-pyridinyl)ethyl]-	198	4.13
	13.80	α-Sitosterol	414	3.40
	13.96	Isobutyl methylphosphonofluoridate	154	6.51
	14.27	l-(+)-Ascorbic acid 2,6-dihexadecanoate	652	13.41
	16.30	Phytol	296	4.55
	20.69	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl) hexa-1,3,5-trienyl] cyclohex-1-en-1-carboxaldehyde	324	4.11
	31.45	Cholesterol	386	4.84

m/z = mass per charge number of ions

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