

Antifungal Potential of Yellow Bur Head *Limnocharis flava* (Buchenau, 1868) Against Pathogenic Oomycete, *Lagenidium thermophilum*

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ABSTRACT

Fungal infection by marine oomycetes is the main problem that hinders crustacean production. Therefore, a study to find an alternative fungal treatment that is safer than chemical treatment is currently needed. One of the potential sources of antifungal properties is macrophytes. *Limnocharis flava*, known as yellow bur head, was found to have antimicrobial properties. Thus, this research was conducted to determine the potential of *L. flava* extract as an antifungal agent against the marine oomycetes *Lagenidium thermophilum* IPMB 1801. In this study, ethanol and methanol solvents were used to extract *L. flava*. The results showed that the methanol extraction yield of *L. flava* is higher (7.03 g, 35.16%) compared to ethanol extract (3.26 g, 16.26%). The antifungal screening test was conducted using the disc diffusion method. Ethanol and methanol extract of *L. flava* had antifungal activities against the hyphal growth of *L. thermophilum*. Continuation from the screening test, the minimum inhibitory concentration for both ethanol and methanol extracts was determined to be at 100 mg/ml respectively. These findings suggest that *L. flava* has the potential to become an antifungal treatment for the control of fungal infections in the crustacean industry.

Keywords: phytobiotic, antibiotic resistance, alternative prevention, disease management, aquaculture

INTRODUCTION

A fungal infection had hindered crustacean production, particularly at eggs and zoea stages. The infection caused the larvae to be vulnerable to other secondary infections (Jithendran et al. 2010). In crustacean culture, the causative agents of fungal infection are *Lagenidium* spp., *Sirolopidium* spp., *Atkinsiella* spp., and *Haliphthoros* spp. (Hatai, 2012). Among them, *Lagenidium* spp. has been reported to cause damage to the culture of crustaceans and molluscs (Jithendran et al., 2010; Hatai, 2012). *L. thermophilum* isolated from black tiger shrimp, *Penaeus monodon* (Muraosa et al., 2006) and mud crab, *Scylla serrata* (Nakamura et al., 1995) is one of the important marine fungal pathogens that infect crustaceans (Hatai et al., 2000; Lee et al., 2016). Previously, there was a mass mortality event of mud crab zoea culture in Universiti Malaysia Sabah and the fungal strains were identified as *L. thermophilum* as the causative agent (Lee et al., 2016; 2017).

The chemical treatment has been widely used for the control of fungal infection. Trifluralin, malachite green and formalin have been used as an antifungal agent against *L. thermophilum* (Lee et al., 2016). However, these compounds may be absorbed into the body and cause internal effects and it poses a risk for the consumers. It is still being used since it has low cost, availability, and efficiency, and lacks proper alternatives (Treves-Brown, 2000). This led to the search for alternative antifungal sources that are natural and safe. Among the alternatives, plant extract has been tested as an alternative treatment against microbial pathogens. Previous studies reported that *Cabomba aquatica* leaves have antifungal effects due to the presence of secondary metabolites such as tannins, steroids, glycosides, flavonoids, phenolic compounds, and alkaloids (Malathy et al., 2015). Ginger *Plagiostachys megacarpa* that were extracted showed an antifungal effect against *L. thermophilum* IPMB 1401 (Hatai et al., 2018). Seaweed extracts are known for functional food and obtain bioactive compounds (Pal et al., 2014). Previous studies of ethanol, methanol, and water extract of *Eucheuma cottonii* and *Caulerpa lentillifera* were reported to have a high antifungal effect against *H. sabahensis* and *L. thermophilum* (Saito & Lal, 2019). Prevention and treatment of infectious diseases using plants is a valid alternative because it contains promising sources of pharmaceuticals. To explore more alternatives, macrophytes may be suitable candidates for the source of antifungal compounds.

Currently, the study on alternative treatments for mud crab fungal from macrophytes extraction is scarce. Macrophytes extraction like the *Limnocharis flava* plant may be a suitable candidate to treat fungal infection. Based on a previous study there is more potential for macrophytes extraction to inhibit fungal growth (Fareed et al. 2008). Therefore, this study increases the value of plants by determining the antifungal effect against *Lagenidium*. Large quantities of *L. flava* plants are easily available from the shallow swamps, ditches, and wet paddy fields. This study contributes to exploring alternative treatments against fungal infections in aquaculture organisms. The objective of this research is to determine the antifungal effect of yellow bur head against *L. thermophilum*.

MATERIALS AND METHODS

Collection and Processing of *Limnocharis flava*

Limnocharis flava was collected from swampy areas at Kota Kinabalu, Sabah. Plants were washed by using tap water until mud and other foreign particles were washed away and removed (Fareed et al. 2008). Then, plants were placed on drying trays and dried at 50°C until fully dried by the drying oven. After drying, the plants were blended into small pieces using an electronic blender, and Panasonic MX-AC2105 mixer grinder (Saito & Lal, 2019). The blended plants were kept inside an airtight plastic bag and refrigerated at 4°C until further use.

Preparation of *Limnocharis flava* Extract

Ethanol and methanol were used to extract *Limnocharis flava* compounds. Ethanol and methanol extraction is done using the maceration process (Azmir et al., 2013). About 20 g of *L. flava* and 200 mL (1:10 w/v) of solvents were added into a 250 mL conical flask and placed in a shaking incubator (Jeio Tech SI 900R shaking incubator) for 3 days at 25°C. After 3 days, the extracts were filtered using Whatman glass fibre filter paper GF/C (circle) 240 mm diameter. The crude extracts were placed into a rotary evaporator at 40°C and 70 hPa. The extraction yield was calculated using the method described by Ngamkhae et al. (2022). The samples were stored in a reagent bottle covered with aluminium foil in the refrigerator at 4°C for further use.

Fungi Strain Selection and Agar Plate Preparation

Peptone Yeast Glucose Seawater (PYGS) agar was applied for various tests throughout this study. The composition of PYGS agar includes 1.2% agar powder, 0.3% glucose, 0.124% peptone, and 0.125% yeast extract in filter seawater. Seawater was filtered through Whatman glass fibre filter paper GF/C (circle) 240 mm diameter. The mixture was mixed by using a magnetic stirrer for 5 minutes until the ingredient dissolved and autoclaved at 121°C for 15 minutes. PYGS agar was left at room temperature for 10-20 minutes before pouring into Petri dishes (100 × 20 mm) in a bio-safety cabinet. The agar plates were allowed to be dried and cooled down in the bio-safety cabinet and treated with Ultraviolet (UV) light for 30 minutes before using the agar plate for fungus subculture or conducting tests. Prepared PYGS agar plates were kept in the refrigerator after cooling and used within three days.

The *L. thermophilum* IPMB 1801 marine fungi obtained from the Borneo Marine Research Institute (IPMB) culture collection were selected for this study. The fungal strain was inoculated on PYGS agar and incubated at 25°C throughout this study. After 3 – 4 days with the appearance of fungal growth, the agar blocks with apparent mycelia masses were transferred onto the new PYGS agar for pure culture (Panchai et

al., 2014). Subculture was done every two to three weeks depending on the fungal growth. The process involves agar blocks at the periphery of the fungi colony being cut into blocks using a sterile blade and then transferred onto the centre of a new PYGS agar plate, invertedly.

Screening for Antifungal Effect

The disc diffusion method (Mostafa et al., 2018) with certain modifications was used in the *L. flava* extracts antifungal effect screening test. The procedures were done in a bio-safety cabinet. It was done by measuring a 10 mm × 10 mm area in the centre of the agar plate to inoculate 10 mm × 10 mm fungal blocks. 5 mm distance away from the 10 mm × 10 mm area. Another 5 mm distance was plotted again. It is 5 mm away from the 10 mm × 10 mm area.

Agar plates were prepared to incubate fungus blocks on it, a 10 mm × 10 mm agar block was measured by using a ruler and cut using a sterile blade at the periphery of the fungus colony with fully covered fungus hyphae. The 10 mm × 10 mm agar block was inverted on the measured 10 mm × 10 mm with careful. The agar plate with fungi is allowed to grow for at least three days until the fungus growth radius reaches 5 mm on all sides.

When the growth radius reached 5 mm, the sterile 8 mm disks that were impregnated with 20 µL test compound (ethanol and methanol extracts) were placed over the plate. After that, the opening of the Petri dish was sealed with parafilm. The agar plates were incubated at 25°C for 7 days and observed daily. The antifungal effect was determined by observing the inhibition activities of the fungus growth towards the left and right side of the Petri dish and comparing it with the top and bottom direction growth whereby there were no test solutions. The growth pattern was compared with the control.

Minimum Inhibitory Concentration (MIC)

Limnocharis flava extract that shows positive antifungal effects undergoes the antifungal bioassay test. The MIC of *L. flava* extracts against fungal strains was determined by using the disc diffusion method (Mostafa et al., 2018). The initial stock was 500 mg/ml of *L. flava* extracts with a 50% concentration of both extracts. The dilutions were done by reducing the concentration to 50 mg/mL, 100 mg/mL, 200 mg/mL, 300 mg/mL, and 400 mg/mL.

MIC values (mg/mL) of extracts and controls (ethanol and methanol) against fungi were tested *in vitro*. Strain samples of *L. thermophilum* IPMB 1801 that were incubated for two weeks were used to test MIC. A total of seven inoculated fungal strains in 10 mm × 10 mm agar block were cut and added into seven PYGS agar plates by using a sterile blade and forceps and incubated at 25°C for 3 days until there was visible hyphae growth. After 3 days, the disc containing extract was placed into the agar plate. Each plate had two discs that contained ethanol and methanol extracts. The

total volume in each disc was 20 μ L. The control discs were added with 20 μ L of ethanol and methanol, respectively. The PYGS agar plate containing the disc was incubated at 25°C for 7 days and observed daily. The MIC values were determined by recording the minimum concentration whereby fungal growth is inhibited. Fungal growth that shows inhibitory activities is considered a positive antifungal effect, while fungal growth that shows no inhibitory activities is considered a negative result.

RESULTS AND DISCUSSION

Antifungal effects had been tested on marine oomycetes *L. thermophilum* IPMB 1801. The extraction yield was recorded in Table 1. *Limnocharis flava* methanol extract (LF-M) has an extraction yield of 35.16% while *L. flava* ethanol extract (LF-E) has an extraction yield of 16.25%. The finding shows that LF-Meth extract has a higher extraction yield compared to LF-Eth extract. The results are comparable to a previous study by Saito and Lal (2019). In both studies, extractions using methanol as the solvent produced a higher percentage of yield while extractions using ethanol as solvent have a lower overall yield percentage despite using different types of plant samples. This shows that the extraction yield increases with the increasing polarity of the solvent used in extraction (Do et al., 2014). The efficiency of the extraction is strongly affected by the extraction method, temperature, extraction time, the composition of phytochemicals, and the solvent used (Ngo et al., 2017; Truong et al., 2019). In the present study, results showed that ethanol and methanol solvents resulted in different extraction yields. This is because differences in the polarity of the extraction solvents could cause a variation in the level of bioactive compounds in the extract, indicating that the extraction efficiency favours the highly polar solvents (Truong et al., 2019). These bioactive compounds are normally created in plants as secondary metabolites with antifungal action. These mixture compounds are significant for the physiology of plants contributing properties that give opposition to microorganisms and different creatures (Castillo et al., 2012). This could be because the plant material contains high levels of polar compounds that are soluble in solvents with a high polarity like methanol. The highest levels of flavonoid, saponin, triterpenoid, phenol hydroquinone, and ninhydrin in *L. flava* extraction, thus resulting in the highest extraction yield of methanolic extract (Jacob et al., 2010; Baehaki et al., 2019; Truong et al., 2019).

Table 1 Extraction yield and antifungal effects of the *L. flava* extracts against *L. thermophilum*

Extraction	Code	Extraction yield (g)	Yield percentage (%)	Antifungal activities on LT1801
<i>L. flava</i> – Methanol	LF – M	7.03	35.16	+
<i>L. flava</i> – Ethanol	LF – E	3.26	16.26	+

+ = Positive effect; – = Negative effect; LT1801 = *L. thermophilum* IPMB 1801

Both tests showed positive results against marine oomycetes (Table 1). The result shows that both extracts could inhibit the growth of hyphae *L. thermophilum* (Figure 1). The ethanol and methanol extract of *L. flava* showed an inhibitory effect on hyphae growth of *L. thermophilum*. Similar cases of the presence of antifungal and antimicrobial activities have been proven in several case studies. For example, a study using dichloromethane and ethanol extracts of *L. flava* had antimicrobial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pyogenes* (Mohd Nazri et al., 2011). In the case of an antifungal activity, the ethanol extract of *L. flava* showed inhibition against fungal *Candida albicans* (Mohd Nazri et al., 2011). Bioactive compounds like flavonoid, saponin, triterpenoid, phenol hydroquinone, and ninhydrin were previously extracted from *L. flava*, and these active compounds are linked to be a part of phytohormones and antifungal compounds that help in the plant growth, nutrition, and development (Jacoeb et al., 2010; Baehaki et al., 2019). These bioactive compounds are normally created in the plants as secondary metabolites which potentially become antifungal agents (Castillo et al., 2012). The presence of antifungal activity from the methanol and ethanol extracts of *L. flava* in this study indicates that the previously described bioactive compounds linked with antifungal compounds can potentially be found in the present study's extracts, however further analysis and more intricate analysis is needed to be conducted to reach a more solid conclusion regarding the antifungal mechanism of *L. flava* methanol and ethanol extracts.

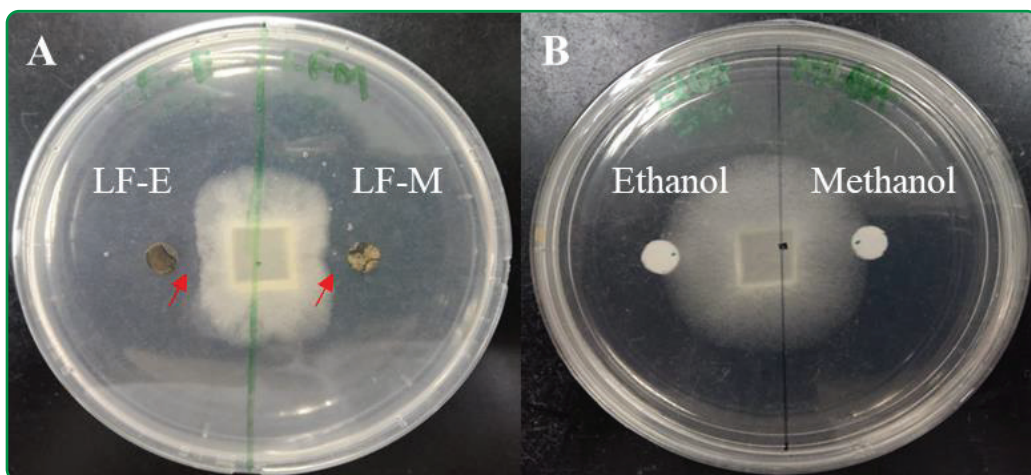


Figure 1 (A) Positive antifungal results from both ethanol (left) and methanol (right) extracts of *L. flava*. Evident inhibition of mycelial masses near the disc is shown by using an arrow (red). (B) The ethanol (left) and methanol (right) extract as control showed no effect against the fungal growth.

Minimum inhibitory concentration (MIC) is the lowest concentration of an antifungal agent expressed in mg/L ($\mu\text{g/mL}$) which, under strictly controlled *in vitro* conditions, completely prevents visible growth of the test strain of an organism after incubation. In the present study, the MIC test was conducted by using the disc diffusion method which is known as the gradient method with few modifications (Mostafa et

al., 2018; Saito & Lal, 2019; Joning et al., 2021). MIC determination using the gradient method is much less complicated than dilution methods (Kowalska-Krochmal and Dudek-Wicher, 2021). The use of disc filter paper impregnated with a predefined concentration gradient of *L. flava* ethanol and methanol extracts makes the method simple, fast, and applicable in routine antifungal diagnostics. *L. flava* of ethanol and methanol extracts showing positive results in screening tests were carried forward to the minimum inhibitory concentration (MIC) test. Ethanol and methanol extract show the lowest MIC at 100 mg/ml against marine oomycetes, *L. thermophilum* (Table 2). In contrast to previous studies by Hatai et al., (2018) showed that the wild ginger, *Plagiostachys megacarpa* extraction at a concentration of 320 µg/mL inhibited both hyphal growth and zoospore production of *L. thermophilum* in 24 h. MIC result from Figure 1 showed that the *L. flava* extraction had an inhibitory effect towards hyphal growth at 100 mg/mL which is lower compared to *P. megacarpa* which is 320 µg/mL. However, it is very difficult to direct comparison because reports on *L. flava* extracts against marine oomycetes species are very limited.

Table 2 Results of Minimum inhibitory concentration test of both *L. flava* extracts against *Lagenidium thermophilum* IPMB 1801

Extracts	MIC Test						
	EtOH	MeOH	50 mg/ mL	100 mg/ mL	200 mg/ mL	300 mg/ mL	400 mg/ mL
LF-M	-	-	-	+	+	+	+
LF-E	-	-	-	+	+	+	+

+ = Positive antifungal effect; - = Negative antifungal effect; EtOH = Ethanol; MeOH = Methanol

In conclusion, *Limnocharis flava* ethanol and methanol extracts were found to have an antifungal effect against *Lagenidium thermophilum* IPMB 1801. This study found that the lowest minimum inhibitory concentration is 100 mg/mL. Therefore, *L. flava* extract has the potential to become an antifungal treatment for the control or prevention of fungal growth and infections in the crustacean industry. However, further tests such as levels of toxicity or characterization of bioactive compounds for both extracts are needed to be conducted in the future. This information is important to find the best alternative method to treat infection of marine oomycetes in crustacean production.

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