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Note

In vitro Inhibitory Effects of Two Bornean Medicinal Wild Gingers against Pathogenic *Lagenidium thermophilum* Infected Mud Crab *Scylla tranquebarica*

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The antifungal activity of two Bornean medicinal wild gingers *Plagiostachys megacarpa* and *Zingiber phillippsiae* were examined against *Lagenidium thermophilum*. The most active extract was *P. megacarpa* at concentration of 320 µg/mL inhibiting both hyphal growth and zoospore production of *L. thermophilum* in 24 h. Toxicity tests were conducted using mud crab (*Scylla tranquebarica*) larva. Bath treatment of *P. megacarpa* at concentrations of 320 and 640 µg/mL for 24 h were highly effective against hyphae and zoospores of the strain and it is non-toxic to mud crab larva. Therefore, crude extracts *P. megacarpa* may be used as alternative treatment for marine Oomycete infection of mud crab.

Key words：Antifungal substances / *Lagenidium thermophilum* / *Scylla tranquebarica* / Borneo / Medicinal wild ginger.

Mud crab *Scylla tranquebarica* aquaculture was one of the most economically important species in hatcheries and local fisheries for seafood production in Sabah, Malaysia. The outbreaks of marine Oomycetes (lower fungi) infection have severely affected the mud crab production in Sabah, Malaysia (Lavilla-Pitogo, & Peña, 2004). The possible and potent causative agents for this issue were the lower fungi of genera *Lagenidium* and *Haliphthoros* (Hatai, 2012).

As an attempt of solving this issue, the formalin, trifluralin, and malachite green were widely studied of their *in vitro* antifungal activities against these fungi (Lio-Po et al., 1982; Kaji et al., 1991; Hamasaki, & Hatai, 1993; De Pedro et al., 2007; Lee et al., 2016a; 2016b). Despite its antifungal potentials to control this infection, their application was always been hesitated due to its toxicity and complication concerning aquatic ecosystems and health issue of treated aquatic life and residents (Schreier et al., 1996). According to Prost and Sopinska (1989), repeated treatment of this lower fungal infection with these chemicals may cause an immunosuppressive effect on treated aquatic organisms. Furthermore, the hazardous residues may be remained in the body or tissues in seafood (Kitancharoen et al., 1997a; Kitancharoen et al., 1997b; Fuangsawat et al., 2011).

The recent discoveries of natural antifungal agents from natural sources have become common. Hussein et al. (2002) showed that *Nigella sativa* extracts and its major carbonaceous constituents, thymoquinone solution has significant antimycotic activity against hyphal growth and zoospores germination of *Saprolegnia parasitica* NJM 8604 and *S. salmonis* NJM 9851. Udomkusonsri et al. (2007) reported that crude ethanolic extracts of *Piper betle* leaves and *Kaempferia galanga* roots had potent anti-oomycetic effects on hyphal growth and zoospores production of *S. parasitica* H2. The ethanolic extracts of *Psidium guajava* and *P. betle* leaves effectively inhibited hyphae growth and zoospores production of *S. diclina* NJM 0208, *Achlya* sp. NJM 0323 and *Aphanomyces invadans* NJM 0002 (Borisutpeth et al., 2009). Apart from that, alcoholic

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Cassia fistula stem-bark extracts was also reported to have antifungal effects on hyphae and zoospores of *S. paracitica* NJM 8604, *S. diclina* NJM 0005 and *A. invadans* NJM 9701 (Borisutpeth et al., 2014). Aqueous extractions of pomegranate (*Punica granatum*) was also reported as potent anti-oomycetic agents against hyphae and zoospore formation in *A. prolifera* BKKU 1125, *Achlya* sp. BKKU 1117 and BKKU 1127, *A. klebsiana* BKKU 1003 and *A. diffusa* BKKU 1012 (Panchai et al., 2015).

In this present study, two medicinal wild gingers, *Plagiostachys megacarpa* A. Julius & A. Takano and *Zingiber phillippsiae* Mood & Theilade were extracted and subjected to antifungal test against *L. thermophilum* IPMB 1401 (Lee et al., 2016a) which isolated from mud crab *S. tranquebarica*. The native species of ginger *P. megacarpa* and *Z. phillippsiae* both belong to the family Zingiberaceae which distributed in Southeast Asia and contained high medicinal value through ethnobotany studies. In North Borneo, local Dusun people eat these gingers to cook and boil with fish. Furthermore, the medicinal properties of these gingers are trusted to cure swollen body and snake bite in their communities. The aims of the present study are: (i) to examine the antifungal activity of Bornean medicinal traditional herbal medicine extracts on hyphae and zoospores of *L. thermophilum*, and (ii) to evaluate their toxicities to newly hatched mud crab *S. tranquebarica* larva.

Plant materials of *P. megacarpa* (BORH1056JK) and *Z. phillippsiae* (BORH1050JK) were collected from Tudan Village, Tuaran District, Sabah, Malaysia and identified by the fourth author. The voucher specimens were deposited in the BORNEENSIS Collection of Institute for Tropical Biology and Conservation, University of Malaysia Sabah.

In the preparation of leaf extracts, the extraction method was according to the modified procedures (Pise, & Sabale, 2014; Gunathilake, & Vasantha Rupasinghe, 2014; Godlewska et al., 2016). The leaves were washed, air-dried (2.2 kg), cut into smaller pieces and boiled in distilled water (18.8 L) for 30 min. The solution was filtered and concentrated by boiling it for 3 h where the volume was reduced to 6.0 L. Subsequently, the aqueous extract was transferred into 12 bottles, each with 500 mL.

The pathogenic fungal strain *L. thermophilum* (IPMB 1401) (Lee et al., 2016a) was routinely maintained at Microbiology and Fish Disease Laboratory, Borneo Marine Research Institute on PYGS agar (0.125% peptone, 0.125% yeast extract, 0.3% glucose, 1.2% agar in 1 L seawater) at 25℃ and sub-cultured to fresh PYGS agar every two wk. The advancing edges of 5-7 day growing whitish fungal colony on PYGS agar were excised and used as inoculums for all experiments.

The plant extracts were diluted to different concentrations (80, 160, 320, 640, 1280, 2560, 5120 and 10240 µg/mL) using sterilized seawater (SSW) in Petri dishes respectively. While, SSW without extracts was served as the control. According to the methods in Lee et al. (2016a; 2016b), agar blocks of the strain IPMB 1401 were excised from the edge of the parent colony using No. 2 cork borer (5 mm in diameter) and subsequently immersed in each Petri dishes containing 30 mL of the test solutions for 1, 2 and 24 h. After each exposure time, two agar blocks were rinsed several times in SSW. Agar block was inoculated onto PYGS agar, the hyphal growth was observed at 48 h incubation at 25℃. Another agar block was transferred to fresh SSW, the zoospore production was observed at 24 h with 25℃ incubation using an inverted microscope Olympus CKX 41.

Healthy newly hatched mud crab larvae (zoea stage) from hatchery were rinsed with SSW containing each 500 µg/mL of streptomycin and ampicillin solution (Lee and Hatai, 2016). The negative control group is SSW. The experiments were divided into two trials with the concentrations of crude extract as 80, 160, 320, 640, 1280, 2560, 5120 and 10240 µg/mL as follows: (i) the experimental group I was *P. megacarpa* extract; and (ii) the experimental group II was *Z. phillippsiae* extract. Total 20 individuals of mud crab larvae were exposed to each 30 mL of the test solutions in Petri dishes for 1, 2 and 24 h. After each exposure duration, the survival of mud crab larva was observed by naked eyes in Petri dishes for their movement and motility under an inverted microscope Olympus. While, inactive and non-motile mud crab larvae were considered dead. These dead ones were investigated if the cause was fungal infection (De Pedro et al., 2007). The number of mud crab larva surviving at each exposure duration was used to calculate the corrected mortality following Abbott's formula (Barnes et al., 1998):

$Pt = ((Po - Pc))/((100 - Pc)) \times 100$

Pt = percentage of corrected mortality (%);

Po = mortality of test group;

Pc = mortality of control group.

Treatment with various concentrations of extracts for 1, 2 and 24 h on *L. thermophilum* at 25℃, is shown in Table 1. The antifungal inhibitory effects of these extracts varied considerably. The *P. megacarpa* extract could inhibit hyphal growth and zoospores discharged at concentration of 320 µg/mL and above for 24 h. While, extracts of *Z. phillippsiae* inhibited hyphal growth and zoospores discharged at concentrations at 640 and 1280 µg/mL and above in 24 h. The vegetative and zoosporic stages of *L. thermophilum* were inhibited after treatment of *P. megacarpa* and *Z. phillippsiae* extracts at concentrations of 320 and 1280 µg/mL, respectively

TABLE 1. Effect of plant leaf extracts on hyphal growth and zoospores production of *L. thermophilum*.

Concentration (μg/mL)	*P. megacarpa*			*Z. phillippsiae*		
	Exposure duration (h)					
	1	2	24	1	2	24
0	+[a]/+++[b]	+/+++	+/+++	+/+++	+/+++	+/+++
80	+/+++	+/+++	+/++	+/+++	+/+++	+/+++
160	+/++	+/++	+/+	+/+++	+/++	+/++
320	+/++	+/+	-/-	+/++	+/++	+/++
640	+/++	+/+	-/-	+/++	+/++	-/+
1280	-/+	-/+	-/-	+/++	+/+	-/-
2560	-/-	-/-	-/-	-/+	-/-	-/-
5120	-/-	-/-	-/-	-/-	-/-	-/-
10240	-/-	-/-	-/-	-/-	-/-	-/-

[a]Hyphal growth on PYGS agar. -: no growth, +: growth;
[b]Amount of zoospores released: + mean little amount, ++ mean fair amount and +++ mean excellent amount.

TABLE 2. The corrected cumulative mortality (%) of mud crab larvae treated by the plant leave extracts.

Concentration (μg/mL)	*P. megacarpa*			*Z. phillippsiae*		
	Exposure duration (h)					
	1	2	24	1	2	24
0	0	0	0	0	0	0
80	0	0	0	0	0	0
160	0	0	0	0	0	0
320	0	0	0	0	0	40
640	0	0	0	0	0	45
1280	0	0	50	30	65	100
2560	60	85	100	55	80	100
5120	80	90	100	70	80	100
10240	100	100	100	100	100	100

in 24 h. Hyphae and zoospores were inhibited after treatment with 2560, 5120 and 10240 μg/mL of treatment levels in all exposure duration, except zoospores production was found at 2560 μg/mL in 1 h for *Z. phillippsiae* extract.

Mud crab larva showed high percentage of cumulative mortality (>50%) when exposed to 2560 μg/mL and above of extracts for 1, 2 and 24 h, is shown in Table 2. Mud crab larva was able to tolerate at 1280 μg/mL of *P. megacarpa* extract for 1 and 2 h, but it showed 50% mortality after exposure for 24 h. Low mortality (<50%) of mud crab larva was observed at 320 μg/mL of *Z. phillippsiae* extract for 24 h. The control and experimental groups of mud crab larva showed no mortality at 80 and 160 μg/mL of both extracts as well as 320 and 640 μg/mL of *P. megacarpa* extract in either 1, 2 or 24 h.

The present study searched for the potential antifungal agents from Borneo traditional herbal medicine that are safe and effective treatments against *L. thermophilum* infection in mud crab *S. tranquebarica*. The results revealed the extracts were able to kill and inhibit hyphal growth and zoospore production of *L. thermophilum*. The efficacy of these extracts against the hyphal growth and zoospore formation of *L. thermophilum* was in the order of *P. megacarpa* > *Z. phillippsiae*.

In the toxicity test, no mortality occurred in mud crab larva for both extracts at concentration of 80 and 160 μg/mL. Extract of *P. megacarpa* has the lowest toxicity on mud crab larva with the concentration of 640 μg/mL and above for exposure within 24 h. These findings showed that *P. megacarpa* inhibited the growth of *L. thermophilum* without threatening the life of mud crab larva. The concentrations at 320 and 640 μg/mL of *P. megacarpa* extract were highly effective against hyphae and zoospores of *L. thermophilum* in 24 h, meanwhile it was well tolerated by mud crab larva. Extract of *P. megacarpa* had a potential as the preventive measurement of mud crab against fungal infection of *L. thermophilum* through bath treatment in crustacean hatchery for 24 h. It was reported that the zoospores were involved in the initiation of aquatic Oomycete infection (Pickering, & Willoughby, 1982; Beakes et al., 1994; Bruno, & Wood, 1999). The bath treatment of this extract might not completely killed all the hyphae and zoospores of this fungus, however it may be used as preventive measurement against initial infection of *L. thermophilum*. In this regard, this extract was very prom-

ising for the treatment of marine Oomycete infection due to its significant antifungal activity against hyphae and zoospores of *L. thermophilum* while still remaining non-toxic to mud crab larva. In addition, extract of *Z. phillippsiae* was also a potent anti-oomycete agent against hyphae of *L. thermophilum*, the exposure condition to the plant leave extracts shown here is a threshold to show antifungal activity. Cheeptham and Towers (2002) stated that the usage of plant extracts as antifungal treatment was limited due to their low percentage yield and source availability. Therefore, it is imperative to investigate the extraction yield of plant extracts and their chemical constituents. Further investigation pertaining to their chemical constituents could identify the antifungal compounds present in the extract. This study is the first report on the *in vitro* effects of the Bornean traditional herbal medicine against pathogenic *L. thermophilum* isolated from mud crab *S. tranquebarica*. It is concluded that, extract of *P. megacarpa* was a promising alternative fungicide to treat marine Oomycete (lower fungi) infection in hatcheries.

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