Evaluation of an anti-parasitic compound extracted from *Streptomyces* sp. HL-2-14 against fish parasite *Ichthyophthirius multifiliis*

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SUMMARY

The present study was conducted to evaluate the anti-parasitic activity of a pure compound from *Streptomyces* sp. HL-2-14 against fish parasite *Ichthyophthirius multifiliis*, and elucidate its chemical structure. By electron ionization mass spectrometry (ESI-MS) and nuclear magnetic resonance spectrum (^{1}H NMR and ^{13}C NMR), the compound was identified as amphotericin B (AmB). The *in vitro* trials revealed that AmB can effectively kill the theronts and tomonts of *I. multifiliis* with the median lethal concentration (LC_{50}) of 0·8 mg L^{-1} at 30 min for the theronts and 4·3 mg L^{-1} at 2 h for the tomonts, respectively. AmB at 5 mg L^{-1} significantly reduced *I. multifiliis* infectivity prevalence and intensity on grass carp (*Ctenopharyngodon idella*), and consequently decreased fish mortality, from 100% in control group to 30% in treated group. The 72 h acute toxicity (LC_{50}) of AmB on grass carp was 20·6 mg L^{-1}, but fish mortality was occurred when exposure to 13·0 mg L^{-1}. These results indicated that AmB was effective in the therapy of *I. multifiliis* infection, but the safety concentration margin is relatively narrow. Further efforts aiming to decrease the toxicity and improve the therapeutic profile remain to be needed.

Key words: ichthyophthiriasis, streptomycete, amphotericin B, anti-parasitics, grass carp.

INTRODUCTION

Ichthyophthirius multifiliis is a common protozoan parasite of freshwater fish, causing 'white spot disease' or 'ichthyophthiriasis' in tropical and subtropical regions with high mortality in aquarium and cultured fish (Shinn *et al.* 2012). Typical pathologic changes of fish infected with *I. multifiliis* are white spots on the gills and skin and increased mucus production (Yao *et al.* 2010). The gills are highly susceptible to infection, and the resulting gill damage contributes considerably to the lethal effects caused by the parasite (Yao *et al.* 2010). There are four key developmental stages within the life cycle of *I. multifiliis*: the sub-epithelial, parasitic trophont (1) which, when mature, exits the host as a free-swimming tomont (2). Tomonts quickly settle on an appropriate substrate, encyst (3) and then undergo binary fission to produce tomites. Released tomites rapidly differentiate into theronts (4) which become infective (Matthews, 2005; Picón-Camacho *et al.* 2012). Both the theront and tomont stages are free living in the life cycle. Killing the parasite at these stages will stop the reproductive cycle and prevent the spread of the disease (Straus and Griffin, 2001).

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The most effective treatment of *I. multifiliis* has been achieved by use of malachite green, and mixtures of malachite green and formalin by immersion (Ekanem *et al.* 2004). However, due to the carcinogenic and genotoxic potentials of malachite green, it has been prohibited for use in the production of consumer fish in most of countries of the world (Heinecke and Buchmann, 2009). Other chemicals, including copper sulphate, acriflavine, potassium permanganate, chloramine-T, sodium percarbonate and toltrazuril have also been used as chemotherapeutants for chemotherapy of ichthiophthiriasis (Tieman and Goodwin, 2001; Buchmann *et al.* 2003; Ekanem *et al.* 2004). These chemicals aimed at interrupting the life cycle by killing the free-swimming stages of the parasite and achieved varying levels of success. Unfortunately, the continuous and long-term reliance on the limited kinds of chemicals has led to the development of drug resistance and causes environmental problems (Ling *et al.* 2010, 2011, 2012). There is, therefore, an urgent need to discover other effective and safe alternative anti-parasitic agents for the therapy of *I. multifiliis* infection.

Natural origin materials might be a reliable source for the discovery of novel and potential anti-parasitic agents (Li *et al.* 2013). In recent years, increased research efforts have been focused on the utilization of natural origin components from plants or microorganisms to treat parasitic diseases in fish

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(Ekanem *et al*. 2004; Chu *et al*. 2010; Yao *et al*. 2010, 2011; Zhang *et al*. 2013). Yao *et al*. (2010, 2011) demonstrated the anti-*I. multifiliis* potential of some natural origin compounds such as sanguinarine, dihydrosanguinarine and dihydrochelerythrine. In another previous study, it was reported that n-butanol extracts of extracellular products from *Streptomyces griseus* SDX-4 were of high efficacy in controlling *I. multifiliis* infections and would be useful in aquaculture (Yao *et al*. 2014). However, the active components responsible for the bioactivity had not been isolated in that study. We have previously demonstrated that a streptomycete strain (*Streptomyces* sp. HL-2-14) from East China Sea in Shanghai city was capable of producing anti-*I. multifiliis* components, and a pure compound was isolated from its fermentation products (unpublished data). The present study was conducted aiming to evaluate the anti-*I. multifiliis* efficacy of the isolated pure compound through *in vitro* and *in vivo* treatment trials, and elucidate its chemical structure by spectrum analyses.

MATERIALS AND METHODS

Uninfected fish

Healthy and uninfected grass carp (*Ctenopharyngodon idella*) with mean weight of 28·5 ± 2·1 g were obtained from a local aquaculture farm and acclimatized in a 1500-L glass aquarium under laboratory conditions (22·0 ± 1 °C, pH 7·1 ± 0·5, dissolved oxygen 6·1–7·2 mg L^{-1}). The aquarium was fitted with an outside biological filter containing pea gravel. The skin surface and gills of 10 randomly sampled fish were examined under a microscope to confirm that fish were not infected with gill parasites or skin parasites. They were fed twice (9:00 and 17:00) per day at 3% body weight. All fish were acclimatized to laboratory conditions for 7 days before the experiment.

Parasites and preparation of theronts and tomonts

A strain of *I. multifiliis* was isolated from grass carp originating from a local fish pond. Laboratory populations were set up and the parasites were propagated on naive grass carp. The procedure was performed according to Heinecke and Buchmann, (2009). For production of theronts and tomonts a heavily infected fish was killed by an overdose anaesthetic (MS-222, Hunan Tiancheng Co. Ltd, China). The fish was placed in a 3 L aquarium containing 2 L municipal water and left in the water for 2–3 h (22–23 °C) allowing trophonts to escape from the epidermis and obtain the tomont stage. The tomonts were harvested from the water during these time intervals using a 200 µl pipette. For theront production, the sacrificed fish was removed after the respective time period and the aquarium was left for 24 h (22–23 °C) for the tomonts to encyst and theronts to develop. A high concentration of theronts suspension was produced by 2 min centrifugating at 1000 rpm. The concentrations of tomonts and theronts were determined by pipetting several microliter droplets of the suspension onto a glass slide and counting the organisms under an inverted microscope with ×40 magnification (Olympus IX71, Japan); the mean count in 10 droplets was extrapolated to determine the final concentration.

Bacteria and isolation of the pure compound

Extracts of fermentation products. *Streptomyces* sp. strain HL-2-14 (isolated from the soil samples in East China Sea) was inoculated in nutrient broth (NB) for 7 days on a reciprocal shaker water bath at 30 ± 1 °C. The combined fermentation liquor (300 L) was filtered, and the supernatant was separated by centrifuging at 3000 rpm for 15 min and then concentrated to dryness (344·8 g) by a vacuum freeze dryer (chemical-free, RiHong Co. Ltd. Changzhou city, China). The dry powder was suspended in distilled water and then was extracted successively in a separating funnel with petroleum ether, ethyl acetate and methanol. Each extract and the remaining aqueous part after solvent extraction were then evaporated to dryness under reduced pressure to give petroleum ether extract (PEE, 20·8 g), ethyl acetate extract (EAE, 61·4 g), methanol extract (ME, 240·5 g) and remaining aqueous extract (AE, 123·1 g). For the investigation of anti-parasitic efficacy, the dried extracts were tested against theronts *in vitro*.

Isolation procedure for pure compound. The ME showing the highest anti-parasitic efficacy against *I. multifiliis* theronts was subjected to further isolation according to the procedure reported previously (Wu *et al*. 2012) with a slight modification. Briefly, parts of the ME (200 g) were put through a silica gelcolumn (silica gel: 100–200 mesh) and eluted with solvent mixture of chloroform–methanol (1:0, 19:1, 9:1, 5:1, 2:1, 1:1, 1:2, 1:10 and 0:1, v/v), affording 641 fractions (300 mL each). Thin layer chromatography (TLC) analysis was performed on silica gel using the same solvent system as the mobile phase. Spots on TLC were visualized under ultraviolet (UV) light (254 and 365 nm) or by spraying the plates with ethanol–sulphuric acid reagent, and fractions showing similar chromatograms were combined eventually yielding seven new fractions A–G (Fr. A, 1–34; Fr. B, 35–96; Fr. C, 97–158; Fr. D, 159–281; Fr. E, 282–314; Fr. F, 315–501; Fr. G, 502–641). All new fractions were allowed to evaporate under vacuum until they got completely dry. A yellow crystal power (514·2 mg) was obtained from Fr. E by successive recrystallization process.

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Anti-parasitic bioassays

For the measurement of anti-parasitic efficacy, *in vitro* trials against *I. multifiliis* theronts and tomonts along with *in vivo* trials against *I. multifiliis* infection were conducted. All trials were performed according to Yao *et al.* (2014). The extracts and the pure compound isolated from strain HL-2-14 were dissolved in dimethyl sulphoxide (DMSO) to get 1·0 g mL^{-1} (sample/solvent) of stocking solutions. Test solutions were obtained by diluting the stocking solutions with aerated and sterile groundwater.

In vitro *trials against theronts*. Trials were performed in 24-well tissue culture plates (Becton Dickinson Labware, NJ, USA). A total of 100 theronts were placed into each well of the plates in which filled with 2 mL different concentrations of test samples. The test concentrations were set as 30·0 and 50·0 mg L^{-1} for the extracts and 0·2, 0·4, 0·8, 1·6, 3·2, 6·4 and 12·8 mg L^{-1} for the pure compound. Anti-parasitic efficacy was determined by microscopic examination of each well at 30 min after treatment. Mortality was considered as the theronts with the absence of motility and integrity. A negative control was included using aerated groundwater containing the same amount of DMSO as the maximum concentration test group (<0·5%). All treatments and control groups were conducted with three replicates.

In vitro *trials against tomonts*. Fifty tomonts were placed into each well of a 6-well tissue culture plate (Becton Dickinson Labware, NJ, USA). Five milliliter of pure compound solutions at concentrations of 1·6, 3·1, 6·3, 12·5 and 25·0 mg L^{-1} were added to each well, respectively. A negative control was included using aerated groundwater containing the same amount of DMSO as the maximum concentration test group (<0·5%). The plates were incubated at 22 ± 0·5 °C. At 2 h after exposure, all solutions in each well were replaced by blank aerated groundwater (containing no test compound). The trial was allowed to stop until the parasites in the controls reached the theront stage. Mortality of theronts in each well (the parasites with the absence of internal cell motility or abnormal cell division and the ones cannot produce the theronts were considered dead) were recorded under microscope view. All treatments and control groups were conducted with three replicates.

In vivo *trials against* I. multifiliis *infection*. Two-hundred uninfected grass carp were transferred to 1000-L tanks for preparation of *I. multifiliis* infection. The tanks were aerated and supplied with groundwater. The temperature was maintained at 22 ± 0·5 °C using a heating device. Approximately 12 000 theronts/fish were put into the 1000-L tanks and incubated for 6 h with gentle aeration to promote infection. After the incubation, the fish were transferred to 50-L tanks with 20 infected fish per tank. Each tank was added with corresponding concentrations of the pure compound up to 1·3, 2·5 and 5·0 mg L^{-1}, respectively. Control tank was also involved with no test sample. Triplicated tanks were used in each concentration and air stones were equipped in there. Daily observation was recorded for fish survival, and the number of trophonts on the fins and skin of fish was scored on day 3 after exposure. The trials ended at day 7.

Toxicity tests

Acute toxicity of the pure compound against the host (grass carp) was tested in 20-L glass tanks, each containing 10 L of test solution and 10 healthy grass carp. Dilutions were prepared from the stock solution as the following concentrations: 10·0, 13·0, 16·0, 19·0, 21·0 and 24·0 mg L^{-1}. The tests were conducted in triplicate, as well as controls (under the same test conditions with no chemicals). Throughout the tests, fish were not fed, and the water quality was in consistence with those in acclimatization period. Fish mortality in the treatment and control groups were recorded after 72 h of exposure; and then median lethal concentration (LC_{50}) was calculated.

Identification of the pure compound

Structure elucidation of the pure compound was performed using electrospray ionization mass spectrometry (ESI-MS; VG Co. Manchester, UK), nuclear magnetic resonance hydrogen spectrum (^{1}H NMR) and nuclear magnetic resonance carbon spectrum (^{13}C NMR) (Bruker, Madison, WI, USA).

Data analysis

The data in this study were analysed with SPSS version 16·0, and presented as mean ± standard deviation (S.D.). The homogeneity of the replicates of the samples was checked by the Mann–Whitney U test. Student–Newman–Keuls test was utilized for multiple comparisons. Probabilities of 0·05 or less were considered statistically significant. The median lethal concentration (LC_{50}) with 95% confidence intervals (CI) was determined using the probit procedure.

RESULTS

Anti-theront efficacy of solvent extracts of strain HL-2-14

The results of anti-parasitic efficacies against *I. multifiliis* theronts of different solvent extracts (PEE,

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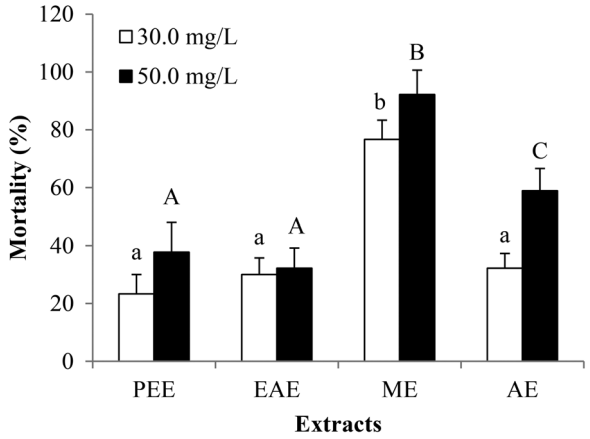

Fig. 1. Anti-theront efficacy of PEE, EAE, ME and AE of Streptomyces sp. HL-2-14 fermentation products against *I. multifiliis* at 30 min. Error bars denoted S.D. of 3 replicates. Statistically significant differences within a test concentration were indicated by the different letters ($P < 0{\cdot}05$).

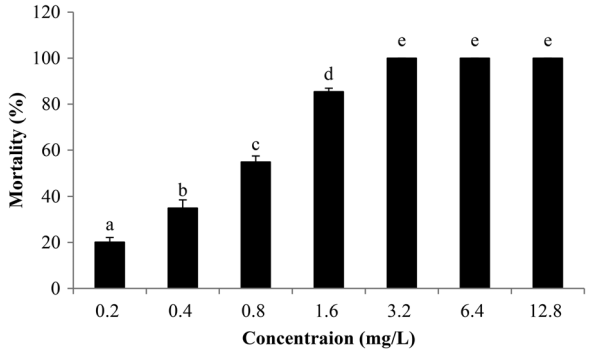

Fig. 2. Anti-theront efficacy of the isolated compound from *Streptomyces* sp. HL-2-14 fermentation products against *I. multifiliis* at 30 min. Error bars denoted S.D. of 3 replicates. Statistically significant differences were indicated by the different letters ($P < 0{\cdot}05$).

EAE, ME and AE) were depicted in Fig. 1, in which indicated ME is the extract having strongest anti-parasitic efficacy. At extract concentration of 50·0 mg L^{-1}, 92·2% of theronts were dead after treated by ME for 30 min, while at the same concentration the theront mortality was 37·8, 32·2 and 58·9% for PEE, EAE and AE, respectively. At the concentration of 30·0 mg L^{-1}, exposure to ME for 30 min resulted in *I. multifiliis* theront mortality by 76·7%. The solvent (DMSO) acted as a control showed no anti-parasitic activity when treated at the highest concentration.

Anti-theront efficacy of the compound

In vitro trials showed that the pure compound was high in anti-parasitic efficacy against *I. multifiliis* theronts (Fig. 2). At the concentrations of 0·2, 0·4, 0·8 and 1·6 mg L^{-1}, the treatment with the pure compound

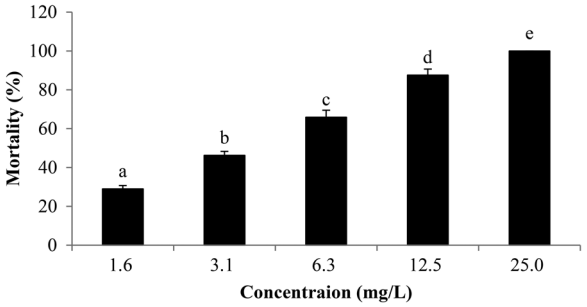

Fig. 3. Anti-tomont efficacy of the isolated compound from *Streptomyces* sp. HL-2-14 fermentation products against *I. multifiliis* after 2 h exposure. Error bars denoted S.D. of 3 replicates. Statistically significant differences were indicated by the different letters ($P < 0{\cdot}05$).

for 30 min led to a theront mortality of 20·1, 34·9, 54·9 and 85·4%, respectively. At the concentrations of 3·2, 6·4 and 12·8 mg L^{-1}, the theronts were 100% dead. The calculated LC_{50} against the theronts was 0·8 mg L^{-1}, with 95% CI of 0·69–0·81 mg L^{-1}.

Anti-tomont efficacy of the compound

Figure 3 showed that the pure compound has a concentration-dependent effect on the survival of tomonts. When treated with the pure compound for 2 h, the tomonts was found 100% dead at 25 mg L^{-1}. At the concentrations of 1·6, 3·1, 6·3, 12·5 mg L^{-1}, the mortality was 29·0, 46·2 65·9 and 87·6%, respectively. The calculated LC_{50} was 4·3 mg L^{-1}, with 95% CI of 3·74–4·77 mg L^{-1}.

In vivo *protecting efficacy of the compound*

Supplementary Table S1 showed that the treatment with the pure compound significantly decreased the prevalence and the burden of *I. multifiliis* on grass carp, as compared to the control. At the control group, 100% of fish was infected with *I. multifiliis* at day 3 post-exposure, the average numbers of trophonts on skin and fins were 341·0 individuals, the fish were 100% dead at day 7. At the highest concentration group (5·0 mg L^{-1}), the prevalence rate of *I. multifiliis* on the fish was significantly reduced to 46·7%, the average numbers of trophonts was 101·0 individuals, only 30·0% fish were dead at the end of the tests.

Acute toxicity

The acute toxicity tests revealed that the pure compound did not exhibit a lethal effect on the fish when exposure at 10·0 mg L^{-1} for 72 h (Fig. 4). Mortality of fish occurred when the concentration reached up to 13·0 mg L^{-1}. At the highest concentration (24·0 mg L^{-1}) for 72 h, 70% of fish were dead. The calculated 72-h LC_{50} was 20·6 mg L^{-1}, with the 95% CI of 19·5–22·0 mg L^{-1}.

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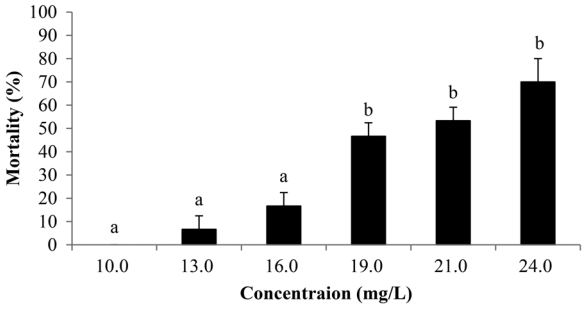

Fig. 4. Acute toxicity of the isolated compound from *Streptomyces* sp. HL-2-14 fermentation products against grass carp (*C. idella*) after 72 h exposure. Fish mortality was not occurred in 10·0 mg L^{-1} group. Error bars denoted standard deviation (S.D.) of 3 replicates. Statistically significant differences were indicated by the different letters ($P < 0{\cdot}05$).

Structure identification

The chemical structure confirmations of the pure compound were accomplished by comparing its ESI-MS, ^{1}H NMR and ^{13}C NMR data with the literature values (Vandeputte *et al*. 1955; Mechlinski *et al*. 1970; Aszalos *et al*. 1985). The compound was identified as a known chemical, amphotericin B (hereafter abbreviated AmB), with the molecular formula of $C_{47}H_{73}NO_{17}$. The chemical structure was shown in Fig. 5.

DISCUSSION

The *in vitro* assay may be an excellent tool for the screening of the anti-parasitic activity of potential drugs (extracts or compounds) (Ling *et al*. 2013). For the *I. multifiliis*, the theronts are the infective stage in their life cycle, it is important to kill theronts in order to control ichthyophthiriasis (Buchmann *et al*. 2001). Therefore, *in vitro* anti-theront trials (theront mortality and survival at various time intervals as parameters) were usually used in evaluation of anti-parasitic activity against *I. multifiliis* in recent years (Shinn *et al*. 2012; Ling *et al*. 2013; Zhang *et al*. 2013). For example, Ling *et al*. (2013) conducted an antiprotozoal screening of traditional medicinal plants against *I. multifiliis* in goldfish based on the *in vitro* anti-theront trials and reported that the crude extract of *Psoralea corylifolia* had the highest activity. Zhang *et al*. (2013) isolated an anti-*I. multifiliis* compound from ethanol extract of *Galla chinensis* according to the results of anti-theront trials. In the present study, the *in vitro* anti-theront trials were also used to investigate which solvent extract showing the strongest parasiticidal effect. The obtained results showed that the ME was the extract showing strongest parasiticidal effect, and was selected for further purification through column chromatography; eventually, a known chemical, i.e. AmB, was obtained.

To the best of our knowledge, this is first time that reports parasiticidal effect of AmB against fish parasites, although Yamasaki *et al*. (2014) has reported that AmB exhibited *in vitro* and *in vivo* activities against a causative pathogen of canine babesiosis, *Babesia gibsoni*. The present results revealed that AmB killed all theronts of *I. multifiliis* at the concentration of 3·2 mg L^{-1} when exposure for 30 min. The calculated 30-min LC_{50} against the theronts was 0·8 mg L^{-1}, with 95% CI of 0·69–0·81 mg L^{-1}. These results suggest that the efficacy of AmB in the treatment of *I. multifiliis* theronts is a substantial improvement over many of the chemicals currently used or tested in an attempt to control this parasite (Shinn *et al*. 2012; Zhang *et al*. 2013). Shinn *et al*. (2012) tested the anti-*I. multifiliis* theronts efficacy of bronopol and reported that bronopol exposure at 100·0 mg L^{-1} for 30 min only killed the theronts by 52·7 ± 21·7%. Shinn *et al*. (2012) also demonstrated that formaldehyde applied at 200·0 mg L^{-1} for 1 h which kills 40% of theronts, hydrogen peroxide applied at 200·0 mg L^{-1} for 1 h which kills 15%; Virkon S at 10·0 mg L^{-1} for 1 h which kills 50%; salt (NaCl) at 5·0 ppt for 24 h which kills 90%, and chloramine T at 20·0 mg L^{-1} for 15 min which kills 20% of theronts. Zhang *et al*. (2013) assessed the anti-parasitic efficacy of pentagalloylglucose from *G. chinensis* against *I. multifiliis* in channel catfish (*Ictalurus punctatus*), and revealed that the mean mortality duration for killing all theronts *in vitro* of pentagalloylglucose was 165·8 ± 5·2 min when applied at 5·0 mg L^{-1}. In addition, in the present study, it was found that the encysted tomonts resisted AmB better than the free-living theronts. This phenomenon was also found in other previous *I. multifiliis* treatment trials with other chemical (Buchmann *et al*. 2003; Zhang *et al*. 2013) or plant extracts (Ling *et al*. 2012; Yi *et al*. 2012). The *in vivo* results showed that treatment of AmB significantly reduced the prevalence of ichthyophthiriasis and the number of trophonts on fins and skin of fish on day 3 (Supplementary Table S1). The mortality of fish was also significantly reduced on day 7, from 100% in control group to 30% in 5·0 mg L^{-1} group. These results suggest that AmB was able to eliminate *I. multifiliis* and continuously immersing with this compound for 3 days is suitable for controlling *I. multifiliis* infection.

The chemical of AmB was firstly isolated from *Streptomyces nodosus* more than half a century ago (Kamiński *et al*. 2014). It exhibiting a broad antimicrobial spectrum and high fungicidal activity is currently the drug of choice in the treatment of severe fungal infections (Hargreaves *et al*. 2006). Structurally, this antibiotic possesses a macrolide ring, containing an internal ester or lactone, a heptane chromophore, and a hydroxyl hydrophilic region. The head of the molecule consists of a

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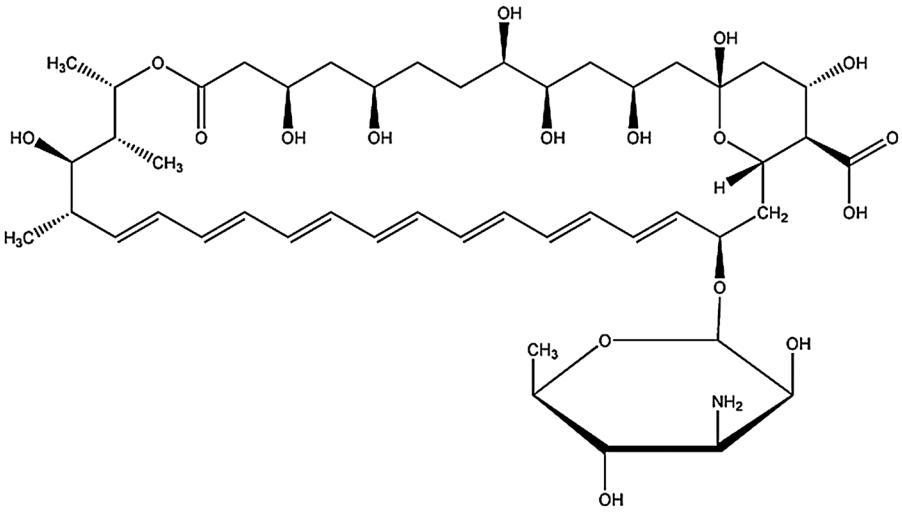

Fig. 5. Chemical structure of AmB.

carboxyl group and an amino sugar, the latter being attached to the macrolide ring through a glycosidic linkage (Larabia *et al*. 2004). According to the general conviction, the action mechanism of the drug is based on the formation of membrane pores that considerably affect membrane barrier function of cells (Arczewska and Gagoś, 2011). The molecules of AmB adopt a quasi-parallel orientation with their polar sides (the polyhydroxyl groups) facing the interior of such formed pores and the hydrophobic parts interacting with the ergosterol in fungal cell membranes (Arczewska and Gagoś, 2011). However, AmB also exhibits undesirable toxicity to mammalian cells (Herec *et al*. 2007). The structure of AmB causes this chemical has a tendency of self-association then leading to the formation of molecular aggregate. The self-associated AmB can form pores in cholesterol-containing membranes, leading to toxicity towards animal cells (Larabia *et al*. 2004). In the present study, the mechanistic studies of AmB against *I. multifiliis* have not been included. The action mechanism of this chemical killing the theronts and tomonts of *I. multifiliis* was speculated to consist in change of membrane cell permeability, but the exact mechanism remains to be further investigated. The acute toxicity tests showed that AmB was relatively toxic to the fish. Mortality of fish occurred when the concentration of AmB reached up to 13·0 mg L^{-1}, while AmB at 5·0 mg L^{-1} reduced the prevalence rate of *I. multifiliis* upon the fish from 100% in control groups to 46·7% (Supplementary Table S1 and Fig. 4). The safe range of AmB concentration is relatively narrow. Efforts aimed to decrease the toxicity to fish and improve the therapeutic profile of the chemical remains to be needed. Chemical derivatization and complexing the chemical within lipid-based formulations (liposomes, complexes and emulsions) are worth to be tried (Hing *et al*. 2000; Larabia *et al*. 2004).

To summarize, the present study evaluated the anti-*I. multifiliis* efficacy of different solvent extracts of *Streptomyces* sp. HL-2-14, and a known chemical AmB was isolated from the ME. AmB could effectively killed theronts and tomonts of *I. multifiliis* in water. Treatment with the compound significantly reduced *I. multifiliis* infectivity prevalence and intensity, and consequently decreased the mortality. However, this compound was relatively toxic to the host (fish). The safe margin of chemical concentration was relatively narrow. Further studies will be needed to decrease the toxicity to fish and improve the therapeutic profile, through chemical techniques such as chemical derivatization.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0031182015000116.

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