Identification of compounds from *Paris polyphylla* (ChongLou) active against *Dactylogyrus intermedius*

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SUMMARY

The present study was designated to ascertain the anthelmintic activity of the rhizomes of *Paris polyphylla* and to isolate and characterize the active constituents. The methanol extract from rhizomes of *P. polyphylla* showed significant anthelmintic activity against *Dactylogyrus intermedius* with the median effective concentration (EC_{50}) 22·5 mg L^{-1}. Based on this finding, the methanol extract was fractionated by silica gel column chromatography in a bioassay-guided fractionation yielding 2 bioactive compounds, the structures of these compounds were elucidated as formosanin C and polyphyllin VII. The *in vivo* tests revealed that formosanin C and polyphyllin VII were significantly effective against *D. intermedius* with EC_{50} values of 0·6 and 1·2 mg L^{-1}, respectively. The acute toxicities (LC_{50}) of formosanin C and polyphyllin VII for grass carp were 2·8 and 2·9 mg L^{-1}, respectively. The overall results provide important information for the potential application of formosanin C and polyphyllin VII in the therapy of serious infection caused by *D. intermedius*.

Key words: *Dactylogyrus intermedius*, *Paris polyphylla*, formosanin C, polyphyllin VII, anthelmintic.

INTRODUCTION

Fish aquaculture in China is an important economic activity that has been growing during the last few years. The main drawbacks for the extensive commercial production of freshwater fish are associated with diseases including bacterial, viral and parasitic infections. The monogenean parasite, *Dactylogyrus intermedius*, is the most common parasite present on the gills of cyprinid fishes, causing serious economic damage to the aquaculture industry (Dove and Ernst, 1998). The parasite is usually attached to the gills of freshwater fish causing irritation, excessive mucus production, accelerated respiration and mixed infection with other pathogens (Dove and Ernst, 1998; Reed *et al*. 2009), leading to serious damage to the host such as loss of appetite, reduced growth performance and high mortalities (Topić *et al*. 2001; İsmail and Selda, 2007).

To cope with the parasitism and deleterious consequences, various parasiticides effective against *Dactylogyrus*, such as formalin, praziquantel, toltrazuril, levamisole, trypaflavine, mebendazole (Schmahl and Mehlhorn, 1985, 1988; Marshall, 1999; Treves-Brown, 1999) have been used to kill the parasite, with varying levels of success. However, the frequent use of these chemical parasiticides has had limited efficacy in reducing monogenean infestations and is often accompanied by serious drawbacks, including the development of drug-resistant parasites, environmental contamination and even toxicity to the host. For these reasons, an effective therapy has to be improved and new drugs are needed. Current research efforts have increasingly focused on developing alternative drug formulations, including medicinal plants. Crude extracts of *Radix angelicae pubescentis, Fructus bruceae, Caulis spatholobi, Semen aesculi* and *Semen pharbitidis* were also found to exhibit a complete elimination of all *D. intermedius* in goldfish (Liu *et al*. 2010). Two alkaloids, chelidonium and chelerythrine from *Chelidonium majus* have been reported to have anthelmintic efficacy against *D. intermedius* (Li *et al*. 2011; Yao *et al*. 2011). Moreover, most of these natural ingredients exhibit higher efficacy than the widely used anthelmintics such as mebendazole (Wang *et al*. 2008). Therefore, traditional medicinal plants could be a reliable source for the discovery of novel and potential antiparasitic agents.

Paris polyphylla, a perennial traditional Chinese medicinal plant, has been widely used as an antifebrile, alexipharmic, detumescent, demulcent, haemostatic and for the treatment of hepatopathy. In our preliminary screen assay using *in vivo* anthelmintic activity, methanol extract from *P. polyphylla* showed a significant effect against *D. intermedius*, which prompted us to conduct a further investigation on

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this plant, aiming to isolate and purify the active compounds responsible for its anthelmintic properties. Additionally, the acute toxicities of the active compounds from *P. polyphylla* were evaluated.

MATERIALS AND METHODS

Parasites and hosts

Healthy grass carp, *Ctenopharyngodon idella*, weighing 13·4 ± 0·6 g were obtained from an aquatic fry farm at Chunlei in Changchun, China, and were maintained in 0·5 m^3 tanks at 25 ± 1 °C (controlled by an automatic aquarium heater) with aeration for 7 days. They were then co-habitated with carp infested with *D. intermedius* which were cultured in our laboratory. This procedure consisted of the collection of eggs, hatching of the eggs and re-infection with *D. intermedius*. The infected fish were prepared according to the protocol described in a previous study (Wang *et al*. 2008). After 21 days of co-habitation, 10 grass carp were then randomly selected, killed by spinal severance and examined for the presence of parasites under a light microscope (Olympus BX51, Tokyo, Japan) at 4 × 10 magnification prior to the experiment. Fish were chosen for the tests when the infection prevalence was 100% and the mean number of parasites on the gills was 30–50.

For the acute toxicity tests, parasite-free grass carp were obtained from a commercial fish farm and maintained in 0·5 m^3 tanks under the same conditions as parasitized fish. On arrival, the absence of parasites was carefully checked by examining 10 randomly selected fish.

In vivo anthelmintic efficacy test

The crude extracts and the pure compounds isolated from *P. polyphylla* were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 0·5 mg L^{-1} (sample/solvent) to prepare stock solutions which were then used for the preparation of the desired concentrations for the *in vivo* anthelmintic efficacy assays. The highest concentration of DMSO in the treatment was less than 1% (initial tests with 1% DMSO showed no anthelmintic activity).

Tests were conducted in each glass tank of 20 litres capacity, filled with 10 litres of aerated groundwater, each containing the test samples and 10 previously infected fish. The pH of the water ranged from 7·0 to 7·5, dissolved oxygen was between 6·5 and 7·5 mg L^{-1}, and the water temperature was constant at 25 ± 1 °C. The test samples were assayed at a different series of concentrations based on initial tests. Negative control groups with no added chemicals were set up under the same experimental conditions. All treatment and control groups were conducted with 3 replicates.

After 48 h of treatment, grass carp in all treatment and control groups were killed by a spinal severance for biopsy. The total number of *D. intermedius* in the gills collected from each fish was counted. *Dactylogyrus intermedius* were considered dead when they appeared lysed or their motility (mouthpiece did not show peristalsis during 2 min) was lost (Wang *et al*. 2006). The anthelmintic efficacy of tested samples was determined by comparison of the number of parasites in the treatment groups with those in the negative control groups, and was calculated using the following equation.

$$E = (C - T) \times 100/C \quad (1)$$

where E is the anthelmintic efficacy, C is the mean number of *D. intermedius* in the negative control, and T is the treatment groups. Concentration–mortality regressions of the extracts and each active compound were estimated by probit analysis using probit procedure, and the 48 h median effective concentration (EC_{50}) and its 95% confidence interval (which is the concentration that eliminated 50% of the parasites compared with the control at 48 h) were calculated. The therapeutic index (TI) was calculated by comparing LC_{50} *vs* the EC_{50}.

Activity-guided isolation of active compounds

The chromatographic separation was monitored by the strategy of bioactivity-guided fractionation (*in vivo* tests guided), only the extracts or fractions that showed strong activity against *D. intermedius* were subjected to further separation and purification.

Preparation of extracts. Dried rhizomes of *P. polyphylla* (5 kg) were extracted using methanol in a water bath at 60 °C for 4 h, followed by filtration. The residue was subjected to 2 successive extractions with methanol. The extracts were combined and concentrated using a rotary vacuum evaporator at 70 °C, resulting in the formation of a paste-like residue (0·94 kg).

The methanol extract (0·9 kg) was suspended in distilled water, and then was extracted successively in a separating funnel with petroleum ether, ethyl acetate, chloroform and *n*-butanol. Each extract and the remaining aqueous part after solvent extraction were then evaporated to dryness under reduced pressure to give the petroleum ether extract (87·3 g), the chloroform extract (120·2 g), the ethyl acetate extract (190·0 g), the *n*-butanol extract (125·5 g) and the remaining aqueous extract (222·8 g). The *in vivo* tests showed that the *n*-butanol extract had the highest in anthelmintic efficacy amongst all extracts. Consequently, it was then subject to further separation.

Fractionation and isolation of pure compounds. The *n*-butanol extract (120·0 g) was subjected to open

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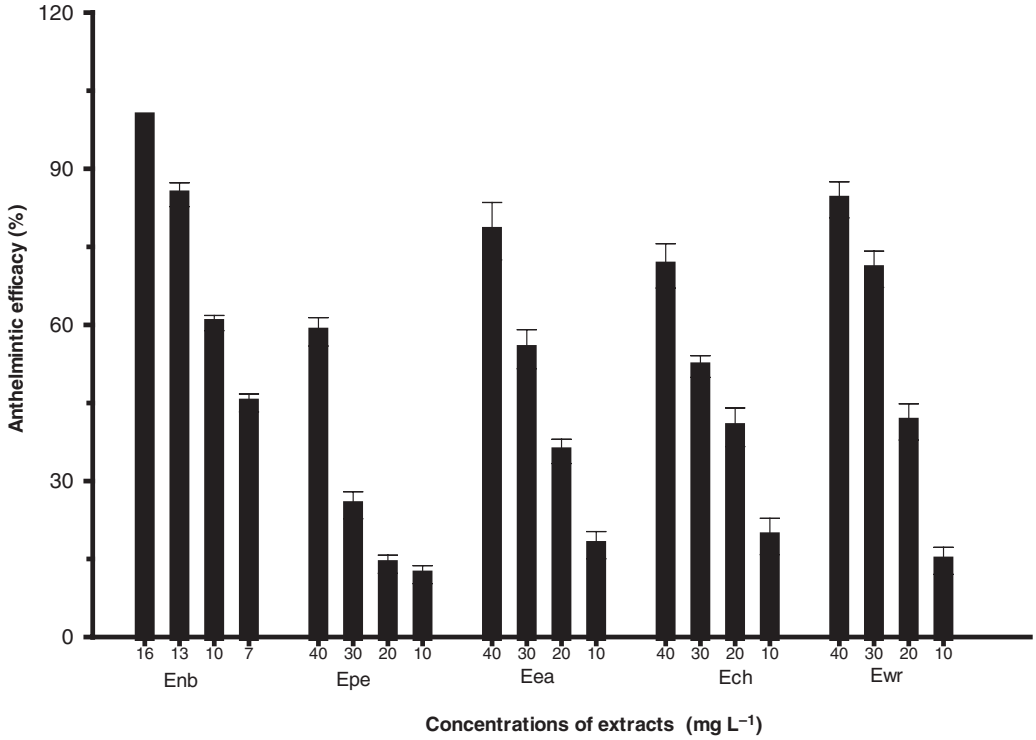

Fig. 1. Anthelmintic efficacy of petroleum ether, ethyl acetate, chloroform, *n*-butanol and the remaining water extract against *Dactylogyrus intermedius* at 48 h.

column chromatography on normal phase silica gel and eluted with a solvent mixture of chloroform–water–methanol solvent system to yield 558 fractions (300 mL each fraction). Thin layer chromatography (TLC) analysis was performed on silica gel using the same solvent system as the mobile phase, spots on plates were visualized under UV light (254 and 365 nm) and fractions showing similar chromatograms were combined to yield 5 main fractions (Fr. A: 1–110 fractions; Fr. B: 111–220 fractions; Fr. C: 221–340 fractions; Fr. D: 341–480 fractions; Fr. E: 481–558 fractions). All of these 5 fractions were concentrated to dryness, and samples of them were dissolved in DMSO for the anthelmintic efficacy assay. Fr. E proved to be the most active fraction and was then applied to reversed-phase high performance liquid chromatography (RP-HPLC) with the following chromatographic conditions: Alltima C_{18} (5 μm, 10 mm × 250 mm) column, water–methanol mobile phase, 5·0 ml min^{-1} flow rate, 30 °C column temperature and 500 μL capacity. Repetition of the chromatographic separations and re-crystallization led to the isolation of 2 active compounds, compound 1 (2·4 g) and compound 2 (1·8 g).

Acute toxicity

The acute toxicity of formosanin C and polyphyllin VII from *P. polyphylla* were assayed for the evaluation of their safety to the host. The tests were conducted in 40 cm × 30 cm × 20 cm glass tanks, each containing 10 L of test solution, and 10 healthy grass carp. The water temperature was 25 ± 1 °C, the pH ranged from 7·0 to 7·5 and the dissolved oxygen was approximately 6·5–7·5 mg L^{-1}. Each sample was assayed at a different series of concentrations ranging between 1·5 and4·5 mg L^{-1} for formosanin C and polyphyllin VII (based on initial tests). Control groups were set under the same test conditions with no added chemicals. The death of fish was recorded when the opercula movement and tail beat stopped and the fish no longer responded to mechanical stimulus. Fish mortalities in the treatment and control groups were recorded after 48 h of exposure. The 48-h median lethal concentration (LC_{50}) and its confidence intervals were calculated using probit analysis.

RESULTS

In vivo anthelmintic efficacy

Selection of extraction solvent. Four solvents, petroleum ether, ethyl acetate, chloroform and *n*-butanol were used for the extraction of the methanol extract. The results of the anthelmintic efficacies for 5 extracts are depicted in Fig. 1, which indicated that the *n*-butanol extract revealed a 100% efficacy at the low concentration of 16·0 mg L^{-1} after 48 h of exposure. This was followed by the remaining aqueous extract and ethyl acetate extract having maximum anthelmintic efficacies of 84·1% (40·0 mg L^{-1}) and 78·0% (40·0 mg L^{-1}), respectively. The chloroform and

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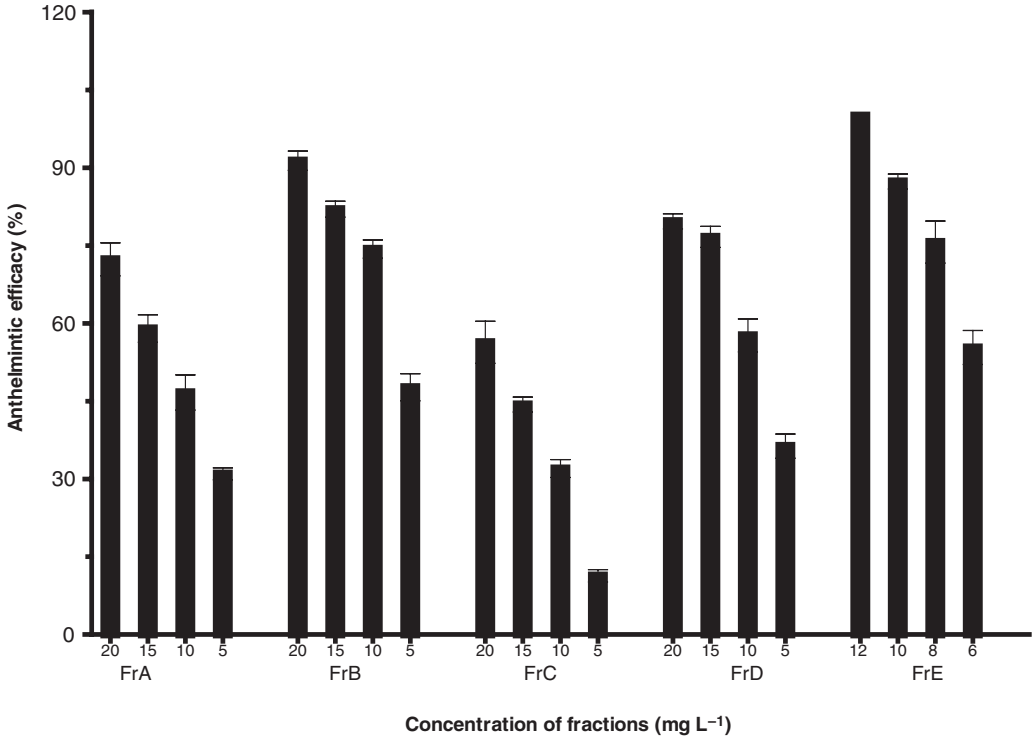

Fig. 2. Anthelmintic efficacy of 5 fractions against *D. intermedius* at 48 h.

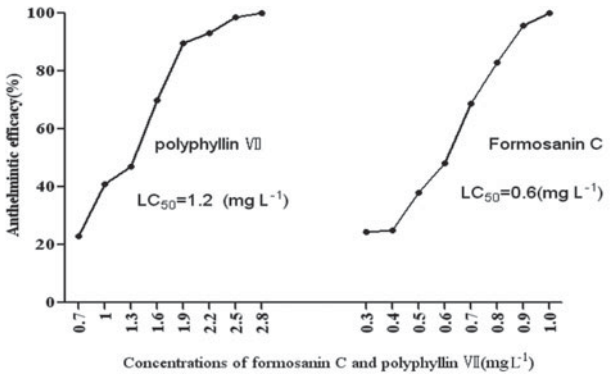

Fig. 3. Anthelmintic efficacy of formosanin C and polyphyllin VII against *D. intermedius* after 48 h of exposure.

petroleum ether extracts exhibited the least activity with the maximum anthelmintic efficacy of 71·3% (40 mg L^{-1}) and 58·7% (40 mg L^{-1}), respectively. Therefore, *n*-butanol was the optimal solvent for the methanol.

Isolation of compounds with anthelmintic activity. Isolation of the *n*-butanol extract led to 5 major fractions. Fr. E was found to possess anthelmintic activity. The *in vivo* test showed that Fr. E (12·0 mg L^{-1}) showed significantly higher activity than the other fractions (20·0 mg L^{-1}; Fig. 2). The anthelmintic efficacy of Fr. E was 100% at a concentration of 12·0 mg L^{-1}. Fr. E proved to be the most active fraction and was then applied to RP-HPLC. Finally, compounds 1 and 2 were obtained from the Fr. E.

Both compound 1 and compound 2 showed remarkable anthelmintic efficacy against *D. intermedius* with a complete removal of the parasites on the gills at concentrations of 1·0 and 2·8 mg L^{-1}, respectively (Fig. 3). The 48 h EC_{50} of the two compounds (compounds 1 and 2) were calculated by the linear equations, and the obtained EC_{50} values were 0·6 mg L^{-1} (0·0007 μmol L^{-1}), 1·2 mg L^{-1} (0·0011 μmol L^{-1}), respectively (Fig. 3).

Identification of the active compounds. The structures of the two compounds (1 and 2) were established by EI-MS, ^{1}H NMR and ^{13}C NMR data by comparison with literature values (Chen and Zhou, 1981; Chen *et al*. 1983; Huang *et al*. 2007). Compound 1 was identified as formosanin C, compound 2 was identified as polyphyllin VII and their structures are shown in Fig. 4.

Acute toxicity

The LC_{50} values of the formosanin C and polyphyllin VII were determined from linear ($y = m + bx$) plots of the probit curves that are presented in Table 1. The probit mortality percentage of the grass carp was plotted against log-concentrations.

For formosanin C, the linear equation $y = -12{\cdot}33 + 15{\cdot}87x$ was derived from the regression analysis of the probit mortality of grass carp in the test solution bioassay. The calculated LC_{50} was 2·8 mg L^{-1} (0·003 μmol L^{-1}) with a 95% confidence interval of 2·65–3·0 mg L^{-1}. For polyphyllin VII, the linear

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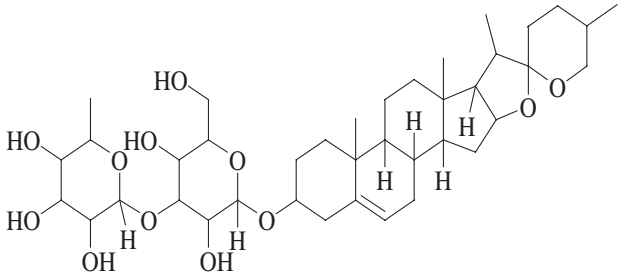
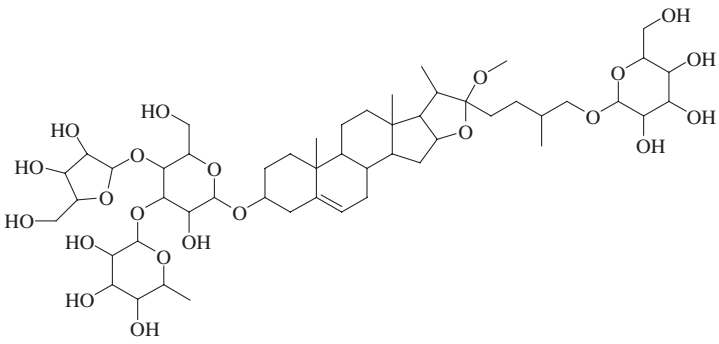
Table 1. Anthelmintic activity and acute toxicity of extracts and active compounds from *Paris polyphylla* after 48 h of exposure

Test samples	Anthelmintic efficacy (mg/l) EC_{50} (95% CL)	Acute toxicity LC_{50} (mg L^{-1})	Therapeutic index (TI)
Methanol extract	22·5 (21·7–24·4)	ND	ND
n-butanol extract	7·8 (7·1–8·5)	ND	ND
Formosanin C	0·6 (0·49–0·71)	2·8 (2·65–3·0)	4·7
Polyphyllin VII	1·2 (1·1–1·35)	2·9 (2·7–3·24)	2·4



Formosanin C



Polyphyllin VII

Fig. 4. Chemical structures of compounds isolated from *P. polyphylla*.

equation $y = -9{\cdot}26 + 13{\cdot}29x$ was derived from the regression analysis of probit mortality with an LC_{50} of 2·9 mg L^{-1} (0·0028 μmol L^{-1}) and 95% confidence interval of 2·7–3·24 mg L^{-1}. No fish mortality occurred in the control groups during the experiments.

DISCUSSION

Currently, most countries have few effective and safe chemical treatments that can be used to control *D. intermedius* (Wang *et al*. 2008). Much effort has been spent on searching for more effective anti-*D. intermedius* drugs. Plant secondary metabolites have been used for centuries in traditional medicine and therefore represent a source of potentially active compounds (Bourgaud *et al*. 2001; Yao *et al*. 2011). Antiparasitic plant-derived compounds have been used as leads to develop semi-synthetic or synthetic drugs with better efficacy and safety (Tagboto and Townson, 2001). Therefore, in the present study, an attempt was made to exploit the active compounds from *P. polyphylla* for their anthelmintic activity against *D. intermedius* by bioactivity-guided isolation. In order to make a full evaluation of the methanol fractions of *P. polyphylla*, 4 extracts by different solvents (petroleum ether, ethyl acetate, chloroform and *n*-butanol) of increasing polarity were assayed for anthelmintic activity. The *n*-butanol extract that exhibited the most significant activity with an EC_{50} value of 7·8 mg L^{-1}, was subjected to bioactivity-guided fractionation and purification. Further fractionation of the *n*-butanol extract led to the isolation of 2 active compounds, which were identified as formosanin C and polyphyllin VII. To our best knowledge, the effects of these two compounds against parasites in fish have not previously been investigated. Therefore, this study is the first

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report of the anthelmintic efficacies of formosanin C and polyphyllin VII. This result extended the general knowledge about the anthelmintic activity of theses two active compounds and the plant's application to control the fish parasite.

Formosanin C, a diosgenin glycoside with 4 sugars, is isolated from *Rhizoma paridis* which has for a long time been used as a folk remedy for snake bite, and as an anti-inflammatory or anti-neoplastic agent. It has already been reported to have some effect on immune responses. Intraperitoneal treatment with 1–2·5 mg kg^{-1} of formosanin C is able to retard the growth of subcutaneously transplanted MH134 mouse hepatoma (Huang *et al*. 1990). It was shown to exert a cytotoxic effect on the mouse hepatocellular MH134 cell line injected subcutaneously into C3H/HeN mice, especially in combination with 5-FU (Wu *et al*. 1990). It was also proved to augment the effect of concanavalin A on lymphocyte proliferation (Chiang *et al*. 1992). In the present study, the anthelmintic activity of formosanin C was reported for the first time, and it was revealed to be significantly effective for the control of monogenean parasites. Recent research indicated that the apoptotic mechanism of formosanin C in human colorectal cancer HT-29 cells involves activation of caspase-2 and the dysfunction of mitochondria (Lee *et al*. 2009). As is well known, mitochondria are the key players that control and regulate apoptosis; mitochondrial dysfunction may lead to both apoptotic and necrotic cell death (Saraste, 1999; Desagher and Martinou, 2000). A direct action on mitochondria might be involved in the eradication of the parasites. This may be the primary mechanism responsible for the activity of formosanin C against *D. intermedius*. However, the detailed mechanism of action regarding the anthelmintic activity of the formosanin C should be further addressed.

The results of the *in vivo* anthelmintic efficacy test showed that formosanin C has strong anthelmintic efficacy against *D. intermedius* with an EC_{50} value of 0·6 mg L^{-1}, and it was 2-fold more efficient than mebendazole, which is frequently used for the control of *Dactylogyrus* (Buchmann *et al*. 1993) (EC_{50} value = 1·25 mg L^{-1}). The 48 h-LC_{50} value of formosanin C was 2·8 mg L^{-1} which is about 5 times the effective one. These findings ensure the safety for the use of formosanin C in the control of *D. intermedius* infection, and suggest that formosanin C has great potential for the development of a new parasiticide. In the case of polyphyllin VII, the median EC_{50} value was nearly 2 times higher than the corresponding toxic dose, indicating that polyphyllin VII is of high risk, which may limit its application in the control of *D. intermedius*.

Our results revealed that formosanin C and polyphyllin VII showed remarkable activity against the monogenean parasite, *D. intermedius*. Both compounds, especially formosanin C, can be chosen as lead compounds for the development of new antiparasitic agents against *D. intermedius*. However, more investigations such as pharmacological evaluations before clinical trials, assessment of the ecological risk posed by practical usage and their detailed mechanism of action must be performed.

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