Screening of plant extracts for anthelmintic activity against Dactylogyrus intermedius (Monogenea) in goldfish (Carassius auratus)

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ORIGINAL PAPER

Screening of plant extracts for anthelmintic activity against *Dactylogyrus intermedius* (Monogenea) in goldfish (*Carassius auratus*)

Ai-Guo Huang · Yang-Lei Yi · Fei Ling · Lin Lu · Qi-Zhong Zhang · Gao-Xue Wang

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Abstract With the aim of finding natural anthelmintic agents against *Dactylogyrus intermedius* (Monogenea) in goldfish (*Carassius auratus*), 26 plants were screened for antiparasitic properties using in vivo anthelmintic efficacy assay. The results showed that *Caesalpinia sappan*, *Lysima chiachristinae*, *Cuscuta chinensis*, *Artemisia argyi*, and *Eupatorium fortunei* were found to have 100 % anthelmintic efficacy at 125, 150, 225, 300, and 500 mg L^{-1} after 48 h of exposure. Crude extract of the five plants were further partitioned with petroleum ether, chloroform, ethyl acetate, methanol, and water to obtain anthelmintically active fractions with various polarity. Among these fractions tested, the ethyl acetate extract of *L. chiachristinae* was found to be the most effective with a 50 % effective concentration (EC_{50}) value of 5.1 mg/L after 48 h of exposure. This was followed by ethyl acetate extract of *C. chinensis* (48 h-EC_{50}=8.5 mg L^{-1}), chloroform extracts of *C. sappan* (48 h-EC_{50}=15.6 mg L^{-1}), methanol extract of *C. chinensis* (48 h-EC_{50}=15.9 mg L^{-1}), and chloroform and petroleum ether extract of *L. chiachristinae* (EC_{50} values of 17.2 and 21.1 mg/L, respectively), suggesting that these plants, as well as the active fractions, provide potential sources of botanic drugs for the control of *D. intermedius* in aquaculture.

Ai-Guo Huang and Yang-Lei Yi contributed equally to this work.

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Introduction

Considered as the fastest-growing food-producing sector in the world, the aquaculture industry has been associated with diseases including bacterial, viral, and parasitic infections. One of the most significant parasitic problems widely reported is the infestation with the Monogenean parasites such as *Dactylogyrus intermedius*, which is one of the ectoparasites distributed in Asia, Central Europe, Middle East, and North America (Paperna 1964). Attaching to the gill epithelia, *D. intermedius* can cause serious damage resulting in pathological changes that interfere with gaseous exchange in fish (Obiekezie and Taege 1991), leading to serious damage to the host including loss of appetite, lower growth performance, and high mortalities.

Several treatments with various chemotherapeutants against Monogeneans have been reported. These agents include praziquantel, mebendazole, trichlorfon, and levamisole (Reimschuessel et al. 2011). Although these methods show promise, there is always the risk of negatively impacting other parts of the aquatic ecosystem. Additionally, with the rise in popularity of "organic" produce and increase fastidiousness of the consumer, the need of a new environment-friendly treatment is thus indisputable.

Recent studies indicate the potential of a group of medicinal plants as potential agents to treat fish parasites. Ekanem et al. (2004) reported that methanol extracts of the seeds of *Piper guineense* (Piperaceae) were active against goldfish monogenean parasites under in vivo and in vitro conditions. Australian tea tree (*Melaleuca alternifolia*) oil treatments lowered the prevalence and significantly reduced the parasite

burden of sticklebacks naturally infected with *Gyrodactylus* spp. (Steverding et al. 2005). Hirazawa et al. (2000) reported on the anthelmintic activity of caprylic acid against infections by the monogenean *Heterobothrium okamotoi* in the tiger puffer *Takifugu rubripes*. In our previous work, crude extracts of several traditional medicinal plants such as *Arctium lappa* L. (Wang et al. 2009), *Dioscorea zingiberensis* C. H. Wright (Wang et al. 2010b), and *Paris polyphylla* (Wang et al. 2010a) can effectively control the *D. intermedius* infection in goldfish (*Carassius auratus*). Considering the popularity of medicinal plants and herbs and their low toxicities, the present work attempts to exploit the anthelmintic activity of 26 plants against *D. intermedius* (Monogenea) in goldfish (*C. auratus*) and investigates the parasitic activities of the different solvent extracts of five plant species

Materials and methods

Parasites and hosts

Healthy goldfish (weight, 4.5±0.3 g) were obtained from a local fish farm (Xi'an, Shaanxi Province, China) and maintained in a 180-L glass aquarium under laboratory conditions (22.0±2 °C, pH 6.9±0.4, with 6.0–7.8 mg L^{-1} dissolved oxygen) for 7 days. They were fed once at 1 % body weight daily with commercial fish pellet feed. Seven days later, the healthy goldfish were cohabitated at a ratio of 20 % with the ones infected with *D. intermedius* which were cultured in our laboratory (Fig. 1). The parasitized fish were prepared according to the procedure described in our previous study (Wang et al. 2009). This procedure includes collecting eggs, hatching eggs, and reinfection. After 21 days, 10 fishes were randomly selected, killed by spinal severance, and checked for presence and intensity of parasites on the gills under a microscope at 10×4 magnification. The fishes were chosen for in vivo anthelmintic efficacy assay when the infection rate was 100 %; the mean number of parasites on the gills was 40–50 per fish.

Plant materials

Fresh plant materials from each of the selected species (see Table 1) were collected in 2012. The specimen identification was confirmed by Prof. X.L. He (Northwest A&F University, Shanxi, China), and voucher specimens have been deposited in the College of Life Science, Northwest A&F University, China. They were washed, cut into small pieces, and then dried in an oven at 50 °C until completely dried. The dried plant materials were then powdered separately and reduced to fine powder using a strainer (30–40 mesh). The powdered sample was freeze dried at −54 °C to ensure desiccation.

Activity-guided screening of plants

The dry powder (100 g) of each species was extracted with methanol (1,000 mL×3 times) for 48 h. The methanol filtrates were separately filtered and evaporated under reduced pressure in a vacuum rotary evaporator. The methanolic extracts were then screened for their anthelmintic activity. Only *Caesalpinia sappan*, *Lysima chiachristinae*, *Cuscuta chinensis*, *Artemisia argyi*, and *Eupatorium fortunei* showed strong anthelmintic activity when compared with others and crude methanolic extracts of these selected plants were further fractionated by solid-phase extraction to isolate allelopathically active fractions. The crude extracts of selected plants were fractionated with petroleum ether, chloroform, ethyl acetate, methanol, and water for 12 h for complete extraction; the process was repeated three times. The ratio of sample to solvent was 1:10 (*m*/*v*). Each extract was subsequently filtered and concentrated under reduced pressure in a vacuum rotary evaporator until the solvents were completely evaporated to get more or less solidified crude extracts. These crude extracts were dissolved in 40 mL of dimethyl sulfoxide (DMSO) to get 200 g L^{-1} (sample/solvent) of stock solutions which were used for preparation of the desired concentrations for bioassay.

In vivo anthelmintic efficacy assay

Tests were conducted in 5-L glass tanks, each containing 2 L of the test solution water and five infected fishes. The water

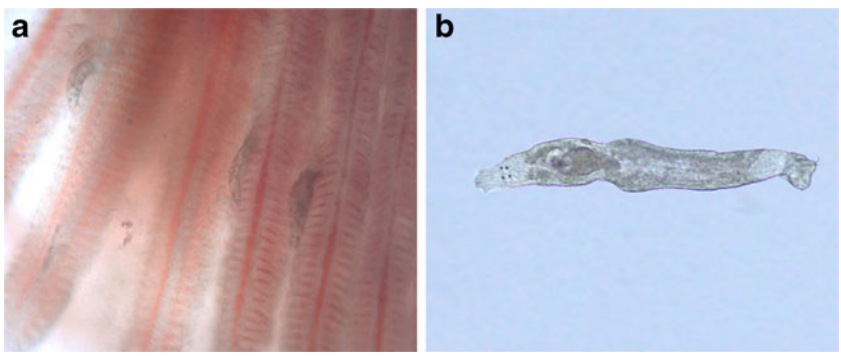

Fig. 1 Photos of *D. intermedius*. **a** *D. intermedius* in fish gills (10×10 magnification). **b** *D. intermedius* detached from fish gills (20×10 magnification)

Table 1 Plants used in this study, the plant part used, the best anthelmintic efficacy, concentration of the best anthelmintic efficacy, and concentration with goldfish died against *D. intermedius* in goldfish

Species	Family	Plant part used	The best AE without fish died (%)	Concentrations causing the best AE (mg/L)	Concentrations causing fish died (mg/L)
Caesalpinia sappan L.	Leguminosae	Rhizome	100	125	250
Lysima chiachristinae Hance.	Primulaceae	Leaves	100	150	300
Clematis chinensis Osbeck.	Ranunculaceae	Roots	100	225	250
Artemisia argyi Levl. et Vant.	Compositae	Leaves	100	300	600
Eupatorium fortunei Turcz.	Compositae	Leaves	100	500	800
Liquidambar formosana Hance	Hamamelidaceae	Fruits	92	45	62.5
Eucommia ulmoides Oliv.	Eucommiaceae	Roots	90	450	475
Pogostemon cablin (Blanco) Benth.	Labiatae	Leaves	86	250	500
Paeonia suffruticosa Andr.	Paeoniaceae	Roots bark	85	400	450
Rubus chingii Hu.	Rosaceae	Fruits	78	400	450
Hedyotis diffusa Willd.	Rubiaceae	Aerial parts	76	450	500
Leonurus japonicus Houtt.	Labiatae	Fruits	72	31.5	31.5
Glycine max (L.) Merr.	Leguminosae	Seeds	68	237.5	250
Gardenia jasminoides Ellis.	Rubiaceae	Fruits	62	450	500
Atractylodes lancea (Thunb.) DC.	Compositae	Rhizoma	58	225	225
Dianthus superbus L.	Caryophyllaceae	Leaves	50	800	800
Cuscuta chinensis Lam.	Convolvulaceae	Seeds	53	300	320
Rosa laevigata Michx.	Rosaceae	Fruits	30	400	450
Celosia cristata L.	Amaranthaceae	Flos	0	–	600
Xanthium sibiricum Patr.	Feverfew	Fruits	0	–	500
Tribulus terrestris L.	Zygophyllaceae	Fruits	0	–	800
Sphora japonica L.	Leguminosae	Fruits	0	–	500
Sophora flavescens Ait.	Leguminosae	Roots	0	–	125
Morus alba L.	Moraceae	Shoots	0	–	500
Triticum aestivum L.	Poaceae	Seeds	0	–	500

AE anthelmintic efficacy, – not analyzed

pH ranged from 7.0 to 7.5, and dissolved oxygen was between 6.2 and 7.8 mg L^{-1} (72–85 % saturation). All tests were performed at 24±1 °C. The concentrations of different series of five crude extracts of the plants were determined based on the initial tests. A control group without extracts was setup under the same experimental conditions as the test groups. Another control, containing the highest percentage of DMSO, was also included to exclude the possible effects of DMSO on the parasites. All the experiments were conducted twice. During the experiments, no food was offered to the fishes. Forty-eight hours later, the surviving fishes in all the treatments were killed by spinal severance and biopsied under a light microscope at 4×10 magnification. The effectiveness of each treatment was confirmed by comparing the number of parasites in each treatment group with that in the control group. Finally, anthelmintic efficacy of each treatment and control group was calculated using the following equation (Wang et al. 2008):

$$AE(\%) = (B-T)/B \times 100$$

where AE is anthelmintic efficacy, B is average number of surviving *D. intermedius* in the negative control, and T is average number of surviving *D. intermedius* in the treatment groups.

Statistical analysis

The data in this study were analyzed by Statistical Product and Service Solutions (SPSS 17.0). The 50 % effective concentration (EC_{50}) with 95 % confidence intervals was determined by Probit analysis.

Results

Screening of plant extracts

As shown in Table 1, of the 26 selected plants, the highest anthelmintic activity was observed in *C. sappan*, *L. chiachristinae*, *C. chinensis*, *A. argyi*, and *E. fortunei*, which

were found to have 100 % anthelmintic efficacy at 125, 150, 225, 300, and 500 mg L^{-1} after 48 h of exposure. Importantly, no fish died at the tested dose of these five plants. High anthelmintic activity against *D. intermedius* was also observed in *Liquidambar formosana*, *Eucommia ulmoides*, *Pogostemon cablin*, and *Paeonia suffruticosa* (anthelmintic efficacy of these plants varied from 80 to 90 %). DMSO, as the negative control, did not show any effects on *D. intermedius*.

The anthelmintic efficacies as well as the EC_{50} value of different extracts of *L. chiachristinae*, *C. chinensis*, *C. sappan*, *A. argyi*, and *E. fortunei* are depicted in Fig. 1. The ethyl acetate extract of *L. chiachristinae* was found to be the most effective with an EC_{50} value of 5.1 mg/L after 48 h of exposure. High anthelmintic activity against *D. intermedius* was also observed in the chloroform and petroleum ether extract with EC_{50} values of 17.2 and 21.1 mg/L. The methanol extract exhibited a 94 % efficacy against *D. intermedius* at 250 mg/L. The water extract exhibited no anthelmintic activity.

In the case of *C. chinensis* (Fig. 2), ethyl acetate extract was the most effective with an EC_{50} value of 8.5 mg L^{-1}, and it exhibited 100 % efficacy against *D. intermedius* at 50.0 mg L^{-1}. The methanol extract also showed high anthelmintic activity, with an EC_{50} value of 15.9 mg L^{-1}, followed by ethyl acetate and petroleum ether extracts, with EC_{50} values of 104.7 and 160.6 mg L^{-1}, respectively. Water extract of *C. chinensis* exhibited the least activity with the maximum anthelmintic efficacy of 56 % at 300.0 mg L^{-1}. However, fish mortality occurred when the concentration reached 400.0 mg L^{-1}.

Regarding *C. sappan* (Fig. 3), the highest anthelmintic efficiency was observed in the chloroform extracts, with an EC_{50} value of 15.6 mg L^{-1}. The water extract was the next most effective, with an EC_{50} of 22.3 mg L^{-1} and anthelmintic efficiency of 95 % at 100.0 mg L^{-1} (without dead fish). The methanol and chloroform *C. sappan* extract showed optimal antialgal activity with 95 of 76.0 % (at 100 mg L^{-1}) anthelmintic efficiency, respectively. The petroleum ether extract showed little anthelmintic activity.

As for *A. argyi* and *E. fortunei* (Figs. 4 and 5), the ethyl acetate *A. argyi* extract displayed optimal anthelmintic activity with an EC_{50} value 70.2 mg L^{-1}. The EC_{50} values for the chloroform and petroleum ether extracts of *A. argyi* were 75.4 and 77.6 mg L^{-1}, followed by chloroform extract of *E. fortunei*, with an EC_{50} value of 84.9 mg L^{-1}. The other fractions led only to weak effects against *D. intermedius* (Figs. 6 and 7).

Discussion

Due to the heavy loss caused by *D. intermedius* on aquaculture and ornamental fish trade, a number of *D. intermedius* treatment methods have been investigated. Development of resistance to commercial drugs by parasites has stimulated the search for new control strategies. Here, we have investigated the anthelmintic effects of 26 plants on *D. intermedius* by in vivo anthelmintic efficacy assay. In order to evaluate their potential as monogenea therapeutics, the in vitro screening

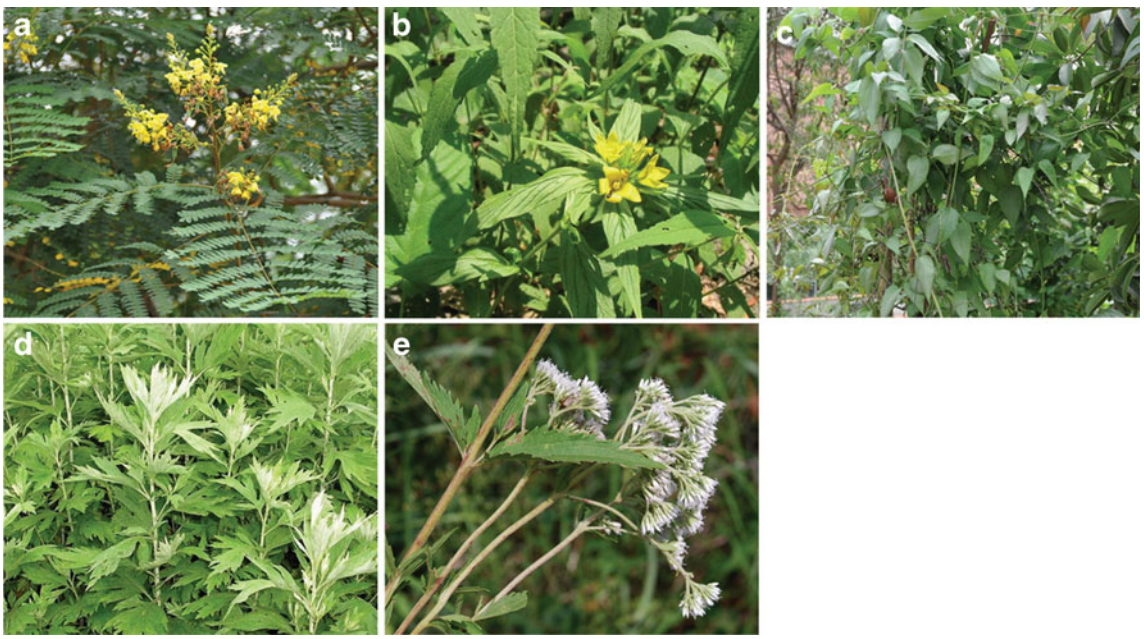

Fig. 2 Photos of plants that have the highest anthelmintic activity. **a** *C. sappan*, photographed by Shouzhou Zhang. **b** *L. chiachristinae*, photographed by Zhoufeng Xu. **c** *C. chinensis*, photographed by Shouzhou Zhang. **d** *A. argyi*, photographed by Huimin Huang, **e** *E. fortune*, photographed by Shouzhou Zhang

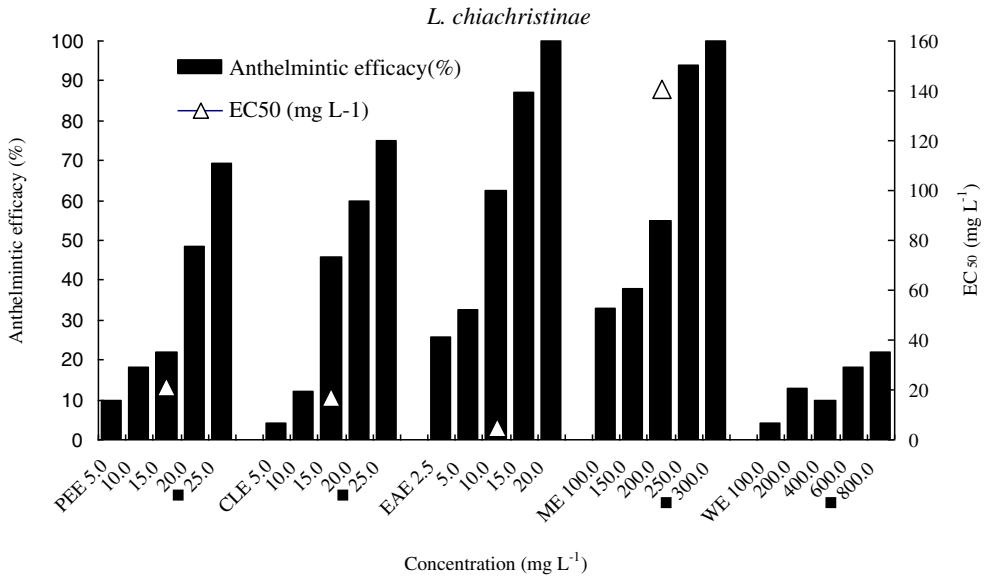

Fig. 3 Anthelmintic efficacy of different extracts of *L. chiachristinae* against *D. intermedius* after 48 h of exposure. *PEE* petroleum ether extract, *CLE* chloroform extract, *EAE* ethyl acetate extract, *MEE* methanol extract, *WAE* water extract. The *star* indicates when fish mortality first occurred

test was carried out in this study aimed to deliver the necessary data for a preselection of those plants that were most promising in further tests to combat *D. intermedius*. Our results clearly showed that the crude extracts of *C. sappan*, *L. chiachristinae*, *C. chinensis*, *A. argyi*, and *E. fortunei* were active. However, to the best of our knowledge, there are no reports evaluating these plants against monogenea; this study is the first report demonstrating antimonogenea activity. In the presence of plant extracts, the infection of *D. intermedius* was strongly suppressed when compared to the control group. Additionally, there was positive correlation between the concentration of the extract and anthelmintic efficiency in all the extracts screened. The plants tested were mainly medicinal plants, which have been widely used in aquaculture in China (Cao et al. 2006). It is tempting to apply these plant extracts in aquaculture farms with the advantages of having no toxic side effects, being economical, and causing no secondary pollution.

Screening of biologically active ingredient against fish parasite from plants has received considerable attention. Kumar et al. (2012) reported that the 96 h of median lethal concentration for piperine against *Argulus* spp. was 52.64 mg L^{-1}. In vitro effect of piperine solution led to 100 % parasite mortality of 9.0 mg L^{-1} in 3 h whereas, under in vivo test, the 100 % antiparasitic efficacy was found at 9.0 mg L^{-1} in 48 h. Ethanol extract of *Artemisia annua* was

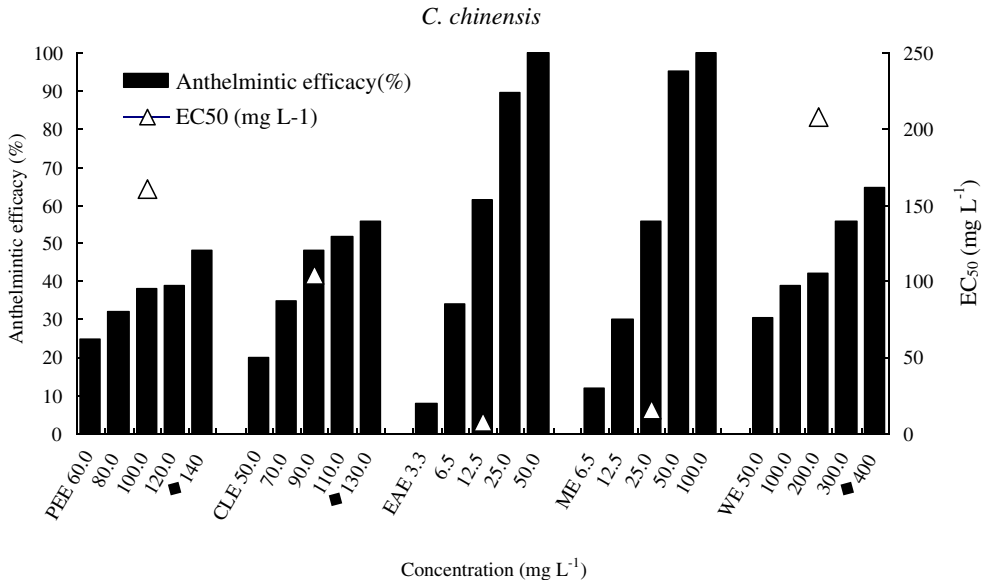

Fig. 4 Anthelmintic efficacy of different extracts of *C. chinensis* against *D. intermedius* after 48 h of exposure. *PEE* petroleum ether extract, *CLE* chloroform extract, *EAE* ethyl acetate extract, *MEE* methanol extract, *WAE* water extract. The *star* indicates when fish mortality first occurred

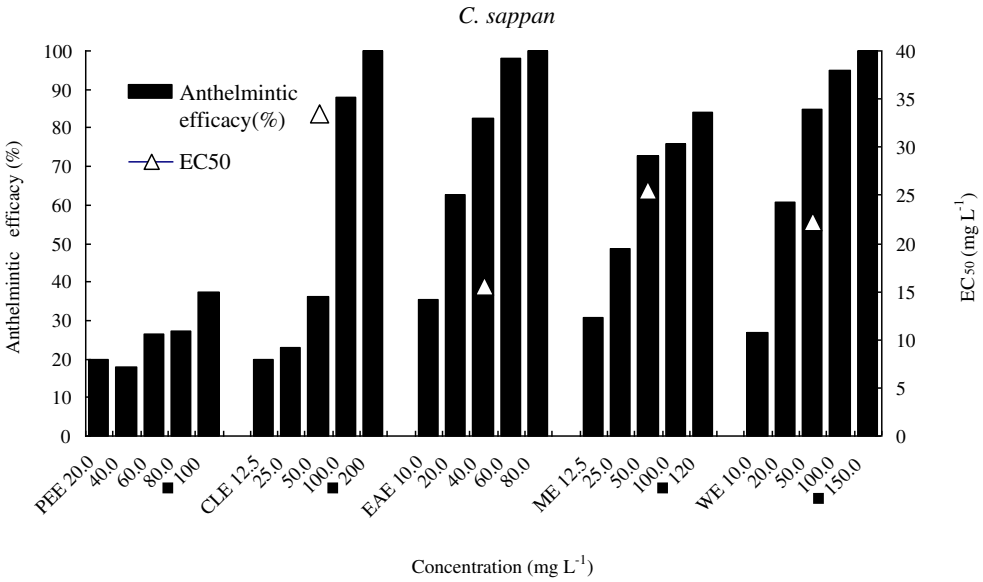

Fig. 5 Anthelmintic efficacy of different extracts of *C. sappan* against *D. intermedius* after 48 h of exposure. *PEE* petroleum ether extract, *CLE* chloroform extract, *EAE* ethyl acetate extract, *MEE* methanol extract, *WAE* water extract. The *star* indicates when fish mortality first occurred

reported to be effective in the dislodgement and mortality of monogenean parasites of juvenile *Heterobranchus longifilis* at concentrations ranging from 50 to 200 mg L^{-1} (Ekanem and Brisibe 2010). The ethanol and *n*-hexane extracts of *Solidago canadensis* are effective against *Trichodina* in *C. auratus gibelio* (Gan et al. 2007). The effects of galangal oil against *Ichthyophthirius multifiliis* infective stage (theront stage) is reported by Potibut et al. (2012), and the lowest effective dose was 30 mg L^{-1}. Yao et al. (2011) studied the anthelmintic activity of *Chelidonium majus* L. whole plant against *D. intermedius*. The ethanol extract from *C. majus* whole plant showed significant anthelmintic activity against *D. intermedius* (EC_{50}=71.5 mg L^{-1}). The crude extracts of *Angelica pubescens* also showed anthelmintic activity against *D. intermedius* in goldfish in vivo with 100 % mortality at a concentration of 120 mg L^{-1} (Wang et al. 2011).

Interestingly, fish exposed to some plant extracts seemed more active and agile in behavior than their counterparts in the control (infected with *D. intermedius*); a similar phenomenon was also observed by Ekanem and Brisibe (2010). This may be explained by the fact that the immune system of these fishes may have been boosted by the plant extracts. Christybapita et al. (2007) suggested that dietary intake of *Eclipta alba* aqueous leaf extract enhances the nonspecific immune responses and disease resistance of *Oreochromis mossambicus*. Additionally, when fishes

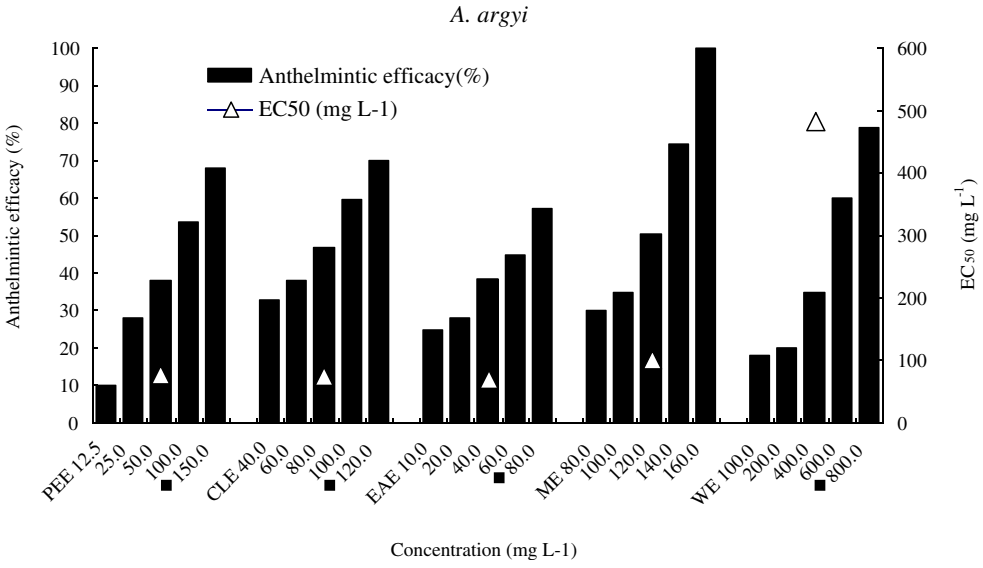

Fig. 6 Anthelmintic efficacy of different extracts of *A. argyi* against *D. intermedius* after 48 h of exposure. *PEE* petroleum ether extract, *CLE* chloroform extract, *EAE* ethyl acetate extract, *MEE* methanol extract, *WAE* water extract. The *star* indicates when fish mortality first occurred

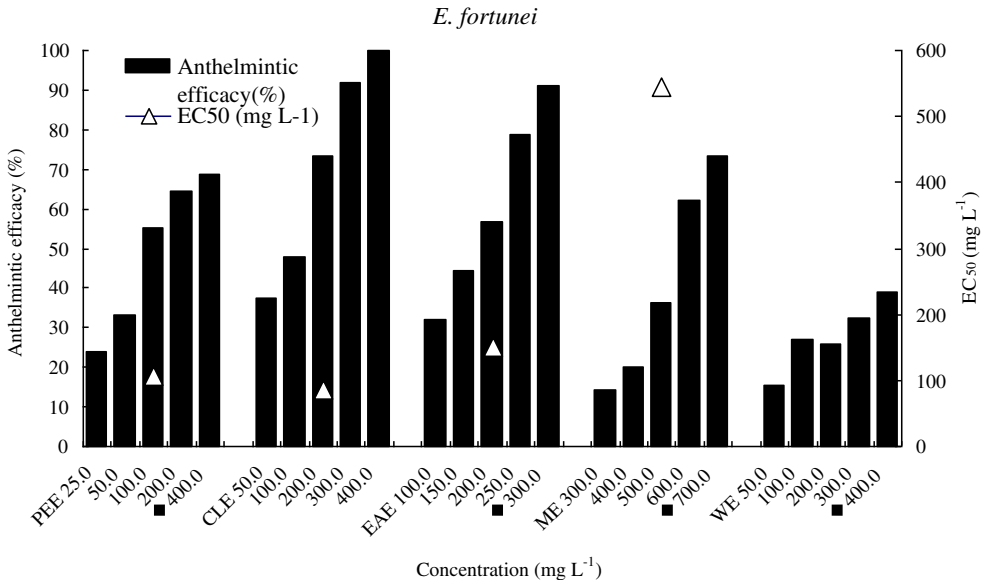

Fig. 7 Anthelmintic efficacy of different extracts of *E. fortunei* against *D. intermedius* after 48 h of exposure. *PEE* petroleum ether extract, *CLE* chloroform extract, *EAE* ethyl acetate extract, *MEE* methanol extract, *WAE* water extract. The *star* indicates when fish mortality first occurred

were free from parasites or the infection level decrease, their immune system and local inflammation will be enhanced after being initially infected with *D. intermedius* (Lu et al. 2013).

L. chiachristinae is a medicinal plant and recorded as Jin-Qian-Cao in Chinese pharmacopoeia. It has been used as a discutientia, antibacterial, and anti-inflammatory agent, and used internally in the treatment of gallstone, icterohepatitis, and fever (Chinese Pharmacopoeia Committee 2005). Lin et al. (1990) reported that the instant granules of *L. chiachristinae* have antimalarial activity against *Diplococcus pneumoniae* in vitro by cylinder plate method. The antibacterial activity against *Staphylococcus aureus* was also reported by Liu and Zou (2002). The main metabolites with pharmacological activity reported from *L. chiachristinae* are quercetin, quercetin glucuronides, and triterpenoid saponin (Liu and Zou 2002; Aoshima et al. 2005). Muzitano et al. (2008) reported that quercetin 3-*O*-α-L-arabinopyranosyl (1→2)-α-L-rhamnopyranoside, quercetin 3-*O*-α-L-rhamnopyranoside, and free quercetin (16 mg/kg body weight) were all able to control the lesion growth caused by *Leishmania amazonensis* and to significantly reduce parasite load in a murine model of cutaneous leishmaniasis. The roots of *C. chinensis* are called Wei-Ling-Xian in Chinese, which is commonly used as analgesic, abirritative, antibacterial, antiphlogistic, anticancer, and a diuretic agent (Chinese Pharmacopoeia Committee 2005). Reports on the chemical components of *C. chinensis* are well documented, with the main focus being on saponins. *C. chinensis* is known for possessing a diverse range of triterpenoid saponin (Mimaki et al. 2004; Fu et al. 2010). Two new triterpenoid saponins isolated from *Glinus oppositifolius* L. were shown to have antiparasitic activity against *Plasmodium falciparum* (Traore et al. 2000). Three saponins isolated from *Hedera helix* L. were shown to have antileishmanial activity (Delmas et al. 2000). The results showed that these saponins exhibited a strong antiproliferative action on all the stages of development of the parasite *Leishmania infantum*. Considering the high antiparasitic activity of quercetin and triterpenoid saponin compounds and the major bioactive constituents of *L. chiachristinae* and *C. chinensis*, we assume that the anthelmintic efficacy of the two plants may be due to the presence of secondary metabolites, particularly the compounds mentioned above.

To conclude, in the present investigation, 26 plants were evaluated for the first time for their anthelmintic activities in vitro against *D. intermedius* (Monogenea) in goldfish. Our results show that the extracts of *C. sappan*, *L. chiachristinae*, *C. chinensis*, *A. argyi*, and *E. fortunei* have the potential for the development of novel therapy for the treatment against *D. intermedius* infection. However, more research is needed to identify specific biocompounds, their mode of action, and effects on the entire aquatic food web. This is crucial in order to develop efficient biological controlling agents for *D. intermedius* consisting of different hosts in various conditions.

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