Screening of Chinese Medicinal Herbs for Anthelmintic Efficacy Against Gyrodactylus kobayashii (Monogenea) in Goldfish (Carassius auratus)

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Abstract
Monogenean infection can cause high mortality and significant financial losses in commercially farmed fish. Existing chemical drugs for the treatment of such infections often have serious drawbacks. In order to find alternative agents of chemical drugs, fourteen medicinal plants were tested for their in vivo anthelmintic activity against Gyrodactylus kobayashii in goldfish (Carassius auratus). Ethanol extracts of Evodia rutaecarpa, Cnidium monnieri and Sophora flavescens had 100% anthelmintic efficacy at low concentrations (100, 100 and 300 mg/L, respectively), after 48 h of exposure. The ethyl acetate extract of C. monnieri was the most effective, with an EC50 value of 11.0 mg/L, after 48 h of exposure, showing 100% anthelmintic efficacy against G. kobayashii at 50.0 mg/L. Higher anthelmintic activity was also observed for remaining extracts of C. monnieri and E. rutaecarpa except for the water extracts of the two plants. The ethyl acetate extract of C. monnieri had the highest therapeutic index (TI, LC50/EC50) value of 31.8, and the lowest EC50, which indicates that this extract was the safest to goldfish among all extracts. For the remaining extracts of C. monnieri and E. rutaecarpa, the 48-h LC50 values were about 10-fold higher than the corresponding EC50 values. This result indicates these extracts have low toxicity in goldfish. The ethyl acetate extract of C. monnieri was the most effective and the safest among the tested extracts. Therefore, the two plants are expected to be alternative agents to control monogenean infection.

Keywords: Disease control, Gyrodactylus kobayashii, Chinese Medicinal Herb, Goldfish, Anthelmintic efficacy

INTRODUCTION
Ornamental fish trade is one of the most rapidly developing areas of the aquaculture industry, and the value of international trade exports of ornamental fish has grown by an average of about 14% per year since 1985 [1-2]. The...
rapid development of the ornamental fish industry has been
overwhelmed by the occurrence of large-scale parasitic
diseases. Goldfish (Carassius auratus) is one of the most popular
ornamental fish worldwide, due to its easy maintenance
and its attractive coloration. The most common ecto-
parasites infecting goldfish are gyrodactylids [3]. Viviparous
gyrodactylids have a direct life-cycle. They can directly
spread among hosts by contact, and undergo continuous
transmission throughout their life-cycle [4,5]. Serious infection
can cause evident clinical symptoms [3]. Monogeneans can
damage the fish epidermis, which results in secondary
infections by other pathogenic microorganisms [6,7].

It is difficult to control monogeneans. Many chemical
drugs have been used against these parasites, but
bring some drawbacks (e.g., low efficacy, toxicity to host
and environmental and human health problems) [8,9].
Additionally, the long-term use of chemical drugs can
lead to drug resistance in parasites [10]. Recently, increasing
attention has been paid to the use of traditional plant-
based medicines to control diseases in aquaculture [11,12].
Zhou et al. [13] showed that herbal medicines were able to
control Gyrodactylus kobayashii (G. kobayashii) in goldfish. Huang et al. [14] screened plant extracts with
anthelmintic activity against Dactylogyrus intermedius
(Monogenea) in goldfish.

In order to find safe and efficient alternatives to treat
monogenean infection in aquaculture, we evaluated the
anthelmintic efficacy of ethanol extracts of 14 plants
against G. kobayashii in goldfish, using in vivo anthelmintic
efficacy assays.

**MATERIAL and METHODS**

**Establishment of a Goldfish-G. kobayashii Model and
Animal Ethics**

Goldfish weighing 3.7±0.7 g were selected from a fish farm
in Anyang city, Henan province, China. All experiments
complied with institutional animal care guidelines and
were approved by the Animal Care Committee of Anyang
Institute of Technology and Academician Workstation of
Animal Disease Control and Nutrition Immunity in Henan
Province (License no. SCXK(AWADCNI)2018-0001). These
goldfish were fed and treated to remove all ectoparasites,
as described previously [13]. A goldfish experimental infection
model using G. kobayashii was performed according to
a method described previously [15]. Uninfected fish were
anesthetized with 0.02% MS-222. In order to allow parasite
transfer between hosts, the caudal fins of uninfected fish
were placed in contact with the caudal fins of heavily-
infected fish reared in our laboratory. Experimentally-
infected fish were then placed in a 1 L container. The success
of the infection was determined by daily examination of
the fins using a stereomicroscope. Ten days after infection,
two parasites were collected from the infected fish for
morphological and molecular identification according
to Li et al. [16] in order to verify that the infective agent
was G. kobayashii. Uninfected goldfish were introduced
periodically into an 80 L aquarium containing infected
goldfish, in order to increase the number of infected fish
for the experiments.

**Preparation of Plant Materials**

The fourteen Chinese medicinal herbs listed in Table 1
were purchased from a drug store and prepared according
to a method described previously [13]. The herbal medicines
were washed thoroughly, air-dried and oven-dried at
45°C for 48 h. In order to ensure the complete removal
of water, dry plant materials were powdered, strained
using a filter net of 30-40 mesh (450-600 µm), and freeze-
dried at -54°C. The powder of all the medicinal plants
was extracted with ethanol. The powder of E. rutaecarpa,
C. monnieri and S. flavescens was extracted for 48 h with
water, methanol, petroleum ether, or ethyl acetate; the
extraction process was repeated three times. To obtain
solidified crude extracts, the extract solutions were filtered,
and then concentrated at reduced pressure in a vacuum
rotary evaporator, until all solvents had evaporated.
Solid extracts then were dissolved in dimethyl sulfoxide
(DMSO) at 0.5 mg/L as stock solutions for anthelmintic
efficacy assays.

**Screening Experiment**

The dry powder (50.0 g) of herbal medicines was extracted
time with 500.0 mL ethanol for 48 h. Ethanol filtrates
were evaporated at reduced pressure in a vacuum rotary
evaporator in order to obtain solidified crude extracts [14].
The solidified crude extract of each plant was dissolved in
DMSO as a stock solution at 0.5 g/mL; these stock solutions
were then screened for anthelmintic efficacy.

In addition, three kinds of herbal medicines (E. rutaecarpa,
C. monnieri and S. flavescens) with higher antiparasitic
activities (Table 1) were fractionated with different solvents
(water, methanol, petroleum ether, and ethyl acetate), and
then tested in anthelmintic efficacy assays. Each solid extract
was dissolved in DMSO to obtain the stock solution at
the concentration of 0.5 g/mL, which was used in anthelmintic
efficacy assays.

**In vivo Anthelmintic Efficacy Assay**

The anthelmintic assay against G. kobayashii in goldfish
was performed according to a previous method [13]. Two
goldfish infected with G. kobayashii were placed in
20x12x10 cm tanks containing 0.5 L of the test solutions
at 23±1°C for 48 h. Two control groups without extract,
or with the highest percentage of DMSO, were set at the
same experimental conditions. All treatments and control
groups were performed using five replicates. Before
manipulation, goldfish were anesthetized with 0.02% MS-
222, and the parasites in the caudal fin were counted
under a stereomicroscope.
The anthelmintic efficacy of each extract was calculated according to the following formula: $E = \frac{(L-L_t)}{L} \times 100\%$ for $L > L_t$, $E = 0$ for $L \leq L_t$. $E$ is anthelmintic efficacy, $L$ is the number of $G. kobayashii$ on the caudal fin before treatment, and $L_t$ is the number of surviving parasites after treatment. Mean anthelmintic efficacy was calculated from five replicates per treatment.

**Acute Toxicity Tests**

The extract with the strongest anthelmintic efficacy in vivo anthelmintic efficacy assay, was tested for safety to goldfish. Acute toxicity tests were conducted in 26.5×16.5×12.5 cm tanks, containing ten healthy goldfish, and 2 L of aerated tap water at 23±1°C. Fish in tanks without plant extracts constituted the control group. Three replicates were set for the group of control fish and for the groups of fish exposed to plant's extracts. Fish mortality in each tank was recorded after 48 h. Fish were not fed during the experiment, and dead fish were continuously removed in order to avoid deterioration of water quality.

**Statistical Analyses**

The homogeneity of replication samples was evaluated through the Mann-Whitney U test. At the 95% confidence level, median lethal concentration ($LC_{50}$, $LC_{90}$) and median effective concentration ($EC_{50}$, $EC_{90}$) were determined using the probit analysis. The therapeutic index (TI) was calculated as $LC_{50}/EC_{50}$. Each statistical analysis was performed using SPSS 19.0. A value of $P<0.05$ was considered significant and $P<0.01$ was considered highly significant.

**RESULTS**

**Anthelmintic Efficacy of Extracts Against G. kobayashii in vivo**

As shown in Table 1, of the 14 medicinal plants selected, only the ethanol extracts of $E. rutaecarpa$, $C. monnieri$ and $S. flavescens$ had 100% anthelmintic efficacy at 100.0, 100.0 and 300.0 mg/L, respectively, after 48 h of exposure. Additionally, the extracts of $Citrus$ reticulate, $Mentha$ haplocalyx, $Punica$ granatum, $Agrimonia$ pilosa and $Omphalia$ lapidescens showed good anthelmintic efficacy, but only at high concentrations (Table 1). There was either very weak anthelmintic activity, or highly toxicity to goldfish, in the remaining ethanol extracts of the herbal medicines tested. In the control groups, the number of $G. kobayashii$ increased on the caudal fin (from 98.2±25, 7 to 160.5±42.7 in the group with no extract, and from 113.1±21.5 to 150.5±31.3 in the DMSO group).

For the different extracts of $E. rutaecarpa$, $C. monnieri$ and $S. flavescens$, their anthelmintic efficacy is showed in Fig. 1, 2, 3, and the corresponding $EC_{50}$ and $EC_{90}$ values are showed in Table 2. The ethyl acetate extract of $C. monnieri$ exhibited 100% anthelmintic efficacy against $G. kobayashii$ at 50.0 mg/L, and was the most effective against $G. kobayashii$, with an $EC_{50}$ value of 11.1 mg/L, and an $EC_{90}$ value of 28.3 mg/L, after 48 h of exposure. In addition, the anthelmintic activity against $G. kobayashii$ was also determined for the methanol and petroleum ether extracts of $C. monnieri$, with $EC_{50}$ values of 23.6 and 44.6 mg/L, and $EC_{90}$ values of 51.0 and 84.0 mg/L, respectively. In all the extracts of $C. monnieri$, the water extract had the weakest anthelmintic efficacy of 42.7% at 1 000.0 mg/L.

The ethyl acetate extract of $E. rutaecarpa$ had good anthelmintic efficacy, with $EC_{50}$ and $EC_{90}$ values of 24.0 and 50.3 mg/L, respectively, after a 48-h exposure. The petroleum ether and methanol extracts of $E. rutaecarpa$ showed 100% anthelmintic efficacy at 150.0 and 120.0 mg/L, with $EC_{50}$ and $EC_{90}$ values of 71.9 and 108.3 mg/L (petroleum ether), and 40.9 and 91.2 mg/L (methanol), respectively. The water extract of $E. rutaecarpa$ had the weakest anthelmintic efficacy of 25.6% at 800.0 mg/L.
the extracts of *S. flavescens* showed effective anthelmintic efficacy against *G. kobayashii* after 48 h of exposure, but at high concentrations, with EC_{50} values of 234.6 mg/L (methanol), 224.4 mg/L (petroleum ether) and 238.6 mg/L (ethyl acetate).

**Acute Toxicity of the Tested Extracts in Goldfish**

The results of the acute toxicity assay of the extracts with higher anthelmintic efficacy at the low concentrations (methanol, petroleum ether, and ethyl acetate extracts of *E. rutaecarpa* and *C. monnieri*) are summarized in Table 3. After 48 h of exposure, the LC_{50} values of the extracts of *E. rutaecarpa* were 759.3 (methanol), 771.9 (petroleum ether) and 235.9 (ethyl acetate) mg/L, which is 18.6, 10.7 and 9.9 times higher than the corresponding EC_{50}, respectively. The methanol, petroleum ether, and ethyl acetate extracts of *C. monnieri* had LC_{50} values of 214.1, 405.3 and 350.0 mg/L, which is 9.1-, 9.1- and 31.8 times higher than the corresponding EC_{50}, respectively. The water extracts of *E. rutaecarpa* and *C. monnieri* exhibited very weak toxicity to goldfish, and killed only two and
one goldfish, respectively, at the concentration of 1000.0 mg/L.

DISCUSSION

Diseases caused by the common ectoparasites, Gyrodactylus, can result in high mortality and huge financial losses in commercially farmed fish \([6,17]\). Additionally, prolonged and frequent use of common chemicals such as formaldehyde, rotenone, and praziquantel results in increasing drug resistance and adverse effects on the environment \([8,10]\). Therefore, there is an urgent need for effective strategies to control Gyrodactylus infections. In our study, the ethyl acetate extract of C. monnieri was the most efficient, having the lowest EC\textsubscript{50} and EC\textsubscript{90} values (11.0 and 28.3 mg/L, respectively) after 48 h of exposure, and it had 100% anthelmintic efficacy against Gyrodactylus kobayashii at 50.0 mg/L. The dried fruit of C. monnieri (L.) Cuss., called “Shechuangzi” in Chinese, is a commonly used traditional Chinese medicine \([18]\). Previous research has found that the ethanol extract had better anthelmintic efficacy against Dactylogyrus intermedius (D. intermedius) in goldfish, and had the minimal effective concentration compared to the chloroform, petroleum ether, and acetone extracts of C. monnieri. This extract had 100% anthelmintic efficacy at 70.0 mg/L after 48 h of exposure. The active compounds of the ethanol extract against D. intermedius were identified as osthol and isopimpinellin \([19,20]\). In the present study, the ethyl acetate extract of C. monnieri was the most effective against G. kobayashii in goldfish at the minimal concentration, showing 100% anthelmintic efficacy at 50.0 mg/L, which was far lower than the concentration of ethanol extract with 100% anthelmintic efficacy (100.0 mg/L). Differences in anthelmintic efficacy of the same extracts may be due to the differences in the evaluation methods. In previous research, anthelmintic efficacy was calculated by comparison with the control group. In our paper, the number of Gyrodactylus was recorded before and after exposure, and anthelmintic efficacy was calculated based on the changes in intensity of Gyrodactylus in goldfish. Besides, the difference in the main parasitic sites may be another cause. The main parasitic site of Dactylogyrus sp. is the gill, which may provide partial protection for this parasite by reducing the exposure to extracts. Nevertheless, Gyrodactylus sp. is mainly parasitic on the fins or the skin, which directly exposes this parasite to the extracts.

"Wuzhuyu", derived from the dried fruit of E. rutaecarpa (Juss.) Benth., is well described in the Chinese medical matter

Table 2. Anthelmintic efficacy (EC_{50} and EC_{90}) of different extracts from Evodia rutaecarpa, Cnidium monnieri and Sophora flavescens against Gyrodactylus kobayashii after 48 h of exposure

<table>
<thead>
<tr>
<th>Plants</th>
<th>Extraction Solvent</th>
<th>EC_{50} (mg/L)</th>
<th>95% CI</th>
<th>EC_{90} (mg/L)</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evodia rutaecarpa</td>
<td>Methanol</td>
<td>40.9</td>
<td>0-56.0</td>
<td>91.2</td>
<td>76.3-131.1</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Petroleum ether</td>
<td>71.9</td>
<td>66.7-76.2</td>
<td>108.3</td>
<td>102.8-115.7</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>Ethyl acetate</td>
<td>24.0</td>
<td>20.8-26.9</td>
<td>50.3</td>
<td>46.0-56.0</td>
<td>0.38</td>
</tr>
<tr>
<td>Cnidium monnieri</td>
<td>Methanol</td>
<td>23.6</td>
<td>15.8-28.4</td>
<td>51.0</td>
<td>47.6-55.8</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>Petroleum ether</td>
<td>44.6</td>
<td>37.8-49.8</td>
<td>84.0</td>
<td>78.5-91.4</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Ethyl acetate</td>
<td>11.1</td>
<td>7.7-13.3</td>
<td>28.3</td>
<td>25.8-31.69</td>
<td>0.94</td>
</tr>
<tr>
<td>Sophora flavescens</td>
<td>Methanol</td>
<td>234.6</td>
<td>161.5-303.0</td>
<td>319.8</td>
<td>266.0-543.6</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>Petroleum ether</td>
<td>224.4</td>
<td>0-292.8</td>
<td>400.0</td>
<td>328.1-714.7</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Ethyl acetate</td>
<td>238.6</td>
<td>0-315.3</td>
<td>416.4</td>
<td>335.9-914.6</td>
<td>0.06</td>
</tr>
</tbody>
</table>

EC\textsubscript{50}, effective concentration with 50% anthelmintic efficacy; EC\textsubscript{90}, effective concentration with 90% anthelmintic efficacy; 95% CI, 95% confidence interval

Table 3. Acute toxicity for goldfish exposed to different extracts from Evodia rutaecarpa and Cnidium monnieri after 48 h of exposure

<table>
<thead>
<tr>
<th>Plants</th>
<th>Extraction Solvent</th>
<th>LC_{50} (mg/L)</th>
<th>95% CI</th>
<th>LC_{90} (mg/L)</th>
<th>95% CI</th>
<th>TI (LC_{50}/EC_{50})</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evodia rutaecarpa</td>
<td>Methanol</td>
<td>759.3</td>
<td>713.5-806.6</td>
<td>854.7</td>
<td>807.3-975.2</td>
<td>18.6</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>Petroleum ether</td>
<td>771.9</td>
<td>719.6-826.5</td>
<td>892.8</td>
<td>835.5-1039.1</td>
<td>10.7</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>Ethyl acetate</td>
<td>235.9</td>
<td>172.6-298.8</td>
<td>385.9</td>
<td>317.2-579.7</td>
<td>9.9</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>&gt;1000.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cnidium monnieri</td>
<td>Methanol</td>
<td>214.1</td>
<td>186.8-240.2</td>
<td>274.6</td>
<td>246.7-344.5</td>
<td>9.1</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>Petroleum ether</td>
<td>405.3</td>
<td>332.0-462.8</td>
<td>552.9</td>
<td>488.5-735.8</td>
<td>9.1</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>Ethyl acetate</td>
<td>350.0</td>
<td>301.5-398.5</td>
<td>453.6</td>
<td>403.7-579.8</td>
<td>31.8</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>&gt;1000.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

LC\textsubscript{50}, 50% lethal concentration; LC\textsubscript{90}, 90% lethal concentration; 95% CI, 95% confidence interval; TI, therapeutic index; –, not calculated
and the Chinese pharmacopoeia, and it is recommended for the treatment of dizziness, headache, emesis, diarrhea and other symptoms [21,22]. Pharmacological studies indicate that the bioactive constituents of *E. rutaecarpa* have many properties, including anti-inflammatory, antihypertensive, antioxidative, antimicrobial and anthelmintic activities [23,24]. For example, Perrett and Whitfield [25] reported that atanine (3-Dimethylallyl-4-methoxy-2-quinoilone), an alkaloid isolated from the hexane extract of *E. rutaecarpa* caused 100% immobility of the cercarial and miracidial larvae of *Schistosoma mansoni* at 100.0 mg/L, after 7 min of exposure. Liu et al. [26] have shown that ethanol extracts of *E. rutaecarpa* are effective at killing intestinal nematode parasites of pigs and leeches *in vitro*. Also, the n-hexane extract of *E. rutaecarpa* has been demonstrated to have strong antifeedant activity against the grain storage insects *Sitophilus zeamais* and *Trichobium castaneum* [26]. Moreover, two alkaloids isolated from the methanol extract of *E. rutaecarpa*, evodiamine and rutacearpine, have shown insecticidal activity against fruit flies (*Drosophila melanogaster*), with LC50 values of 3.9 and 3.6 mg per adult, respectively [27]. In the present study, the ethyl acetate extract of *E. rutaecarpa* showed good anthelmintic efficacy, with EC50 and EC90 values of 24.0 and 50.3 mg/L, after 48 h of exposure. Although the active compounds against *G. kobayashii* that are contained in the ethyl acetate extract have not been identified, some of the compounds described above are thought to jointly or independently control *G. kobayashii* infection.

The therapeutic index (TI), a parameter for the quantitative relationship between efficacy and safety, is used to evaluate the potential application of the extracts in aquaculture [28]. TI is calculated as the ratio of LC50 to EC90, and higher TI values reflect safer effects. In our study, we found that the ethyl acetate extract of *C. monnieri* had the highest TI value of 31.8, and the lowest EC50, which indicated this extract was safest to goldfish among all extracts and had the potential to be used in aquaculture [29]. Additionally, the methanol extract had a higher TI value of 18.6, but the EC50 was also higher compared with the ethyl acetate extract for *E. rutaecarpa*. The 48-h LC50 values of the remaining extracts of *C. monnieri* and *E. rutaecarpa* were about 10-fold higher than the corresponding EC50 values, which reflects low toxicity to goldfish. The bioactive ingredients that play a major role in anthelmintic activity are necessary for further bioassay-guided isolation and identification. In addition, field trials need to be performed before the use of extracts from *C. monnieri* and *E. rutaecarpa* in aquaculture.

Among the 14 Chinese medicinal herbs screened, the extracts of *C. monnieri* and *E. rutaecarpa* had the highest anthelmintic efficacy against *G. kobayashii* in goldfish. The ethyl acetate extract of *C. monnieri* was the most effective and the safest of all the extracts tested. *C. monnieri* and *E. rutaecarpa* may become novel therapeutic agents against *G. kobayashii* infection.

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COMPETING INTERESTS

The authors declare they have no competing interests.

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