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NOTE

Anthelmintic activity of saikosaponins a and d from radix bupleuri against *Dactylogyrus* spp. infecting goldfish

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ABSTRACT: Disease caused by the parasitic helminths *Dactylogyrus* spp. results in significant economic damage to the aquaculture industry. Treatment using common chemicals (e.g. formalin) is usually dissatisfactory due to environmental problems, risk of residues, toxicity to fish, and the possibility of anthelmintic resistance. The search for an alternative drug is thus becoming more urgent. This study was designed to evaluate *in vivo* the anthelmintic efficacy of total saponin (TS), saikosaponin a (SSa), and saikosaponin d (SSd) from radix bupleuri (i.e. the dried root of *Bupleurum* sp.) based on our previous screening works, with the aim of determining which has commercial potential. Results showed that median effective concentration (EC_{50}) values for TS, SSa, and SSd were 2.01, 1.46, and 0.74 mg l^{-1}, respectively. The acute toxicities against goldfish *Carassius auratus* for TS, SSa, and SSd were also determined, with median lethal concentration (LC_{50}) of 8.99, 11.20, and 1.54 mg l^{-1}, respectively. The resulting therapeutic indices (TIs) indicated that SSa (TI = 7.67) is a potential therapeutic agent for treating *Dactylogyrus* infection.

KEY WORDS: Anthelmintic efficacy · *Dactylogyrus intermedius* · *D. vastator* · Chaihu · *Bupleurum chinense* · *B. scorzonerifolium* · *Carassius auratus*

INTRODUCTION

Dactylogyrus is the largest genus of parasitic helminths, with 971 species, approximately 95% of which parasitize the gills of fishes in the family Cyprinidae (Gibson et al. 1996, Dove & Ernst 1998). Heavy infections with *Dactylogyrus* may result in significant damage to feral and cultured cyprinids, including ornamental fish. The disease caused by *Dactylogyrus* spp. (dactylogyriasis) is a significant threat to the aquaculture industry because of the direct life cycle of the helminth, which comprises an obligate adult stage, fertilized egg, and free-swimming larval stage (Ji et al. 2012). The fertilized eggs hatch into free-swimming larvae and are then carried to a new host by water currents as well as their own ciliated movement (Klinger & Floyd 2013). Large numbers of the parasite attach to the gills of fish, causing epithelial proliferation, excessive mucus secretions, and accelerated respiration, and mortality occurs because of osmoregulatory imbalance (Steverding et al. 2005, Reed et al. 2012). Moreover, secondary infection by bacteria and fungi is common on tissue damaged by monogeneans (Klinger & Floyd 2013).

The effective treatment of *Dactylogyrus* has been achieved by use of formalin when administered as a short-term or prolonged bath (Thoney & Hargis 1991). However, the treatment is usually not satisfactory due to high stocking density and poor water quality. In addition, fish need to be carefully monitored during treatment because they do not tolerate formalin well (Klinger & Floyd 2013). Other chemi-

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cals, such as praziquantel (Schmahl & Mehlhorn 1985), mebendazole (Treves-Brown 1999), potassium permanganate, and hydrogen peroxide (Reed et al. 2012), have been used successfully for *Dactylogyrus* removal; however, some fishes may be intolerant of high doses or long baths required for eliminating *Dactylogyrus*. Moreover, the threats of drug resistance, risk of residues, and environmental problems caused by the frequent application of these chemotherapies have led to the need for other alternative drugs (Klinger & Floyd 2013, Tu et al. 2013).

Recently, interest in the use of medicinal plants to control monogenean diseases in fish has increased due to their demonstrable efficacy and low environmental hazard. Steverding et al. (2005) explored the possibility of using Australian tea tree *Melaleuca alternifolia* oil to treat *Gyrodactylus* spp. infection in three-spined stickleback *Gasterosteus aculeatus*. More recently, several researchers reported anthelmintic activity of garlic extracts/compounds against *Gyrodactylus* sp. in guppies *Poecilia reticulata* (Schelkle et al. 2013, Fridman et al. 2014); in addition, Militz et al. (2013) demonstrated the potential of dietary supplementation of garlic to prevent monogenean infection in *Neobenedenia* sp. In our previous works, a large number of medicinal plants were screened for anthelmintic activity, and some showed high activities against *D. intermedius* in goldfish (Liu et al. 2010, Wang et al. 2010, Ji et al. 2012, Lu et al. 2012, Tu et al. 2013), especially the methanol extract of radix bupleuri (Wu et al. 2011).

Radix bupleuri ('Chaihu' in Chinese) is the dried root of *Bupleurum chinense* or *B. scorzonerifolium*. It is one of the most common components of Chinese traditional medicine prescriptions for the treatment of chronic hepatitis, intermittent fever, inflammatory disease, and ulcers of the digestive system (Pistelli et al. 1996, Guo et al. 2000, Zhu et al. 2009). Phytochemical and pharmacological studies have confirmed that saikosaponins are responsible for the main pharmaceutical activities of radix bupleuri. Saikosaponin a (SSa) and saikosaponin d (SSd), as the main constituents of saikosaponins, are known for their bioactivities, including anti-allergic action, analgesic effects, anti-inflammatory activity, hemolytic activity, plasma cholesterol-lowering action, and protective action for hepatic injuries (Aoyagi et al. 2001, Zhu et al. 2009). However, no information on the efficacy of saikosaponins for treating parasitic infection has been available until now. Here we investigated *in vivo* the anthelmintic activity of methanol extract, total saponin (TS), and SSa, SSc, and SSd from radix bupleuri against *Dactylogyrus* spp. in goldfish.

MATERIALS AND METHODS

Fish and parasites

A batch of goldfish *Carassius auratus* (3.01 ± 0.61 [SD] g) was obtained from a fish farm (Nanyang, Henan, China, 112° 16′ 31″ N, 32° 54′ 7″ W) and held in several 200 l glass aquaria with aquarium filters and air stones (water temperature 20.0–22.0°C, pH 6.7–7.2, dissolved oxygen 5.8–7.4 mg l^{-1}). Thirty fish were checked randomly to examine for the presence of parasites and bacteria. Two species of *Dactylogyrus* were found on the gills of fish, and no bacteria or other parasites were detected. Two species of *Dactylogyrus* were isolated from the gills of goldfish and identified based on haptor morphometrics following the protocol of Šimkaová et al. (2007). Briefly, each *Dactylogyrus* species was fixed in a mixture (1:1) of glycerine and ammonium picrate (GAP) and observed using a light microscope (Olympus BX51+ DP 70). For scanning electron microscopy (SEM), the parasites were subjected to proteolytic digestion (Paladini et al. 2009) on 5 × 5 mm glass slides to obtain tissue-free opisthaptors, and after 30 min, the digestion was punctuated by the addition of distilled water for 3 to 5 times. The opisthaptors were air-dried, sputter-coated with gold, and then examined using a JEOL JSM-6360LV scanning electron microscope. After haptor morphological observation, the infection rate and intensity of *Dactylogyrus* were determined according to Wang et al. (2008). Thirty fish were randomly sampled and killed by spinal severance. Eight branchial arches per fish were placed on glass slides, and the number of parasites was recorded under a light microscope at 40× magnification. All sampled fish (N = 30) were infected with *Dactylogyrus* (infection rate = 100%), and the mean number of parasites per fish was 94.

Preparation of methanol extract

The methanol extract of radix bupleuri was prepared according to Wang et al. (2010). Radix bupleuri was purchased from Xi'an Wanshou Chinese Medicinal Herbs Markets and identified as the dried root of *Bupleurum chinense* by Prof. X. P. Song (Northwest A&F University, Shaanxi, China). The dried plant material was reduced to powder using a commercial electric stainless steel blender, and the powder was sieved afterwards (30–40 mesh, 0.42–0.60 mm). The dried powder (1000 g) was extracted with 2.5 l of methanol in a 65°C water bath under reflux for 4 h,

and the process was repeated 3 times. The extract was subsequently filtered and concentrated under reduced pressure in a vacuum rotary evaporator until the solvent was completely evaporated to form solidified crude extracts (113.93 g). Crude extract (10 g) was dissolved in 0.02% dimethyl sulfoxide (DMSO) to obtain 500 mg l^{-1} of stocking solution for the anthelmintic efficacy assay.

Preparation of TS and HPLC analysis

The crude extract (90.70 g) was dissolved with distilled water using an ultrasonic method, and the solution was subjected to D201 resin column chromatography, washed with 2% NaOH (4 l) and H_2O (10 l), and eluted with 85% ethanol to yield about 15.0 g TS of *B. chinense*. The extract was dissolved in HPLC-grade methanol and was then analyzed by a Wondasil C_{18} packed column (Shimadzu LC-15C) at a flow rate of 1 ml min^{-1} and detection wavelength of 210 nm. The acetonitrile–water mobile phase (42:58, v/v) was employed for the analysis. SSa, SSc, and SSd of AR grade were purchased from Aladdin Industrial. The structure of the 3 chemicals is shown in Fig. 1. The stock solutions of TS, SSa, SSc, and SSd (each 50 mg l^{-1}) were prepared as described above for the anthelmintic efficacy assay.

In vivo anthelmintic effect against *Dactylogyrus*

The anthelmintic assay against *Dactylogyrus* was performed according to the method of Wang et al. (2008). The test container was a plastic pot with 2 l of aerated tap water each containing 5 goldfish. After acclimatization for 48 h, the fish were exposed to different concentrations of the methanol extract, TS, SSa, SSc, or SSd. A negative control group with no extracts and DMSO was set up under the same conditions as the tests; in addition, a positive control group with 0.02% DMSO but no constituent from radix bupleuri was included in order to remove the possible effects of DMSO on *Dactylogyrus*. The anthelmintic assay was performed in triplicate, and dissolved oxygen, pH, and temperature of the water in all pots were monitored. After 48 h, the number of parasites per fish was recorded as described above, and the anthelmintic efficacies of each treatment and the control groups were calculated according to the following formula (Ji et al. 2012):

$$AE(\%) = (B - T) / B \times 100\% \quad (1)$$

where AE is anthelmintic efficacy, B is the mean number of surviving parasites in the negative controls, and T is the mean number of surviving parasites in the treatments.

Acute toxicity test against goldfish

An aqueous static renewal 48 h bioassay was performed to evaluate the acute toxicity of the methanol extract, TS, SSa, SSc, and SSd against goldfish (DeLorenzo et al. 2006, Wu et al. 2011, Tu et al. 2013). Fish were placed into several plastic pots (5 fish pot^{-1}) and exposed to different concentrations of the methanol extract, TS, SSa, SSc, or SSd. A negative control with no extracts and DMSO, and a positive control with 0.02% DMSO were set up in this test. The test was replicated 2 times, and water quality was monitored as in the previous experiment. Mortality was observed daily in each pot, and the death of a fish was determined when opercular movement and tail beat stopped and the fish no longer responded to a mechanical stimulus (Wu et al. 2011).

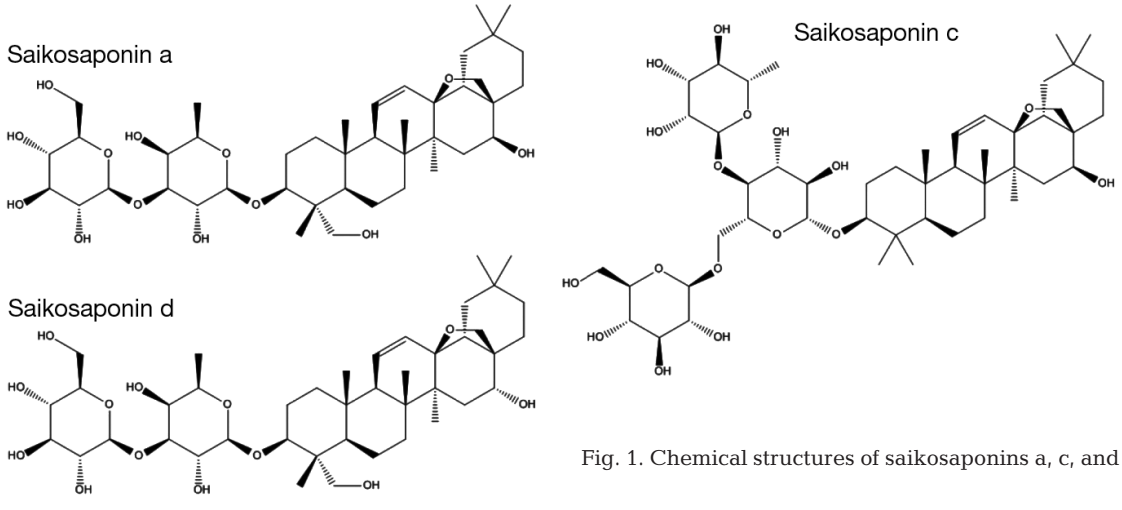

Fig. 1. Chemical structures of saikosaponins a, c, and d

Table 1. Acute toxicity (48 h EC_{50} and EC_{90}) of methanol extract, total saponin, and saikosaponins a, c, and d from the dried root of *Bupleurum* sp. (i.e. radix bupleuri) against *Dactylogyrus* spp. (mean infection intensity: 94 dactylogyrids per fish). EC_{50} (EC_{90}): 50% (90%) effective concentration; CL: confidence limit; –: not examined

Constituent	EC_{50} (mg l^{-1}) (95% CL)	EC_{90} (mg l^{-1}) (95% CL)	χ^2	p
Methanol extract	8.48 (6.79–9.86)	21.08 (19.50–23.17)	1.09	0.90
Total saponin	2.01 (1.97–2.21)	3.40 (3.22–3.64)	7.17	0.21
Saikosaponin a	1.46 (1.38–1.53)	2.04 (1.94–2.17)	3.10	0.38
Saikosaponin c	>3.00	–	–	–
Saikosaponin d	0.74 (0.62–0.84)	1.07 (0.95–1.34)	9.17	0.27

Table 2. Acute toxicity (48 h LC_{50} and LC_{90}) of the methanol extract, total saponin, and saikosaponins a and d from the dried root of *Bupleurum* sp. (i.e. radix bupleuri) against goldfish *Carassius auratus* (N = 10 for each treatment). LC_{50} (LC_{90}): 50% (90%) lethal concentration; CL: confidence limit; –: not examined

Constituent	LC_{50} (mg l^{-1}) (95% CL)	LC_{90} (mg l^{-1}) (95% CL)	χ^2	p
Methanol extract	37.54 (36.02–39.00)	40.57 (39.09–45.23)	0.82	0.85
Total saponin	8.99 (7.92–10.07)	10.97 (9.92–13.73)	0.34	0.84
Saikosaponin a	11.20 (9.98–12.56)	14.25 (12.82–17.55)	1.48	0.69
Saikosaponin d	1.54 (1.30–1.78)	2.02 (1.79–2.61)	0.29	0.87

The dead fish was removed immediately to prevent deterioration of water quality.

Statistical analysis

The data were analyzed using the Predictive Analytics Software Statistic v. 18.0. The Mann-Whitney *U*-test was used to determine the homogeneity of the replicates of the samples. The lethal concentration for 50 and 90% of fish (LC_{50}, LC_{90}) and the effective concentration for 50 and 90% of parasites (EC_{50}, EC_{90}) with 95% confidence intervals (CI) were calculated using the PROBIT procedure. The therapeutic index (TI) was calculated as LC_{50}/EC_{50}.

RESULTS AND DISCUSSION

The disease caused by an important external monogenean parasite, *Dactylogyrus*, accounts for significant damage to fish populations. In June 2013, a large number of goldfish heavily infected with *Dactylogyrus* were found in a fish farm in Nanyang, Henan province, and almost 70% of fish died within less than 1 mo. Based on haptor morphological observation under light and SEM, we identified 2 species of *Dactylogyrus*, viz. *D. intermedius* and *D. vastator* (see Fig. S1 in the Supplement; www.int-res.com/articles/d111p177_supp.pdf). To our knowledge, few reports have provided descriptions of the attachment apparatus in these 2 monogeneans in goldfish. In addition, we noted that the ratio of the number of *D. intermedius* to that of *D. vastator* infecting a fish was 3.3.

At present, many chemicals are no longer recommended to treat monogenean infections because of their side effects; therefore, the search for an effective drug against the disease has become urgent. In our previous studies, the methanol extract of radix bupleuri was found to be the strongest anthelmintic effective against *Dactylogyrus* infection (Wu et al. 2011), suggesting one or several active constituent(s) in this extract. The present study was designed to determine anthelmintic activity of the main constituents of radix bupleuri against *Dactylogyrus* in goldfish.

First, this study was performed to evaluate the anthelmintic effect of TS against *Dactylogyrus* infection. The results showed that TS exhibited strong anthelmintic activity (EC_{50} = 2.01 mg l^{-1}; Table 1, Fig. 2). The analysis of HPLC for TS demonstrated that SSa, SSc, and SSd were its main constituents, comprising 29.76, 5.35, and 35.47% of TS, respectively (Fig. S2 in the Supplement). Following this, we determined the anthelmintic activity and toxicity to goldfish of 3 saikosaponins. The results showed that SSd was the most effective for eliminating *Dactylogyrus* infection, with EC_{50} of 0.74 mg l^{-1} (Table 1, Fig. 2); however, an aqueous static renewal bioassay demonstrated that SSd was also the most toxic to goldfish, with 48 h LC_{50} of 1.54 mg l^{-1} (Table 2). SSa was safer for goldfish (EC_{50} = 11.20 mg l^{-1}) than SSd, although the anthelmintic activity of SSa (EC_{50} = 1.46 mg l^{-1}) was slightly lower than that of SSd (Tables 1 & 2). In order to identify which saikosaponin was a suitable drug candidate, the TI was used to characterize the balance between safety and efficacy. TI, as an important parameter, is the quantitative relationship between efficacy (pharmacology) and safety (toxicology) that can be calculated using various pairs of pharmacological and toxicological end points (Muller & Milton 2012). In this study, TI was represented as a ratio of LC_{50} to EC_{50}. The TIs for the

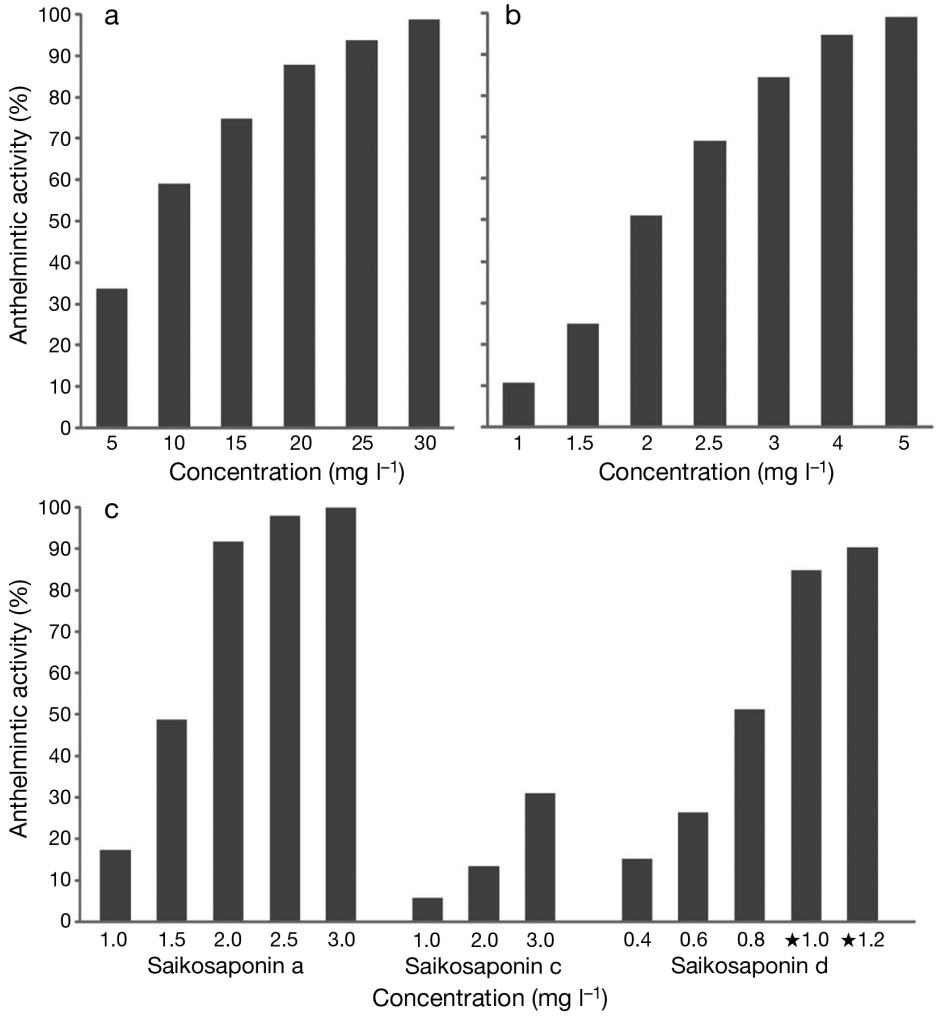

Fig. 2. Anthelmintic efficacy of (a) methanol extract, (b) total saponin, and (c) saikosaponins a, c, and d from the dried root of *Bupleurum* sp. (i.e. radix bupleuri) against *Dactylogyrus* spp. infection in goldfish *Carassius auratus* after 48 h of exposure. Mean infection intensity: 94 dactylogyrids per fish; star indicates that the constituent caused fish mortality

methanol extract, total saponin, and saikosaponins a and d were 4.42, 4.47, 7.67, and 2.12, respectively. Based on the data, SSa has the potential to be used commercially because of its higher TI.

As shown in Fig. 1, the chemical structure of SSa was very similar to that of SSd, with only 1 difference in bond angle. We assume that the difference in chemical structure induced a significant difference in anthelmintic activities and toxicities to goldfish. This interesting phenomenon was also noted by Chiang et al. (2003), who reported that SSd possessed stronger cytotoxicity against human hepatocellular carcinoma cells than SSa did. An earlier study (Motoo & Sawabu 1994) showed that the concentration of SSd required for 50% inhibition of cell growth of PLC/PRF/5 cells (human hepatoma cell lines) was lower than that of SSa. However, the reason for this phenomenon remains unclear and needs to be investigated.

In our anthelmintic assays, the effect of the methanol extract of radix bupleuri against *Dactylogyrus* infection was evaluated. Table 1 shows that EC_{50} of the extract was 8.48 mg l^{-1}, which differs from the value calculated in our previous study (EC_{50} = 3.50 mg l^{-1}; Wu et al. 2011). We assume that the goldfish were infected with different mean numbers of dactylogyrids, which resulted in the different values of EC_{50}. Conversely, LC_{50} of this extract against goldfish was 37.54 mg l^{-1}, which was close to the value calculated in the previous study (LC_{50} = 35.20 mg l^{-1}; Wu et al. 2011), because the same number of fish were used for the acute toxicity in these 2 experiments. Therefore, we suggest that a parameter represented as a ratio of EC_{50} to the mean number of parasites infecting a fish be used as a uniform criterion. According to this criterion, EC_{50} per parasite in this study was calculated to be 0.090, which is very close to that calculated in the previous study (0.088).

In summary, our results demonstrated that among the 3 main constituents in TS of radix bupleuri, SSa was more suitable as a potential therapeutic agent to treat *Dactylogyrus* spp. infection in goldfish because of its high TI (7.67).

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