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Short communication

Systematic screening identifies synergistic combinations of traditional Chinese medicines and ingredients against *Dactylogyrus* infections using a goldfish model

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ABSTRACT

Combinatorial therapy is a promising strategy for infectious diseases because it may result in improved efficacy and reduced side effects. No synergistic drug combinations, however, have yet been identified for control of fish infectious diseases based on systematic screening. In this study, 10 kinds of traditional Chinese medicines (TCMs) demonstrating a strong anthelmintic effect against *Dactylogyrus* were selected for systematic screening, and a total of 45 pairs of TCMs extracts were examined. Eleven synergistic anthelmintic pairs were identified, and 7 of which contained rhizome of peltate yam (RPY). Furthermore, the synergy assessment of anthelmintic compounds from the TCMs demonstrated that gracillin (GR)—the main active compound of RPY—has the highest synergy rate (71.4%). The results suggested that RPY can be referred as a promiscuous synergizer which non-specifically increases the effects of many other TCMs. The discovery of promiscuous synergizer can accelerate the search for synergistic combinations. Besides, this work also provides a feasible means to prioritize combination tests via measuring the background ratio of different interaction types.

1. Introduction

Dactylogyrus spp. are common ectoparasites of the gills of fish, and some of them can result in serious damage in aquaculture (Wootten, 1989). Heavy infection with certain species of *Dactylogyrus* can cause epithelial proliferation, excessive mucus secretions and accelerated respiration, and then high mortality because of severe respiratory distress (Steverding et al., 2005, Reed et al., 2009). To date, many chemicals have been evaluated for anthelmintic efficacy against *Dactylogyrus*, including formalin (Klinger and Floyd, 2013), praziquantel (Stetter et al., 2003), and mebendazole (Treves-Brown, 1999); however, a majority of them are restricted in many countries due to the development of pesticide resistance and concerns of drug residues and environmental contamination (Reverter et al., 2014). Therefore, therapeutic agents with novel modes of action are urgently needed to control *Dactylogyrus* infection.

The use of therapeutic combinations with different mechanisms or modes of action has special potential for many infectious diseases (Greco et al., 1995; Chou, 2006; Cokol et al., 2011). Major benefits of drug combinations can include a substantially-reduced chance of evolving drug resistance, improved efficacy and reduced side effects (Fitzgerald et al., 2006; Tan et al., 2012). Until now, however, only few reports are available on drug combinations used to control fish diseases, and furthermore, there is no approach developed for screening synergistic drug combinations in the treatment of fish diseases, especially fish parasitic diseases.

Currently, there is more and more interest worldwide in the use of traditional Chinese medicines (TCMs) for the prevention and treatment of various diseases including fish parasitic diseases (Reverter et al., 2014). Besides, more importantly, TCM is characterized by the use of herbal formulae (*Fu-Fang*), which are usually consisted of two or more medicinal herbs and capable of systematically treating various diseases via potentially synergistic herb interaction (Williamson, 2001; Li et al., 2010, 2011). Recently, Jiang et al. (2014) have confirmed that both the extract combinations and the active compound combinations between rhizome of peltate yam and folium ginkgo were effective against *Dactylogyrus* infections, indicating the use of botantical combinations as a possible means of controlling fish parasitic disease. In the previous study, however, systematic screening of combinations was not used to explore the possible synergistic interaction between anthelmintic extracts or compounds. In this study, a systematic screening of binary combinations was performed to determine the synergistic interaction between the 10 TCMs. In addition, active compounds from the TCMs were used to verify the potential synergistic effects.

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2. Materials and methods

2.1. Fish and parasites

A batch of goldfish (*Carassius auratus*) ($n = 20{,}000$), with body weight of 3.5 ± 0.5 g, was purchased from Nanyang, Henan Province, China and transported by road (6 h) in several foam tanks (18.0–20.0 °C and more than 90% oxygen saturation) to the Aquatic Animal Diseases Lab of Northwest A&F University. Prior to the next experiment, 30 fish were selected randomly to examine for the presence of parasites, and no parasites were found on gills of the fish. After acclimatization in several aquaria (water temperature 20.0–24.0 °C, pH 7.0–7.5, dissolved oxygen 6.0–7.2 mg L^{-1}) for 10 days, the remaining fish were cohabitated with the ones infected with *Dactylogyrus intermedius* (healthy fish / infected fish = 4:1), which has been maintained by serial transmissions on goldfish since 2008 using the method described by Wang et al. (2008). After that, ten fish were randomly selected and examined for the presence and intensity of *D. intermedius* on the gills every four days. The fish were killed by spinal severance for biopsy, and the lamella branchialis were placed on glass slides. The number of parasites was counted under a light microscope ×40. The fish were used for the next assay when the infection rate reached 100% and the mean number of the parasites on gills was 30–40 per fish (35.66 ± 2.77, $n = 50$).

2.2. Preparation of plant extracts and compounds

A total of ten kinds of TCMs (Table 1) were purchased from a Chinese medicine shop in Yangling, Shaanxi, China, and they were identified by Prof. X.P. Song (Northwest A&F University, Shaanxi, China). The voucher specimens (No. 2014001–No. 2014010) were deposited in Aquatic Animal Diseases Lab, Northwest A&F University. The remaining samples were oven-dried at 45 °C for 48 h, and then crushed to pass through a sieve (0.42–0.60 mm) to get into fine powder using a commercial electric stainless steel blender. The powder (100.0 g) of each TCM was extracted with 1 L ethanol in a water bath at 65–70 °C for three times (each for 2 h), and then dried to form solid crude extracts following a method described by Wang et al. (2010a). The active compounds of these TCMs (Table 1) were purchased from Sigma-Aldrich Co., LLC. The extracts and the compounds were dissolved in dimethyl sulfoxide (DMSO) or methanol to obtain stock solutions (20 mg mL^{-1}), respectively, and then stored at 4 °C before use.

2.3. Anthelmintic activity assay of the extracts

An anthelmintic activity assay against *D. intermedius* was adapted from the method described by Wang et al. (2010a). For each extract, five infected goldfish were transferred into each 5-L plastic pot containing 2 L of filtered aquarium water. After acclimatization for 48 h (water conditions were the same as previously mentioned), the fish were exposed to a series of concentrations of the extracts, respectively. The control group without any extracts but DMSO or methanol was set up under the same conditions in order to discard the possible effects of DMSO or methanol on the parasites. All tests were performed in triplicate. After 48 h exposure, the mean 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 Eq. (1). EC_{50} value of each extract with 95% confidence intervals CIn was calculated using the probit procedure of SPSS 18.0.

$$\mathrm{AE}(\%) = (\mathrm{B}-\mathrm{T})/\mathrm{B} \times 100\% \tag{1}$$

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

2.4. Synergy and antagonism assessment

The Loewe additivity model was used to examine the interactions between the ten TCMs. First, the anthelmintic effects of 10 extracts from the TCMs in binary-combination ($n = 45$) were determined. For each extract–extract interaction assay, eight concentrations of two extracts were combined in a two dimensional grid ($8 \times 8 = 64$ concentration combinations), where the concentration of each extract was nonlinearly increased along each axis (0, 1, 2.5, 3, 4, 5, 10 and 15 mg L^{-1}). Subsequently, "isophenotypic" curves describing extract concentration combinations on anthelmintic efficacies were performed using a method of Cokol et al. (2011). Briefly, the grid points were imputed using Akima interpolation (Akima, 1970), and the contours were plotted. The grids were then rescaled from 0 (low anthelmintic efficacy) to 1 (highest anthelmintic efficacy) and the longest isophenotypic curve described by the greatest number of data points was plotted for assessment. According to Loewe additivity, isophenotypic curves were expected to be parallel to the diagonal for independent drug pairs, concave for synergistic drug pairs, and convex for antagonistic drug pairs (Cokol et al., 2011).

Similarly, an experiment was conducted to assess synergy for each of three anti-parasitic compounds (cinnamaldehyde, osthole and gracillin) using the above method. The combination index (CIn) values were calculated from the optimal combination at 50% effective concentration with the lowest total concentration.

CIn values can be calculated for drugs using the following Eq. (2) (Chou, 2006):

$$\mathrm{CIn} = (\mathrm{D})_1/(\mathrm{Dx})_1 + (\mathrm{D})_2/(\mathrm{Dx})_2 \tag{2}$$

where $(\mathrm{Dx})_1$ is the dose of Drug 1 alone that inhibits x%, $(\mathrm{Dx})_2$ is the dose of Drug 2 alone that inhibits x%, $(\mathrm{D})_1$ is the dose of Drug 1 in the combination that inhibits x%, and $(\mathrm{D})_2$ is the dose of Drug 2 in the combination that inhibits x%. CIn values <1 indicates synergism; >1 indicates antagonism; $=1$ indicates additive effect. To be somewhat conservative, CIn values <0.8 and >1.2 were chosen as indicative of synergy and as indicative of antagonism, respectively.

Table 1

List of all traditional Chinese medicines used in this study, their abbreviations (Ab.), plant species, anthelmintic compounds, median effective concentrations (EC_{50}s) and references.

Traditional Chinese medicines	Ab.	Plant species	Anthelmintic compound(s)	Ab. of compound(s)	EC_{50} of compound(s)	Reference
Pink plumepoppy	PP	*Macleaya microcarpa* Fedde	Sanguinarine	SA	0.37 (0.30–0.41)	Wang et al., 2010a
Rhizome of peltate yam	RPY	*Dioscorea zingiberensis* C.H. Wright	Gracillin	GR	0.43 (0.35–0.62)	Wang et al., 2010b
Fructus bruceae	FB	*Brucea javanica* Merr.	Bruceine A	BA	0.49 (0.43–0.57)	Wang et al., 2011
Cortex cinnamomi	CC	*Cinnamomum cassia* Presl	Cinnamaldehyde	CA	0.57 (0.47–0.66)	Ling et al., 2015
Caulis spatholobi	CS	*Spatholobus suberectus* Dunn	Ononin	ON	0.62 (0.44–0.67)	Han, 2011
Folium ginkgo	FG	*Ginkgo biloba* L.	Ginkgolic acids[a]	GAs	0.76 (0.59–0.82)	Wang et al., 2006
Fructus cnidii	FC	*Cnidium monnieri* Cuss.	Osthole	OS	0.81 (0.73–0.98)	Wang et al., 2008
Radix bupleuri	RB	*Bupleurum chinense* DC.	Saikosaponin D	SD	1.40 (1.29–1.56)	Zhu et al., 2014
Cortex pseudolaricis	CP	*Pseudolarix kaempferi* Gord.	Epicatechin	EP	4.22 (3.90–4.61)	Ji, 2013
Rhizoma dryopteris crassirhizomatis	RDC	*Dryopteris crassirhizoma* Nakai	Kaempferitrin	KA	2.71 (2.61–2.90)	Jiang et al., 2013

[a] Ginkgolic acids are composed of five different 6-alkylsalicylic acids with alkyl (C13:0, C15:0, C15:1, C17:1, C17:2).