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Efficacy of a therapeutic bath with selected antiparasitic drugs on a *Dactylogyrus anchoratus* infection in juvenile common carp (*Cyprinus carpio*)

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Abstract: The study aimed to assess the effects of a therapeutic bath of five different antiparasitic drugs, in different baths and durations: fenbendazole (25 mg l^{-1}, 12 h and 2 × 12 h), formaldehyde (0.17 ml l^{-1}, 15 min), ivermectin (0.031 mg l^{-1}, 1 h), mebendazole (1 mg l^{-1}, 12 h) and levamisole (50 mg l^{-1}, 2 h and 3 × 1 h) on the reduction on the intensity and prevalence of a monogenean infection (*Dactylogyrus anchoratus*) in juvenile carp. The best effect on reducing the parasite number was achieved with the bath in formaldehyde (0.17 ml l^{-1}, 15 min) and fenbendazole (25 mg l^{-1}, 2 × 12 h with 24 h break), where the infection was reduced by more than 90%. Registered veterinary medicinal products (VMPs) with the active substance of fenbendazole can successfully replace the use of unregistered formaldehyde in the treatment of monogenean infections.

Keywords: fenbendazole; formaldehyde; ivermectin; levamisole; mebendazole

The prosperity of intensive aquaculture directly depends on the health conditions of the fish. Prevention and treatment in aquaculture preceded by a professional health examination are a necessary part of the technological proceedings. A frequent diagnosis in intensive carp farming includes parasite infections. Large losses in the breeding, especially of juvenile carp, are often caused by infections with flatworms of the class Monogenea, *Dactylogyrus* sp. and *Gyrodactylus* sp. (Stoskopf 1993; Svobodova et al. 2007; Noga 2010; Palikova et al. 2019). The infection can cause great damage to the fish's gills and skin which causes breathing problems and secondary infections. Resolving such a situation requires medical intervention. When choosing any treatment for animals intended for human consumption, the veterinarian must follow the applicable laws and directives (in the Czech Republic by Act No. 378/2007 Coll. on Medicinal products and Act No. 166/1999 Coll. on Veterinary care; in the EU by Directive 2001/82/EC of the European Parliament and the Council on the Community code relating to veterinary medicinal products). For fish intended for human consumption, only veterinary medicinal products (VMPs) whose pharmacologically active substances are classified in terms of maximum residue limits (MRLs) can be used for treatment throughout the EU (Decree No. 470/2009). Veterinarians may use a drug "off-label" on their own responsibility, in accordance

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with Decree No. 344/2008 Coll. on the Use, transcription and issue of veterinary medicinal products. Then, it is possible to choose, for the treatment, a VMP intended for another animal species or another indication or a human medicinal product, as appropriate medicine prepared in the pharmacy according to the prescription "magistraliter". When using the "off-label" drug, it is necessary to meet the specific withdrawal period of 500 daily degrees.

The effective therapy of a Monogenean infection in Czech aquaculture breeding can be undertaken only with the "off-label" procedure. There is no registered antiparasitic VMP for use in fish in the Czech Republic. When selecting drugs for our testing, VMPs registered for animals intended for human consumption other than fish available on the market in the Czech Republic were chosen. Another criterion for the choice of drug was the possibility of its use in the form of a therapeutic bath. Often, sick fish do not eat and a therapeutic bath is an effective treatment that will affect all fish equally. For the treatment of parasitic worm infections, various active antiparasitic substances, such as formaldehyde, fenbendazole, ivermectin, mebendazole, levamisole and others, are recommended (Svobodova et al. 2007; Noga 2010; Kolarova et al. 2015; Alves et al. 2019; Palikova et al. 2019). VMPs with active substances of fenbendazole, ivermectin, mebendazole and levamisole are registered for other animals intended for human consumption and are available on the market in the Czech Republic. Formaldehyde is used as disinfectant, but if it is used as a medicine, it is also subject to the rule of compliance with a withdrawal period of at least 500 daily degrees.

Fenbendazole (FBZ) is a benzimidazole commonly used in mammals intended for human consumption (cattle, sheep, goats, pigs) with a wide anthelmint effect. There are several registered VMPs with FBZ as an active substance. For the use of FBZ in fish, it is necessary to choose the "off-label" approach and observe a minimum protection period of 500 daily degrees. In fish, the oral administration of FBZ is recommended to reduce nematode infections (Stoskopf 1993; Noga 2010; Palikova et al. 2019). To reduce monogenean infections in fish, an FBZ bath treatment at a dose of 25 mg l^{-1} for 12 h is also recommended (Noga 2010; Kolarova et al. 2015; Kolarova et al. 2017; Palikova et al. 2019).

Formaldehyde (FAD) (used as a 36–38% aqueous solution of formaldehyde = formalin, CH_2O) is a disinfectant substance, which does not fall into the category of VMPs. Formalin is an irritant and sensitising substance, belonging to carcinogen group 1. In the case of formalin, it is not necessary to determine the MRL, at this time, because formalin is listed in the List of substances for which it is not necessary to determine the MRLs [Table 1 of Annex Commission Regulation (EU) No. 37/2010]. FAD may be applied "off-label" to animals intended for human consumption. When using formalin for treatment in fish, a withdrawal period of at least 500 daily degrees must be observed. Formalin is effective in fish in the form of a short-term or long-term therapeutic bath for ectoparasitic infections of protozoa, monogenea, digenea, and crustaceans. The recommended dose for the short-term therapeutic bath (30–60 min) is in the range of 0.17–0.25 ml l^{-1}, depending on the water temperature. For the long-term therapeutic bath (unlimited in time), a dose of 0.017–0.025 ml l^{-1} is recommended (Stoskopf 1993; Noga 2010; Kolarova et al. 2015; Kolarova et al. 2017; Palikova et al. 2019). FAD was chosen as a substance that is widely used in aquaculture that has a common proven effect on monogenean infections.

Ivermectin (IVM) is an avermectin commonly used in mammals intended for human consumption (cattle, sheep, pigs, and horses) with a wide antiparasitic effect. IVM is an active substance of many registered VMPs used as a treatment of ecto- and endoparasitic worm infections. For the use of IVM in fish, the "off-label" approach should be chosen and a minimum protection period of 500 daily degrees should be observed. IVM is a potent anti-parasitic agent which is used in the aquaculture industry in some countries. For treatment of monogenean infections in fish, a 12 h bath at a concentration of 0.031 mg l^{-1} is recommended (Santamarina et al. 1991; Pike and Wadsworth 2000; Kolarova et al. 2015; Palikova et al. 2019). A significant disadvantage of IVM is its environmental toxicity, being distributed rapidly and widely throughout ecosystems, bio-accumulated at high levels, and persisting as a long-term residue (Wang et al. 2020). The therapeutic antiparasitic effect of this substance is excellent, but IVM is dangerous for the environment.

Mebendazole (MBZ) is a benzimidazole used in mammals intended for human consumption (deer, fallow deer, moufflon, roebuck, chamois)

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with an anthelmintic effect. MBZ is used in fish for the treatment in a prolonged bath at 1 mg l^{-1} (Noga 2010; Kolarova et al. 2015; Palikova et al. 2019). MBZ is an active substance of a few registered VMPs only. For the use of MBZ in fish, the "off-label" approach should be chosen and a minimum protection period of 500 daily degrees should be observed.

Levamisole hydrochloride (levamisole HC, LHC) is an imidazothiazole used in mammals intended for human consumption (pigs, poultry) with anthelmintic and immunostimulatory effect. LHC is an active substance of a few registered VMPs. For the use of LHC in fish, it is necessary to choose the "off-label" approach and observe a minimum protection period of 500 daily degrees. In fish, a bath treatment at a concentration of 50 mg l^{-1} for 2 h is recommended (Stoskopf 1993; Kolarova et al. 2015; Palikova et al. 2019; Kibenge et al. 2021). Levamisole has also been shown to be a suitable immunostimulant for fish (Mulero et al. 1998; Morrison et al. 2000; Gopalakannan and Arul 2006; Li et al. 2006; Hang et al. 2014; Kowalska et al. 2015; Alves et al. 2019; Biller et al. 2019).

The aim of this study was to verify the effect of four selected antiparasitic agents (FBZ, IVM, LHC, MBZ) in fish in the relevant VMPs available on the EU market as products for animals intended for human consumption other than fish, and which are recommended for treatment of infections with parasitic worms of the class Monogenea. For comparison, the disinfectant substance (FAD), commonly and effectively used in the treatment of monogenean infection in fish, was also included in the test.

MATERIAL AND METHODS

Experimental fish

A total of 96 common carp (*C. carpio*) juveniles with a total length of 106.8 ± 13.00 mm and body weight of 22.5 ± 8.07 g, naturally infected with a pure line of *D. anchoratus* (ectoparasites from the class Monogenea) were used for testing.

The fish came from the breeding facility of the Faculty of Fisheries and Protection of Waters at the University of South Bohemia (Czech Republic). They were kept in laboratory conditions their whole life.

Parasitological examination

The fish were killed by stunning and cutting the spinal cord in accordance with the national and international guidelines for the protection of animal welfare (EU-harmonised Animal Welfare Act of the Czech Republic). A parasitological examination was performed for six carp from each tested group during the experiment. A smear from one half of the carp body as well as a smear from four gill arches of one side were examined by light microscopy. The prevalence and intensity of the *D. anchoratus* infection was mentioned.

Tested antiparasitic substances

The VMP medicinal product containing fenbendazole (FBZ) was Panacur 25% p.o. susp. (25 mg fenbendazole in 1 ml of VMP), produced by Intervet International B.V. (Boxmeer, the Netherlands). The pure formaldehyde 36–38% (FAD) chemical substance was purchased from Penta Chemicals Unlimited (Prague, Czech Republic). The VMP containing ivermectin (IVM) was Bio-mectin inj. (10 mg ivermectin in 1 ml of VMP), produced by Vetoquinol Ltd. (Prague, Czech Republic). The VMP containing mebendazole (MBZ) was Rafendazol plv. (8 mg mebendazole and 10 mg rafoxanidum in 1 g of VMP), produced by BIOPHARM Ltd. (Rajec Jestrebi, Czech Republic). The VMP containing levamisole hydrochloride (LHC) was Levamisole hydrochloride KELA 80% mg g^{-1} plv. (800 mg of LHC in 1 g of product, that is equivalent to 678 mg of levamisole in 1 g of product; with the additives: colloidal anhydrous silica and lactose monohydrate) was purchased from KELA Laboratoria n.v. (Hoogstraten, Belgium).

Test conditions

The fish of each group were held in 300 l glass aquaria. Tap water was used for the control groups and for the preparation of the antiparasitic baths. The physical and chemical water quality parameters were monitored in all the tested groups during the experiment: temperature of 21.5 ± 1.5 °C, acid neutralisation capacity, $ANC_{4.5}$, 1.12 mmol l^{-1}; total ammonia, 0.01 mg l^{-1}; NO_3^-, 5.11 mg l^{-1}; NO_2^-, 0.006 mg l^{-1}; PO_4^3, 0.03 mg l^{-1}; chemical oxygen demand, COD_{Mn} 0.84 mg l^{-1}; oxygen saturation 95–99%; pH 7.7–7.9.

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Experimental design

In this study, the effects of five different antiparasitic substances with different bath durations were compared. An examination of the carp was performed to determine the prevalence and intensity of the *D. anchoratus* infection on the gills. The carp of the tested groups were examined immediately after bathing and at various time intervals after bathing, always in a number of six fish from each group (Table 1). The bathing time schedule was prepared so that each test group could be compared with the control group at the appropriate time. Therefore, a total of five control groups (C1–C5) were used. The experimental design is given in detail in Table 1.

Ethics statement

All the experimental manipulations complied with the valid legislative regulations in the Czech Republic (Law No. 166/1996 and No. 246/1992); the permit was issued to No. 68668/2020-MZE-18134.

Statistical analyses

The results of the infection intensity are presented as the mean ± standard deviation of a number of individuals of *D. anchoratus* from the gill arches from the left side of the carp. The reduction in the infection intensity is presented by %, compared to the appropriate control. The prevalence is presented in %. The intensity of the infection was analysed using Statistica v12.0 for Windows (StatSoft, Inc., Czech Republic). The *D. anchoratus* counts did not meet the criteria required for parametric analysis. Therefore, a Kruskal-Wallis (KW) non-parametric analysis of variance (ANOVA) was used to examine the effects of the therapeutic bath with the selected antiparasitic drugs on the number of parasites in the fish. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

There was no infection in the skin of the control and experimental fish. During the examination of the fish, only gill infections of *D. anchoratus* were diagnosed.

Using two bath treatment methods in FBZ (FBZ1 and FBZ3), a statistically significantly higher reduction of *D. anchoratus* parasites was demonstrated compared to the respective control. The FBZ1 bath also caused a statistically significantly higher reduction of the parasites during the control 24 h after the end of the bath (FBZ2). A higher reduction of parasites, statistically significant in comparison with FBZ1 and FBZ2, was recorded after repeated bathing FBZ 12-hour bath with a break of 24 h (FBZ3) (Figure 1A, Table 2).

A therapeutic bath in IVM (IVM1) and examination after 3 days after the IVM1 bath (= IVM2) demonstrated a statistically significant reduction ($P < 0.05$)

Table 1. Experimental design

Tested substance	Code in test	Concentration	Bath duration	Examination time	Appropriate control
Fenbendazole	FBZ1	25 mg l^{-1}	12 h	immediately after bath	C2
Fenbendazole	FBZ2	25 mg l^{-1}	12 h	24 h after bath	C3
Fenbendazole	FBZ3	25 mg l^{-1}	2 × 12 h with 24 h break	immediately after the last bath	C4
Formaldehyde	FAD	0.17 ml l^{-1}	30 min	immediately after bath	C4
Ivermectin	IVM1	0.031 mg l^{-1}	1 h	immediately after bath	C1
Ivermectin	IVM2	0.031 mg l^{-1}	1 h	36 h after bath	C5
Mebendazole	MBZ1	1 mg l^{-1}	12 h	immediately after bath	C2
Mebendazole	MBZ2	1 mg l^{-1}	12 h	1 day after bath	C3
Levamisole HC	LHC1	50 mg l^{-1}	2 h	immediately after bath	C1
Levamisole HC	LHC2	50 mg l^{-1}	2 h	36 h after bath	C5
Levamisole HC	LHC3	50 mg l^{-1}	3 × 1 h once in day	immediately after last bath	C5

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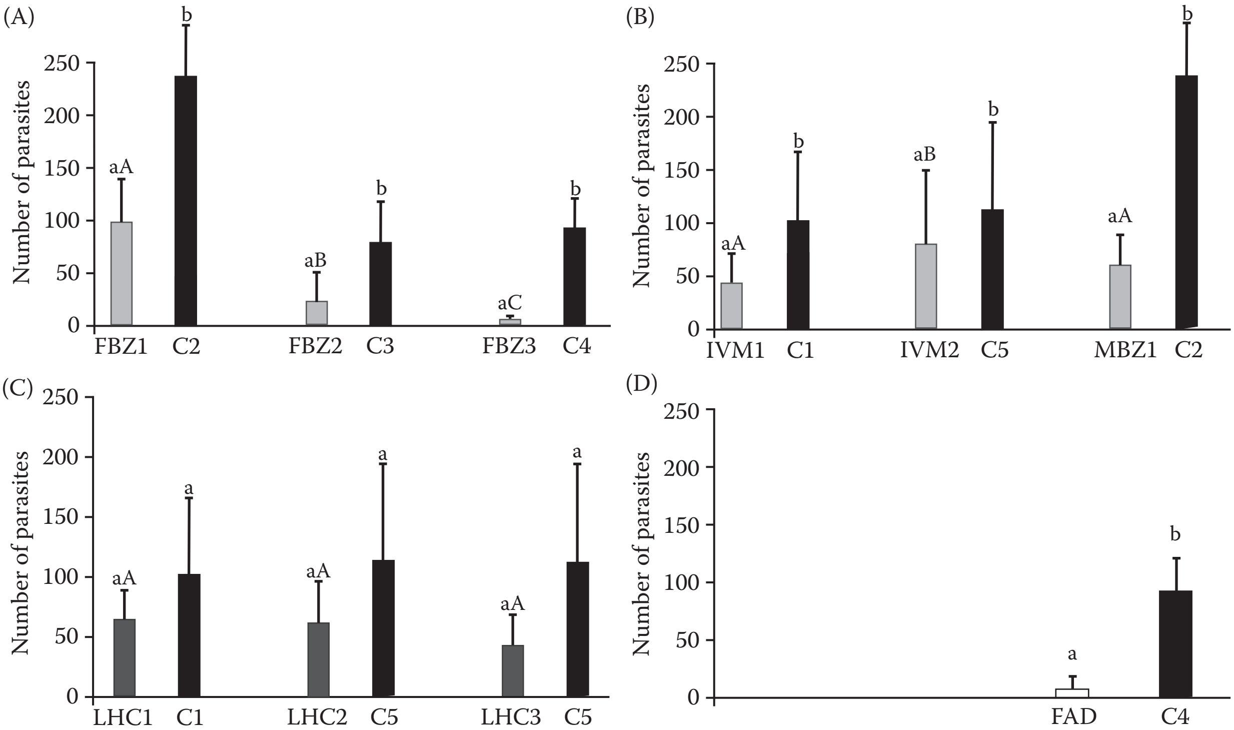

Figure 1. Mean number of *D. anchoratus* in the juvenile carp (*C. carpio*) in the gills
(A) After the fenbendazole (FBZ), (B) ivermectin (IVM) and mebendazole (MBZ); (C) levamisole hydrochloride (LHC); and (D) formaldehyde (FAD) bath treatments compared to the appropriate control (see Table 1). Values with different lowercase letters are significantly different ($P < 0.05$) among the experimental group and control at the given sampling time. Values with different uppercase letters are significantly different ($P < 0.05$) among the experimental groups

Table 2. Number of *D. anchoratus* in the juvenile carp (*C. carpio*) in the gills (mean intensity ± standard deviation), reduction in the intensity compared to the appropriate control (%) and infection prevalence (%)

Code of substance	Number of *D. anchoratus*	Infection reduced (by %)*	Prevalence (%)
C1	102.2 ± 63.4	0.0	100.0
C2	238.0 ± 185.4	0.0	100.0
C3	81.0 ± 39.6	0.0	100.0
C4	93.8 ± 27.8	0.0	100.0
C5	112.2 ± 81.6	0.0	100.0
FBZ1	99.20 ± 40.55	58.3	100.0
FBZ2	23.70 ± 27.10	70.9	83.3
FBZ3	6.80 ± 4.36	92.7	100.0
FAD	6.50 ± 11.62	93.1	66.7
IVM1	43.80 ± 25.19	57.1	100.0
IVM2	79.80 ± 69.36	28.9	100.0
MBZ1	60.80 ± 27.68	74.5	100.0
LHC1	64.00 ± 25.57	37.4	100.0
LHC2	60.80 ± 36.22	45.8	100.0
LHC3	42.8 ± 26.11	61.9	100.0

*Infection reduction in comparison to the appropriate control

C1–5 = control; FAD = formaldehyde; FBZ = fenbendazole; IVM = ivermectin; LHC = levamisole HC; MBZ = mebendazole

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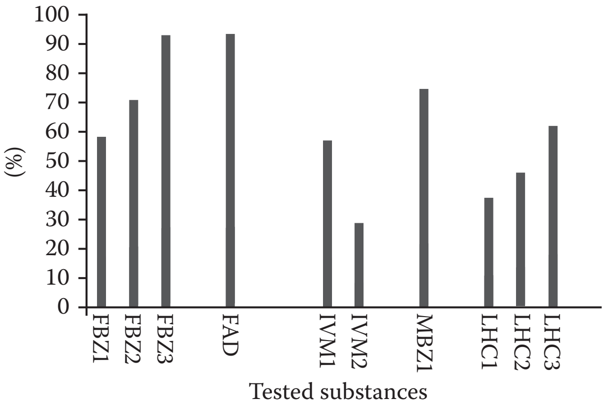

Figure 2. Reduction in the infection by %, compared to the appropriate reduction in the infection in the control (C = 0%)

For the tested substances and bath design, see Table 1

of the parasite intensity in the treated fish compared to the appropriate controls (Figure 1B, Table 2).

The MBZ bath (1 mg l^{-1} for 12 h) reduced the intensity of *D. anchoratus* in carp by 74.5% however, no statistically significant difference was found, when compared to the respective control (Figure 1B, Table 2). The antiparasitic effect 24 h after the end of the 12 h mebendazole bath (MBZ2) could not be correctly evaluated because most of the fish died.

Baths in LHC (1–3) did not show any statistically significant differences between themselves or compared to the respective controls (C1 and C5) (Figure 1C, Table 2).

There was a statistically significant reduction in the infection intensity in the FAD bath compared to the control (Figure 1D, Table 2).

The best effect in reducing the parasite intensity was achieved with the FAD bath (0.17 ml l^{-1}, 15 min) and the FBZ3 bath (25 mg l^{-1}, 2 × 12 h with 24 h break), where the infection was reduced by more than 90% (Table 2, Figure 2). The other tested VMPs did not achieve an efficiency of 80% (Table 2, Figure 2). The lowest prevalence of a parasitic infection was found after the FAD bath (66.7%), followed by the FBZ2 bath (83.3%) (Table 2). The prevalence of the other VMPs remained 100%, as in all the controls.

DISCUSSION

In our testing using FBZ for the treatment of the *D. anchoratus* parasitic infection in carp in the form of a 12-hour bath, a repeated administration at a dose of 25 mg l^{-1} was the most effective compared to the other tested VMPs. Only FAD had a comparable effect, which can only be used in an "off-label" mode (Figure 1D, Table 2). The effectiveness of FBZ in the treatment of monogenea infections has been confirmed by many authors. Smahl and Benini (1998) achieved a good effectivity with FBZ in an intermittent bath treatment (3 × 2 µg ml^{-1} for 6 h at 36-hours intervals) of microsporidian spore (*Glugea anomala*) in sticklebacks (*Gasterosteus aculeatus*). The repeated oral administration of FBZ was successfully used to reduce *Dactylogyrus* sp. infections on the gills of rohu (*Labeo rohita*) (Gupta et al. 2020). Our previous testing proved the safety of FBZ used as a bath for fish (Kolarova et al. 2022).

The experience with the antiparasitic effect of MBZ on monogenean infections in fish is not convincing. Buchmann and Bresciani (1994) studied the *in vitro* effect of MBZ (1 and 10 mg l^{-1} for up to 42 h) on the gill parasitic monogenean *Pseudodactylogyrus bini* and demonstrated that MBZ at 10 mg l^{-1} for 36 h disorganised the internal structure of the parasite. Goven and Amend (1982) reported a good therapeutic effect of an MBZ bath (2 mg l^{-1}) on a monogenean infection caused by *Gyrodactylus* sp., but not on *Dactylogyrus* sp. Katharios et al. (2006) demonstrated no significant effect of an MBZ bath (400 mg l^{-1} for 1 h) in the control of the monogenean *Microcotyle* sp. in the gills of cultured red porgy (*Pagrus pagrus).* Alves et al. (2019) tested the efficacy of an MBZ bath (125, 150, 175 and 200 mg l^{-1}) on monogenean infections in tambaqui (*Colossoma macropomum*) without any therapeutic effect. In our testing, the MBZ bath (1 mg l^{-1} for 12 h) reduced the number of *D. anchoratus* in carp by 74.5% however, but without a statistically significant difference from the respective control. Due to the death of most fish treated in this way within 24 h after the end of the bath, MBZ cannot be classified as a promising antiparasitic.

In vitro tests with 200 mg l^{-1} of IVM were 100% effective against monogenean parasites (*Anacanthorus spatulatus*, *Notozothecium janauachensis*, *Mymarothecium boegeri* and *Linguadactyloides brinkmanni*), but the same concentration caused *in vivo* lethargy, signs of hypoxia and 100% mortality within 2 h in tambaqui (Alves et al. 2019). Santamarina et al. (1991) reported that an IVM concentration of 0.031 mg l^{-1} was effective against 6 to 8 speci-

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mens of *Gyrodactylus* sp., but it was proven to be highly toxic for rainbow trout, *Oncorhynchus mykiss*, which caused fish death within minutes. Reducing the concentration to 0.025 mg l^{-1} avoided any signs of acute toxicity, but also decreased the efficacy.

All the monogenean parasites (6 to 8 specimens of *Gyrodactylus* sp.) died in the *in vitro* test with 100 mg l^{-1} of LHC, however, the *in vivo* test with rainbow trout infected by *Gyrodactylus* sp. was not effective (Santamarina et al. 1991). Alves et al. (2019) indicated that LHC (50, 75, 100 and 125 mg l^{-1}) was 100% effective against monogenean parasites (*A. spatulatus*, *N. janauachensis*, *M. boegeri* and *L. brinkmanni*) in *in vitro* assays and 88.2% effective in *in vivo* tests on tambaqui fingerlings naturally parasitised by monogeneans and bathed in LHC (125 mg l^{-1}, 24 h).

The FAD bath was, according to our expectations, the most effective compared to the other four tested antiparasitics. Many authors reported a successful and relatively safe FAD treatment for antiparasitic infections: Fajer-Avila et al. (2003), in the case of a monogenean infection in bullseye pufferfish (*Sphoeroides annalatus*); Pahor-Filho et al. (2017) in the case of a polyparasitic infection in marine and estuarine fish in southern Brazil. Zhou et al. (2021) tested the safety and antiparasitic efficacy against monogenean *Gyrodactylus kobayashii* in goldfish (*Carassius auratus*) with 35 natural saponins compared with the efficacy and safety of formaldehyde. Only seven substances (extract of *Dioscorea collettii*, var. *hypoglauca*, *D. spongiosa*, *D. bulbifera*, *Cyanchum otophyllum*, *Smilax china*, *Solanum nigrum* and *Tupistra chinensis*) showed a better result than formaldehyde.

In conclusion, we found FBZ to be a promising antiparasitic for the treatment of monogenean infections in fish which is available for veterinary practice as a VMP. FBZ has shown to have a similar antiparasitic effect as FAD and its use appears to be safe for fish (Kolarova et al. 2022). Registered veterinary medicinal products (VMPs) with the active substance of fenbendazole can successfully replace the use of unregistered formaldehyde in the treatment of parasitic diagnoses.

Conflict of interest

The authors declare no conflict of interest.

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