IN VITRO AND *IN VIVO* STUDIES OF THE ANTIPARASITIC EFFECT OF ASPIRIN AGAINST *DACTYLOGYRUS EXTENSUS* (MONOGENEA) INVASION IN CARP (*CYPRINUS CARPIO*)

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ABSTRACT

Aspirin is one of the most widely used medications globally as an analgesic and antipyretic drug. Aspirin use as an antiparasitic against fish parasites has never been tested. The parasite *Dactylogyrus* is considered a serious threat to freshwater aquaculture in relation to considerable losses. The present study is aimed to evaluate the antiparasitic effect of aspirin against *Dactylogyrus extensus* on *Cyprinus carpio* and alteration in gill histopathology. *In vitro*, aspirin exhibited antiparasitic activity with median effective concentration (EC50) values at one and six minutes of 8.137 g/L^{-1} and 1.629 g/L^{-1}, were assessed for the first time. *In vivo* antiparasitic efficacy of aspirin to *D. extensus* was found to be 46.49%. Severe lesion profile was observed in histopathological evaluations of the gills.

ZUSAMMENFASSUNG: *In-vitro*- und *in-vivo*-Studien zur antiparasitären Wirkung von Aspirin gegen die Invasion von *Dactylogyrus extensus* (Monogenea) bei Karpfen, (*Cyprinus carpio*).

Aspirin ist eines der weltweit am häufigsten verwendeten Medikamente als Analgetikum und Antipyretikum. Die Verwendung von Aspirin als Antiparasitikum gegen Fischparasiten wurde nie getestet. Der Parasit *Dactylogyrus* gilt aufgrund erheblicher Verluste als ernsthafte Bedrohung für die Süßwasseraquakultur. Die vorliegende Studie zielt darauf ab, die antiparasitäre Wirkung von Aspirin gegen *Dactylogyrus* extensus auf *Cyprinus carpio* und Veränderungen in der Kiemenhistopathologie zu bewerten. In vitro zeigte Aspirin antiparasitäre Aktivität mit medianen Effektivkonzentrationswerten (EC50) von eine und sechs minute von 8,137 g/L-1 und 1,629 g/L-1, was zum ersten Mal bewertet wurde. Die antiparasitäre *In-vivo*-Wirksamkeit von Aspirin gegenüber *D. extensus* betrug 46,49%. Bei histopathologischen Auswertungen der Kiemen wurde ein schweres Läsionsprofil beobachtet.

REZUMAT: Studii *in vitro* și *in vivo* ale efectului antiparazitar al aspirinei împotriva invaziei cu *Dactylogyrus extensus* (Monogenea) la crap (*Cyprinus carpio*).

Aspirina este unul dintre cele mai utilizate medicamente de tip analgezic și antipiretic. Utilizarea aspirinei ca antiparazitar împotriva paraziților peștilor nu a fost niciodată testată. Parazitul *Dactylogyrus* este considerat o amenințare pentru acvacultura de apă dulce ducând la pierderi considerabile. Prezentul studiu își propune să evalueze efectul antiparazitar al aspirinei împotriva lui *Dactylogyrus extensus* de la *Cyprinus carpio* și alterarea histopatologiei branhiale. *In vitro*, aspirina a prezentat activitate antiparazitară cu valori medii ale concentrației efective (EC50) în un și şase minute de 8,137 g/L-1 și 1,629 g/L-1, care a fost evaluată pentru prima dată. Eficacitatea antiparazitară *in vivo* a aspirinei la *D. extensus* a fost de 46,49%. Leziuni severe au fost observate în evaluările histopatologice ale branhiilor.

INTRODUCTION

Monogeneans are members of the parasitic flatworms' class, infecting marine and freshwater fish (Reed et al., 2012). Direct life cycle of the monogenean and their rapid reproduction results in an increase in the incidence of severe diseases in fish cultures. Dactylogrid monogeneans are found on the gills of freshwater fish. The pathogenesis of the Dactylogyrid family is directly related to loss of epithelium in the gills due to feeding by parasites (Jalali and Barzegar, 2005; Andrade-Porto et al., 2017; Hu et al., 2017). Histopathological changes such as telangiectasia and edema as well as hyperplasia and fusion of the secondary lamellae were previously described in fish infected by *Dactylogyrus* species (Fujimoto et al., 2014; Santos et al., 2017). Disrupted respiration function due to the damaged gill tissue can lead to the death of fish (Pimentel-Acosta et al., 2019). Additionally, monogeneans may provide the entry point for secondary infections due to their feeding and attachment form, increasing disease outbreaks (Dove and Ernst, 1998; Zhang et al., 2014).

Aquaculture is a recognized fast-growing food production segment in the overall agri-food sector and is characterized by a dynamic performance over the last 30 years. Today aquaculture meets half of the global fish consumption. However, diseases are a serious threat to sustainable aquaculture, leading to serious economic losses. Discernibly, increased diseases can slow progress of aquaculture. Therefore, control of diseases including parasites in aquaculture is of crucial importance for its future. A wide range of chemicals and drugs have been used to control parasites in order to elevate efficiency of fish culture. These compounds have human and environmental safety concerns, resulting in some limitations of their use. In terms of fish, numerous anti-parasitic chemicals, drugs or herbs were evaluated for their effects on fish (Carraschi et al., 2017; de Oliveira et al., 2017; Guimarães et al., 2007). Aspirin containing an active substance of acetylsalicylic acid (ASA) is commonly used as an analgesic, antipyretic and anti-inflammatory drug for medical treatment. Previous studies on aspirin were to analyse its analgesic effects on fish. Lopez-Luna et al. (2017) reported that immersion in aspirin solutions showed an analgesic effect to noxious chemicals in larval zebrafish. Although the analgesic effects of aspirin were tested in fish, the pathological effects of immersion for aspirin are mostly unknown (Diggles et al., 2017). ASA has been studied for its ecotoxicological effects in relation to pharmaceutical drugs in aquatic ecosystems and histological alterations in fish tissues (Bottoni et al., 2010; Nunes et al., 2015). ASA was tested to evaluate the inhibitory effects on acute stress response of tilapia and related hormones (Van Anholt et al., 2003). ASA was found to control mycelial growth of the fungus *Saprolegnia* which frequently occurs in freshwater fish with negative impact on aquaculture (Sundari et al., 2016). To our knowledge, aspirin has never been tested for its antiparasitic capacity in fish. Thus, considering the advantages of aspirin such as ease of availability and low price, it was worth studying its potential as an antiparasitic for fish *in vitro* and *in vivo*.

The aim of the present study was to assess the median effective concentration (EC50) of aspirin for *D. extensus in vitro* and *in vivo* and antiparasitic efficacy of aspirin in carp (*Cyprinus carpio*) infected with *D. extensus* as well as to analyze the alteration of gill architecture in aspirin-treated carp by histopathology.

MATERIAL AND METHODS

Fish and parasites. Fish were used within ethical framework as approved by the ethics committee of the Ankara University with the reference number 2019-7-72. Carps weighing around 60 g were obtained from laboratory stock maintained in the one-loop aquaponics systems producing carp and mint (*Mentha* spp.) at Ankara University, Department of Fisheries and Aquaculture. Fish were kept in aerated water at 20-22°C at a stocking density of 35 kg/m^3 in fiberglass fish tanks. The dissolved oxygen and the pH were around 5.80 mg/L^{-1}

and 6, respectively. Fish were fed with a standard pellet at a daily rate of 2% body weight. Fish were routinely examined for the presence of parasites. The microscopic examination of mucus on the gill filaments with a light microscope showed the parasite presence. Monogenean gill parasites were confirmed by their morphology and their sclerotised structures using microscopy (Soylu and Emre, 2007; Dzika et al., 2009). The species of the parasite found on the gills of *C. carpio* was identified as *Dactylogyrus extensus.*

For the *in vitro* and *in vivo* experiments, the samples were collected by scraping mucus from the gills and placing into glass microscope slides with 200 µl wells. Mucus scrapes were examined to count the parasites using microscopy for *in vitro* tests. For *in vivo* tests, the mucus scrapes from the gills surface were weighed using analytical balance with 0.0001 g sensitivity. During the procedure required to obtain the mucosa, fish were kept under light anaesthesia.

Aspirin. Antiparasitic capacity of aspirin against *D. extensus* was tested *in vitro* and *in vivo*. Aspirin tablets (each tablet containing 100 mg acetylsalicylic acid) were obtained from a local pharmacy, Ankara, Turkey. Aspirin tablets were dissolved in distilled water to obtain the concentrations of 2.5, 5, 10 and 50 g/L aspirin.

***In vitro* aspirin test against *D. extensus*.** Individual parasite samples were exposed *in vitro* to solutions of aspirin at the following concentrations:2.5, 5, 10 and 50 g/L. Mucus on the gill filaments of carp infected by *D. extensus* were gently scraped to a glass microscope slide by a micro spatula and submerged in the different concentrations of aspirin. Parasite motility was continually checked and dead/live parasites counted every minute. Parasites showing no reaction to disturbance by a thin needle were considered dead. Parasite samples maintained in water were used as control. The same procedure was also applied to control parasites. Each replicate contained five parasites and experiments were run in duplicates for each concentration of aspirin. Mortality rates were analyzed to assess the median effective concentration (EC50) with Probit analysis.

***In vivo* antiparasitic efficacy tests.** *In vivo* tests for aspirin were carried out with carp infected with *D. extensus*. Aspirin was administered by immersion. In *in vivo* tests, aspirin concentration was selected based on *in vitro* EC50 results and aspirin EC50 (2.08 g/L) for five min. was diluted to 1/100, corresponding to 20 mg/L. In preliminary *in vivo* tests, EC50 value at a concentration of 2.08 g/L for *D. extensus* was not tolerated by carp, therefore in immersion tests, EC50 value for five min. was diluted at a ratio of 1:100.

Ten carps invaded by *D. extensus* on the gill filaments were used for immersion treatments. The carps were randomly divided into two groups of fish. Experimental group (N = 10) were immersed in the aspirin solution (20 mg/L for 5 min). The immersion procedure was carried out in a 20 L glass aquaria containing 10 L of aspirin solution. Fish were treated one by one. The scrapes of mucus samples (approximately 0.001 g) from the gills‘ surface of carp were collected to assess the number of *D. extensus* on the gills for pre- and posttreatment. The antiparasitic efficacy of the aspirin was calculated as percent reduction of parasites after treatment. Following immersion, five fish were separated for gill histopathology.

Histopathological analysis. Histopathological analysis was done for the gills infected by *D. extensus* (positive control) and aspirin-treated fish gills infected by *D. extensus*. The fish gills not exposed to parasites were evaluated as blank control. Gill tissue samples were fixed in 10% buffered formalin. The fixed tissues were washed in tap water and dehydrated with ascending concentrations of ethanol. After dehydration, tissues were cleared in xylene and embedded to paraffin. Histological sections (five µm) were stained with haematoxylin and eosin (H+E), and examined by light microscopy (Bullock, 1978; Culling, 1974).

Statistics. All data from *in vitro* tests of concentrations were used in calculation of median concentration (EC50). EC50 values at the 95% confidence level (95% CL) were evaluated by Probit (Finney, 1971). Other data were compared using variance analysis ANOVA at a significance value of 5%. All statistical analysis was performed with SPSS26.

RESULTS AND DISCUSSION

***In vitro* parasite mortality.** Aspirin EC50 for *D. extensus* was 8.137 g/L at one min. exposure; 6.009 g/L at two min.; 3.941 g/L at three min. and 3.379 g/L at four min., and 2.089 g/L at five min (Tab. 1).

In vitro mortality of *D. extensus* showed a concentration- and exposure time-dependent manner for aspirin $p < 0.05$ ($F_{crit} = 2.66130452$). A 100% cumulative mortality was reached in five min. for a concentration of 5 g/L^{-1} aspirin while for the concentration of 50 g/L the cumulative mortality of 100% was observed in one min. (Tab. 1, Fig. 1)

Table 1: Median effective concentrations (EC50) values of aspirin for *D. extensus*.

Estimated parameters by Probit	Exposure time (minutes)				
	1	2	3	4	5
EC50 g/L	8.137	6.009	3.941	3.379	2.089
Lower and upper bounds	5.47-10.08	4.89-10.19	2.86-5.25	2.51-4.60	1.62-2.92
Chi-Square	0.21	0.13	1.77	1.50	0.55
P value	0.90	0.93	0.41	0.47	0.75

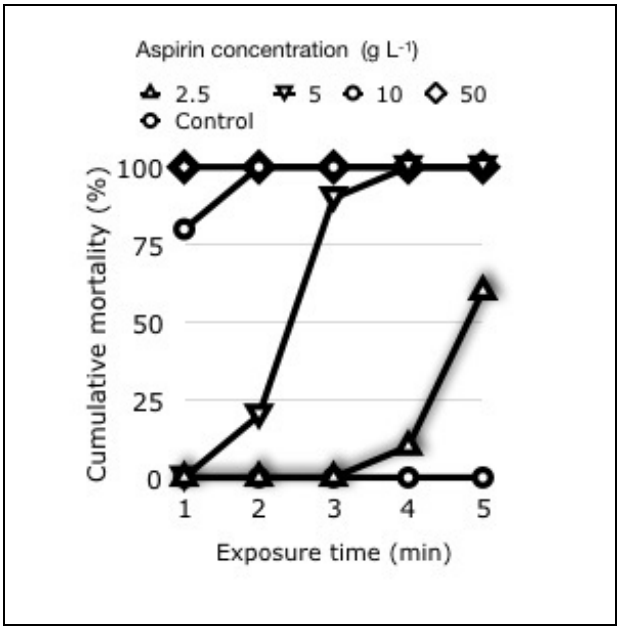

Figure 1: Cumulative mortality of *D. extensus* (monogenean) exposed to aspirin solution of different concentrations.

***In vivo* antiparasitic efficacy.** *In vivo D. extensus* found on the gills of carp were significantly decreased by aspirin exposure at a concentration of 20 mg/L for five min. ($p < 0.05$). The aspirin treatment reduced *D. extensus* on the gills by 46.49%. The mean intensity of *D. extensus* in the mucus of gill (0.001 g) was 45.6 ± 5.49 before treatment and decreased to 21.2 ± 2.80 after treatment with aspirin.

Histopathological analysis. In the untreated carp gills, infestation with *D. extensus* was recorded, resulting in: intense hyperplasia in primary and secondary lamellae; occasional edema and necrosis of the secondary lamellae and telangiectasia in secondary lamellae (Fig. 2). In the negative control group, carp without *D. extensus* infection gills tissue showed a normal structure. Treatment with aspirin at 20 mg/L for five min. completely destroyed *D. extensus* in the gills. Severe lesions such as necrosis in secondary lamellae, hyperplasia, and hyperemia in lamellae, intense hyperemia in the blood vessels of the gills, subepithelial edema and intense hyperplasia of the primary lamella ends were obvious in the gills of carp treated with the aspirin solution differently from the gills invaded by *D. extensus*.

In vitro the median effective dose (EC50) of aspirin was 8.137 g/L at one min. for *D. extensus*. The EC50 values of aspirin were time-dependent, decreasing to 2.089 g/L at five min. Data on the EC50 values aspirin for the monogenean parasites of fish do not exist. The data on aspirin EC50 are difficult to compare with other widely used anti-parasitics because of differing time intervals. The maximal period assessed for aspirin EC50 was five min. here as all parasites were dead at six min. in each concentration tested. The incubation time for other widely used anti-parasitics were longer than the maximal period that we detected for EC50 values in this study. For example, Hu et al. (2017) reported that the traditional anthelminthic drug praziquantel EC50 for *D. intermedius* was 2.69 g/L at 24 hours. Formalin EC50 for *D. minutus* at 16 min. was reported as 0.114 mg/mL, indicating rapid effect of aspirin despite higher concentration than formalin (Tancredo et al., 2019). Compared with an aquatic organism, *Daphnia magna* (zooplankton) aspirin LC50 for *D. magna* was found to be 310 mg/L at 48 hours (Bang et al., 2015). Therefore, it can be considered that aspirin affected *D. extensus* at short-term with relatively higher concentrations. *In vitro* the 50 g/L concentration of aspirin had the most rapid efficacy for *D. extensus*, killing all parasites in one min. whereas at the concentration of 2.5 g/L in six min. The effect of aspirin on *D. extensus* was dose-dependent and time-dependent. Similar pattern of time- and concentration dependent was also pointed out for other Monogenean skin parasites (*G. bullatarudis* and *G. turnbulli*) exposed to salt and gill parasite (*D. minutus)* exposed to formalin (Schelkle et al., 2011; Tancredo et al., 2019).

The treatment strategy to remove monogeneans requires first *in vitro* tests (Tavares-Dias and Martins, 2017; Gonzales et al., 2020). These are followed by the *in vivo* experiments. Here, EC50 value determined for five min. *in vitro* tests was applied to *in vivo* tests by diluting at a ratio 1:100. *In vivo* application of EC50 of aspirin for *D. extensus* (as previously assessed *in vitro* EC50 = 2.08 g/L at five min. for *D. extensus*) was found to be 46.49% effective, showing a much higher killing capacity. In the literature, the discrepancy between *in vitro* and *in vivo* results was reported for various antiparasitic applications. Relatively lower efficacy of treatments was attributed to the protective effect of mucus or scales and the location of parasites on the gill tissue in *in vivo* tests (Rintamäki-Kinnunen and Valtonen, 1996; Schelkle et al., 2011; Kumar et al., 2012). *In vivo* aspirin treatment conducted at 1:100 dilution of EC50 eliminated nearly half of *D. extensus* from the gills. Mucus on the gill surface had no protective effect for the parasites in case of aspirin treatment. Important here is that while aspirin treatment was effective in removing parasites, severe destruction of gill tissue occurred.

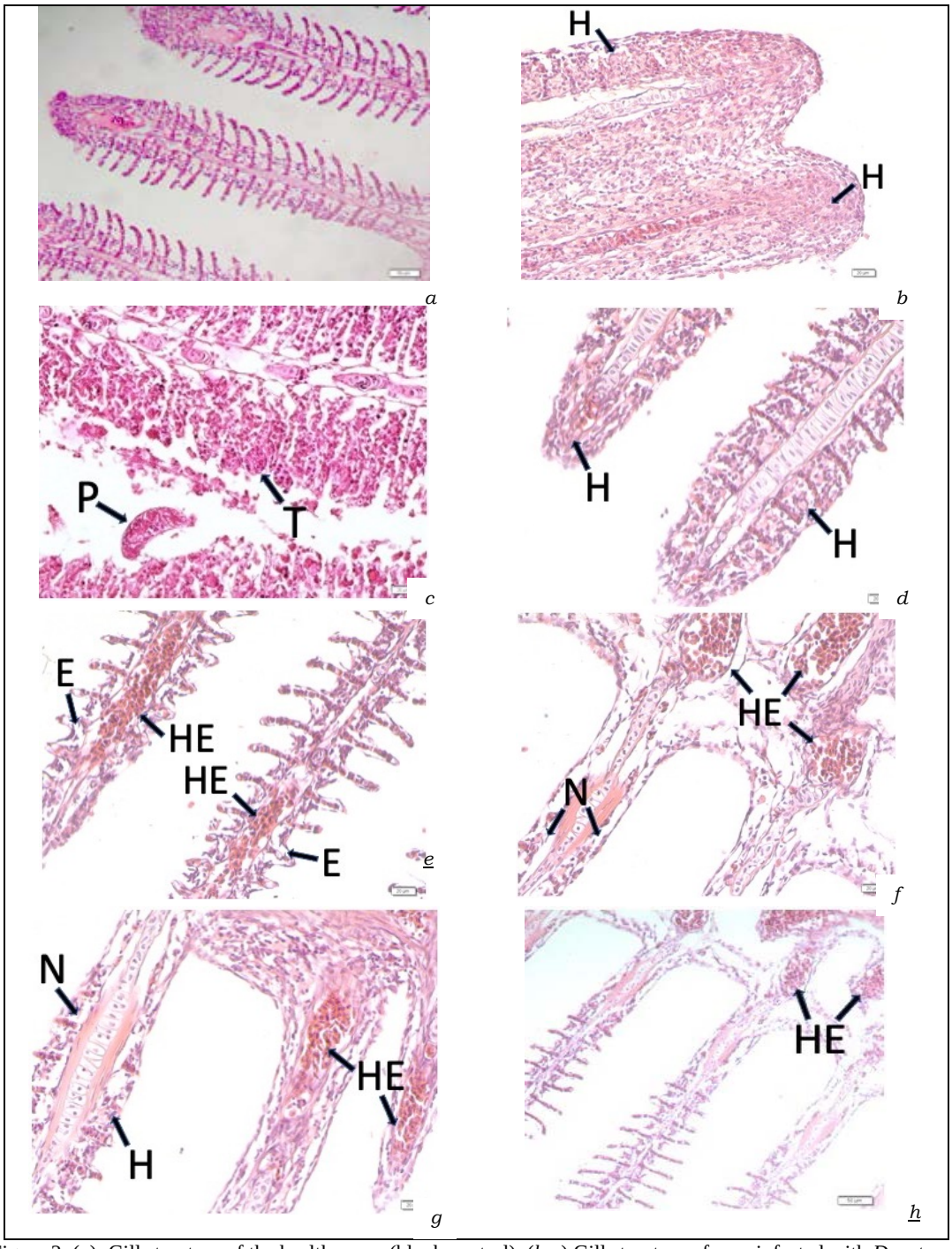

Figure 2. (*a*): Gill structure of the healthy carp (blank control); (*b-c*) Gill structure of carp infected with *D. extensus* (positive control). Dense epithelial cell hyperplasia (H) in primary and secondary lamellae (*b*): Dense epithelial cell hyperplasia (H) in primary and secondary lamellae (*c*): Talengiectasis (T) in secondary lamellae and parasite (P) attached to the lamellae; (*d-h*) Gill structure of aspirin-treated fish. (*d*): Intense hyperplasia (H) of the primary lamella ends and between the second lamella (*e*): Subepithelial edema (E) and hyperemia (HE) in secondary lamellae (*f*): Hyperemia (HE) of blood vessels feeding lamellae and necrosis at the base of the primer and secondary lamellae (*g*): Necrosis (N) in secondary lamellae, hyperplasia (H) and hyperemia (HE) in lamellae (*h*): Intense hyperemia (HE) in the blood vessels of the gills.

Histopathological analysis showed degenerative changes in the gills of aspirin treated fish. The lesion profile involves intense hyperplasia, necrosis, obvious telangiectasia, and edema of some secondary lamellae in gills of carp infected by *D. extensus* was similar to histological alterations reported for carp (*C. carpio)* infected by *Dactylogyrus* genus (Jalali and Barzegar, 2005). The histopathological changes observed in *D. extensus* infected fish in this study are in line with the changes commonly seen in diseased gills expressed by Gjessing et al. (2019) and also similar to the pathology of monogenean infections in fish as studied by Raissy and Ansari (2011) and Santos et al. (2017). The major lesions observed in post-treatment gill samples included intense hyperaemia in blood vessels feeding lamellae and secondary lamella. The other lesions in post treatment gill tissues appear to be specifically associated with the parasite, complying with the study of Strzyżewska-Worotyńska et al. (2017). Nunes et al. (2015) reported that in *Salmo trutta fario* exposed to ASA (100 μg L^{-1} ASA for 28 days), histopathological changes were epithelial lifting, fusion of the secondary lamellae as well as sporadic necrosis, emphasizing non-specific and reversible characters of the lesion. These types of lesions are assumed to form as a barrier to reduce pollutant uptake by minimization of the surface area of the gills without any effect on respiration function (Fernandes and Mazon, 2003). However, in our study, in relation to high concentration of aspirin, post treatment lesions in the gills were degenerative tissue changes, usually irreversible. The appearance of damaged gill tissue can be directly linked to acid properties of aspirin (Nunes et al., 2015). Here the observed gill tissue alterations are considered not reversible. It can be pointed out that osmoregulation capacity of fish can be affected by toxicants in water through altered gill tissue (Bernet et al., 1999). Thus, homeostasis can be challenged by aspirin treatment in relation to disrupted osmoregulation.

CONCLUSIONS

D. extensus is sensitive to aspirin, showing *in vitro* aspirin EC50 of 8.137 g/L at one minute exposure. The mortality of *D. extensus* was distinctly time- and dose-dependent manner *in vitro*, and mortality decreased at lower concentrations of ASA. Antiparasitic efficacy of ASA as applied by exposure of carp at a concentration of 20 mg/L for five minutes can be considered very high. Short term exposure of carp to ASA caused irreversible alterations in gill tissue with severe lesions, making it difficult to recommend as an antiparasitic for gill parasites of fish.

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