Int. J. Aquat. Biol. (2023) 11(1): 1-10
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com

Original Article

Use of ethanol (95%) extract of *Anacardium occidentale* (Linnaeus 1753) to control *Centrocestus formosanus* (Nishigori 1924) infection in *Xiphophorus hellerii* (Heckel 1848)

Udaya Priyantha Kankanamge Epa*, Asha Srimali Premarathna

Department of Zoology and Environmental Management, University of Kelaniya, Daulagama, Kelaniya 11600, Sri Lanka.

Abstract: *Centrocestus formosanus* (Nishigori 1924) is a trematode parasite introduced into many parts of the world through the aquarium fish trade. Its infection causes high fish mortalities and economic losses to fish farmers worldwide. In this study, the efficacy of ethanol (95%) extract of *Anacardium occidentale* (Linnaeus 1753) apple to control *C. formosanus* infection in *Xiphophorus hellerii* (Heckel 1848) was investigated. According to Probit analysis, the estimated 96 h LC_{50} of plant extract for *X. hellerii* was 387.28 mgL^{-1}. Infected *X. hellerii* was treated with concentrations of 300, 320, 340, 360, and 380 mgL^{-1} plant extract with exposure periods of 24 and 48 h. The behavior and mortality of treated and non-treated fish were observed for two weeks. Mortality and parasitic intensity of treated fish were significantly lower than that of non-treated fish during the experiment and recovery period. The parasitic intensity in treated fish decreased significantly with increasing concentration of plant extraction in 24 h and 48 h exposure. The lowest dose of *A. occidentale* apple extract needed to reduce more than 70% of metacercariae infected to gills of *X. hellerii* within 24 h was 340 mgL^{-1}. According to the findings, *A. occidentale* apple extract can effectively control *C. formosanus* infections in aquarium fish.

Article history:
Received 25 October 2022
Accepted 2 December 2022
Available online 25 February 2023

Keywords:
Aquarium fish
Parasites
Trematode
Infection

Introduction

Centrocestus formosanus (Nishigori 1924) (Trematoda, Heterophyidae) is a small intestinal parasite of fish-eating birds and mammals. It uses fish as a second intermediate host in completing its complex life cycle (Hernandez et al., 2003; Ortega et al., 2009, Pinto and Melo, 2011; Paula-Andrade et al., 2012). Infestations of *C. formosanus* in aquarium fish have been reported in Asia (Gjurcevic et al., 2007; Rim et al., 2013), Europe (Gjurcevic et al., 2007; Pinto and Melo, 2010), and other parts of the world (Mitchell et al., 2000; Hernandez et al., 2003; Salgado-Maldonado et al., 2005; Paula-Andrade et al., 2012). Its infection leads to pathogenic conditions and contributes to high mortalities (Mitchell et al., 2000; Mitchell et al., 2005) in aquarium fish, including, *Aplocheilus panchax* (Mardhavi, 1980), *Cyprinus carpio* (Hernandez et al., 1998), *Danio rerio*, *Hypostomus plecostomus*, *Trichogaster trichopterus*, *Poecilia sphenops* (Scholz and Salgado-Maldonado, 2000), *Carassius auratus* (Gjurcevic et al., 2007), *Xiphophorus maculatus*, *Poecilia reticulata*, *Cichlasoma nigrofasciatum* and *Nimbochromis venustus* (Ortega et al., 2009).

Chemical substances, including acriflavine, chloramine, formalin, malachite green, methylene blue, etc., are widely used to control infectious parasitic fish diseases (Madsen et al., 2000). However, resistant genes and residues of drugs remaining in the animal body lessen the wide usage of such chemicals in controlling fish diseases (Swain and Sahoo, 2003). Further, using chemicals is expensive and discouraged by most ornamental fish-importing countries due to their adverse impacts on human health and the environment. Recently, there

*Correspondence: Udaya Priyantha Kankanamge Epa
E-mail: epa@kln.ac.lk

DOI: https://doi.org/10.22034/ijab.v11i1.1760
DOR: https://dorl.net/dor/20.1001.1.23830956.2023.11.1.1.1

has been increased research into utilizing traditional plant-based medicines to control bacterial and protozoan infections in cultured fish species (Castro et al., 2008), mainly because phytomedicines are safe, widely available, and inexpensive. However, plant extracts to treat parasitic infections in fish have rarely been reported.

Anacardium occidentale (Linnaeus 1753), cashew, is widely used in traditional medicine in Asia. Its leaves, bark, roots, and fruit are reported to have anti-microbial (Goncalves and Gobbo, 2012), anti-fungal (Queiroz et al., 2011), and anti-filarial (Suresh and Ray, 1990) constituents. The antiparasitic activity of *A. occidentale* on gastrointestinal nematodes in sheep has also been previously reported (Nery et al., 2010). Apple of *A. occidentale* is considered a by-product of the cashew processing industry. The present study aimed to investigate the efficacy of ethanol (95%) extract of *A. occidentale* apple to control *C. formosanus* infected swordtail, *Xiphophorus hellerii* (Heckel 1848). *Xiphophorus hellerii* is a small, popular ornamental freshwater fish of the Family Poeciliidae.

Materials and Methods

Preparation of plant extraction: *Anacardium occidentale* apples in the fully ripened stage were collected from home gardens in the Puttalam district (8.0408°N, 79.8394°E), Sri Lanka, washed thoroughly, cut into small pieces and dried in the shade. The dried *A. occidentale* apples were ground using a mixer grinder (Braun AG; Type: MX 32). The resultant powder (370 g) was exhaustively extracted by a soxhlet apparatus using 100 mL of 95% ethanol. After the extraction, ethanol was evaporated using a rotary evaporator attached to a vacuum pump (IKA^R RV 10D S96). The extract's stock solution (100 g.L^{-1}) was prepared using deionized water and stored at 4°C.

Parasitic survey: A disease outbreak that caused mass mortalities of *X. hellerii* broodstock was observed in a fish breeding center at Ginigathhena, Rathnapura (6.9857°N, 80.4883°E) in January 2020. Hundred *X. hellerii* with 3.88±0.12 cm length and 95.22±1.31 g weight were randomly caught from infected broodstock ponds and were anesthetized using MS 222 (*Tricaine methanesulphonate*) and examined for external parasites (Buchmann, 2007). Wet mounts of gill tissues were observed under an optical microscope (Olympus CX21FS1) to determine whether parasites were present. Many metacercariae cysts, oval in shape, were randomly distributed along the entire length of the gill filaments of infected fish. The metacercariae were identified morphologically using histological slides and photographs (Hernandez et al., 2003; Gjurcevic et al., 2007; Han et al., 2008).

Toxicity assay of plant extraction on fish*:* *Xiphophorus hellerii* (length and weight of 3.55±0.15 cm and 88.42±0.36 g, respectively) were randomly collected from the infected broodstock tanks and used to estimate LC_{50} of *A. occidentale* apple extract. The acute toxicity tests were performed according to the procedure for static non-renewal technique (USEPA, 2002*).* According to the results of the range-finding test, the concentration of *A. occidentale* ethanol (95%) extract to be used for the static nonrenewal acute toxicity test ranged 200-400 mgL^{-1}. Static non-renewal 96 h toxicity bioassay was carried out with varying extracts of 270, 300, 330, 360, and 390 mg.L^{-1}. Three replicates for each treatment and control were used in the study. Glass tanks (40x20x20 cm) were filled with 10 L of aged tap water, and the stock solution of plant extract was added in required volumes to get a concentration series of 270, 300, 330, 360, 390 mg.L^{-1}. Water was pre-aerated for 15 min to full oxygen saturation before different volumes of plant extracts were added. Ten infected broodstock fish were introduced into all the experimental tanks. Tank water was well-aerated and fish were not fed during the experimental period. The experiment duration was 96 h and at each 24 h period, the total number of dead fish if any, was recorded and carcasses were discarded immediately. The concentration at which 50% of fish mortality occurred after 96 h was taken as the median lethal concentration (LC_{50}) for 96 h.

Table 1. Median lethal concentration (LC_{50}) of ethanol (95%) extract of *Anacardium occidentale* on *Xiphophorus hellerii.*

Exposure period	LC_{50} ($mg.L^{-1}$)	Standard deviation	95% confidence interval limits
24	540.18	229.65	430.94-8801.35
48	478.31	182.00	408.52-1137.37
72	395.54	113.98	367.08-475.95
96	387.28	126.92	358.79-470.04

Determination of the anti-parasitic activity of plant extraction: The experiment was conducted in glass tanks filled with 10 L of aged tap water. The stock solution of ethanol (95%) extract of *A. occidentale* was added in required volumes to get a concentration series of 300, 320, 340, 360, and 380 mgL^{-1}. Tanks were randomly arranged, with three replicates for each treatment and control. Water was pre-aerated for 15 min to full oxygen saturation before the different volumes of plant extract were added. Ten infected *X. hellerii* (length 3.65±0.15 cm and weight 83.62±0.26 g*)* were introduced into each aquarium. Tanks were well-aerated, and fish were not fed during the experimental period. The treatment was conducted for 24 and 48 h of exposure times. After exposure, the treated and non-treated fish samples were anesthetized (MS 222), and their gills were removed. The gills were observed under a high-power microscope (Olympus, CX21 FS1), and the number of metacercarial cysts in all the gill filaments was counted. The total number of metacercarial cysts present in a fish was taken as the parasitic intensity of that fish. The percentage reduction of parasitic intensity was calculated compared to the parasitic intensity of non-treated fish [(% reduction of parasitic intensity = average number of parasites in control fish – average number of parasites in a fish after the treatment / average number of parasites in control fish) x 100]. A concentration of plant extract was considered adequate if more than 70% of metacercariae were killed at a concentration not toxic to *X. hellerii*.

After the experiment, the treated and non-treated fish were transferred into another set of glass tanks with aged tap water. Water was continuously aerated, and fish were fed a commercially available diet daily (2% of body weight). Excess feed and fecal matter were siphoned out every day. Fish were observed for recovery for two weeks, and any dead fish were counted and discarded.

Histopathological studies: Random samples of gills of fish treated with plant extract and non-treated fish were fixed in 10% phosphate-buffered formalin. Samples were processed following standard procedure and 5 µm thick tissue sections (Leica microtome RM2235) were stained with Hematoxylin and Eosin stains (Eagderi et al., 2013).

Data analysis: The data were subjected to the Anderson-Darling test to confirm the normality. The interaction between ethanol (95%) extract of *A. occidentale* and exposure time in reducing parasitic intensity was determined by two-way ANOVA. The mean parasitic intensity and mortality of treated and non-treated fish in 24 and 48 h exposure and water quality parameters in experimental tanks were compared using one-way ANOVA followed by Tuckey's pairwise test. The parasitic intensity of fish in experimental tanks after 24 and 48 h exposure was compared using paired t-test. All the statistical tests were conducted using MINITAB 14 statistical software.

Results

Toxicity assay of plant extraction on fish: The 96 h LC_{50} value of ethanol (95%) extract of *A. occidentale* on X. hellerii at the 95% confidence level was 387.28 $mg.L^{-1}$ (Table 1). At exposure to high concentrations of plant extract, the symptoms of toxicity observed include initial inactivation, air gulping, slow opercula rate, and setting at the bottom motionless. Then they exhibited body imbalance and surface floating, followed by death. Mortality of

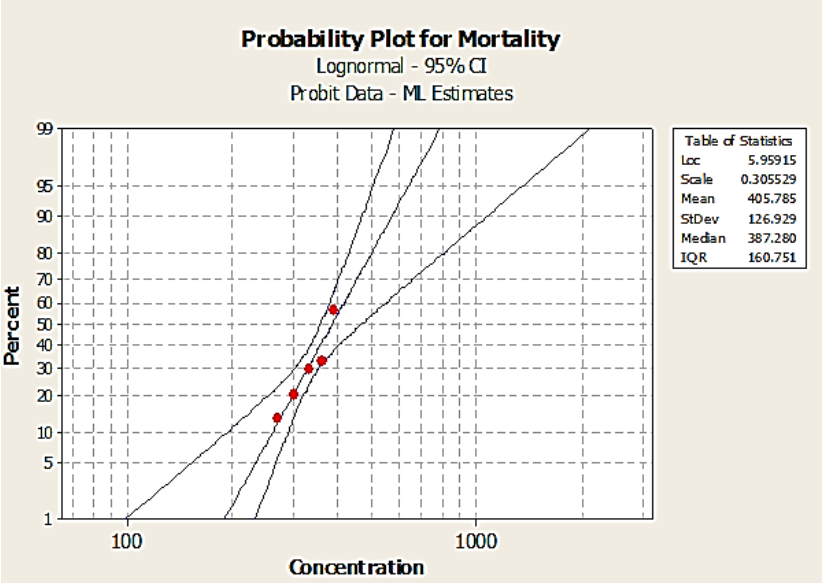

Figure 1. Probability plot for mortality obtained from Probit analysis for 96 h exposure of *Xiphophorus hellerii* to different concentrations of ethanol (95%) extract of *Anacardium occidentale.*

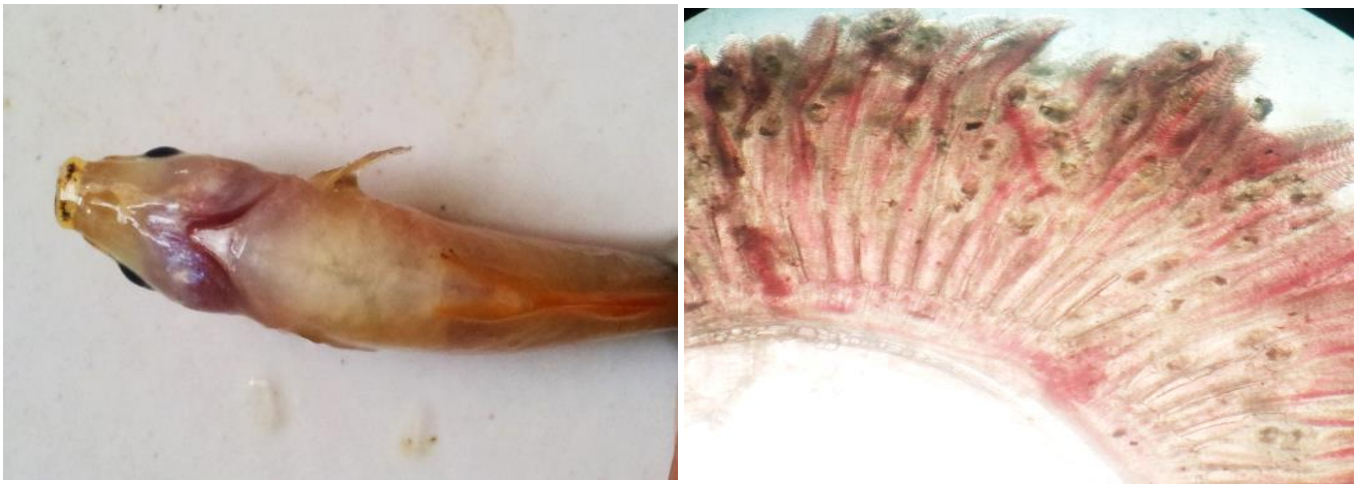

Figure 2. (Left: a) The swollen, reddish-colored opercula of an infected *Xiphophorus* helleri, and (right: b) Metacercarial cysts in a gill of an infected *Xiphophorus hellerii* (100x H&E).

X. hellerii increased with increasing concentration of ethanol extract of *A. occidentale* according to the curve obtained from Probit Analysis for 24, 48, 72 and 96 h exposure (Fig. 1).

Symptoms of infection and identification of metacercariae: *Xiphophorus hellerii* of the broodstock tanks showed symptoms of parasitic infection such as lethargy, respiratory difficulties such as air gulping, gathering near air stones, excess mucus on swollen gills, pale coloration in gills and hemorrhagic areas on the opercula (Fig. 2a). The opercula were bulged out in some fish, even exposing gills. The prevalence of infection was 100%, and the mean intensity of encysted metacercariae was 496.8±127.9 (range 223-558) cysts per fish (Fig. 2b). The same gill arch showed various developmental stages of metacercarial cysts, indicating continuous infection. The cyst contained larvae with two suckers, 32 circumpolar spines around the oral sucker arranged in two rows, and an X-shaped excretory bladder occupying a greater portion of the posterior body. A moderately small ventral sucker was positioned at about one-third of the anterior region (Fig. 3a).

Histopathologically, the changes were characterized by moderate to severe hyperplasia of

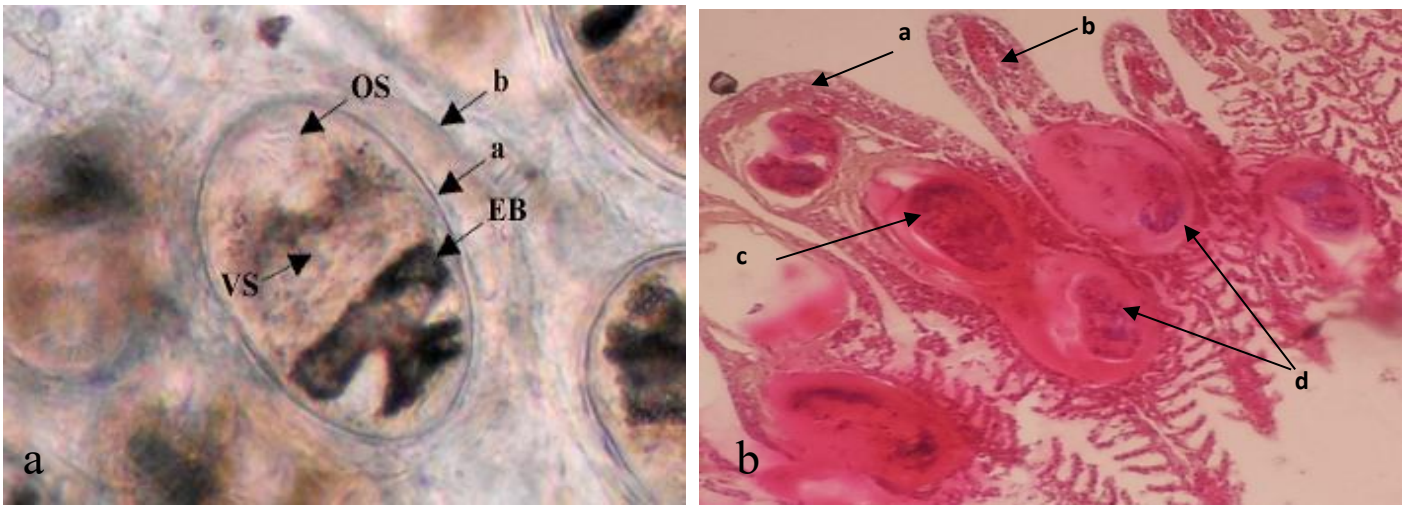

Figure 3. (a) *Centrocestus formosanus* metacercaria in a primary gill filament of *Xiphophorus hellerii*. (OS) oral sucker, (EB) X-shaped excretory bladder, (VS) ventral sucker, (a) Wall of the cyst, (b) Thick capsule secreted by host tissues (400x H&E), and (b) Gills of *X. hellerii* show extensive hyperplasia due to the presence of metacercarial cysts of *C. formosanus*; a. Hyperplasia due to cysts; b. The cartilage of the primary gill lamella; c. A newly recruited cyst; d. Cysts at a stage of higher development (400x H&E).

Table 2. Mean ± SD and % reduction (within brackets) of metacercariae of *C. formosanus* infected gills of *Xiphophorus hellerii* treated with ethanol (95%) extract of *A. occidentale* and control fish.

Period	The concentration of ethanol (95%) extract of *A. occidentale*					
	Control	300 mg.L^{-1}	320 mg.L^{-1}	340 mg.L^{-1}	360 mg.L^{-1}	380 mg.L^{-1}
24 h	523.3±60.7^{a1}	376.3±32.0^{b,1} (30%)	202.4±12.0^{c,1} (32%)	137.9±14.1^{c,1} (73%)	117.5±21.3^{c,1} (77%)	120.7±5.99^{c,1} (77%)
48 h	531.5±57.1^{a1}	152.1±9.33^{b,2} (71%)	117.8±7.16^{b,2} (77%)	81.0±7.37^{b,2} (84%)	73.7±14.2^{b,2} (86%)	71.5±4.22^{b,2} (87%)

Different superscript letters in a row denote significant differences in parasitic intensity in treated and control fish (P<0.05). Different superscript numbers in a column represent significant differences between different exposure times (P<0.05) indicated by paired t-test (P<0.05).

the primary gill lamellae's cartilage that enveloped the metacercarial cysts (Fig. 3b). The enclosed larvae in some cysts showed specific features of adult fluke, and they were surrounded by a thick capsule secreted by the host tissue. Newly recruited cysts did not have such a thick capsule, and the larvae did not show features of an adult fluke.

The antiparasitic activity of plant extraction: The mean parasitic intensity of treated fish was significantly lower than that of non-treated fish (Table 2) after 24 h and 48 h exposure times (P<0.05). There was a significant interaction between the concentration of plant extract and exposure time in removing metacercariae from infected fish (P<0.05). The parasitic intensity of fish treated with 300 mg.L^{-1} plant extract after 24 h was significantly lower than that of control fish and higher than that of all the other treatments. The parasitic intensity of fish in all the treatments after 48 h was not significantly different (P>0.05).

Many inactive metacercariae were observed under a high-power microscope in the wet mounts of the gills in the treated fish. In contrast, the gills of control fish contained highly active metacercariae. There was a linear relationship between the parasitic intensity of fish and the concentration of plant extraction after 24 (Fig. 4a) and 48 h (Fig. 4b) exposure times (Regression analysis, R^2>0.5). The mean parasitic intensity of fish was significantly lower in 48 h exposure than 24 h exposure in all the treatments (P<0.05, paired t-test). There was a slight increase in the parasitic intensity of fish in control aquaria after 48 h compared to 24 h. This difference was not significant (P>0.05, paired t-test).

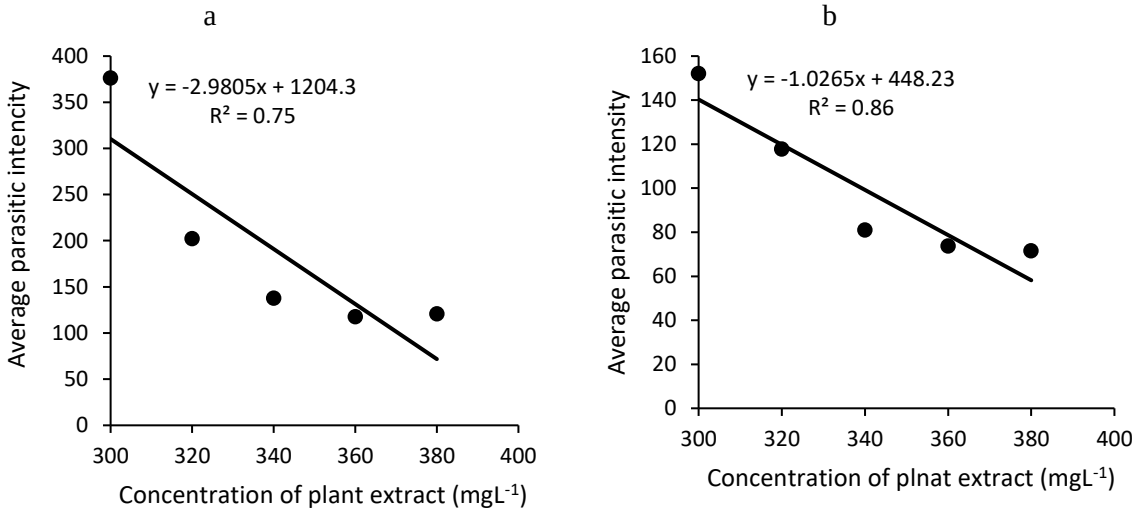

Figure 4. (a) The mean parasitic intensity of fish vs. the concentration of ethanol (95%) extract of *Anacardium occidentale* (a) after 24 h exposure time, and (b) after 48 hour exposure time.

Table 3. Mean±SD and range of mortality (within brackets) of *Xiphophorus hellerii* treated with ethanol (95%) extract of *Anacardium occidentale* and control fish.

Period	The concentration of ethanol (95%) extract of *A. occidentale*					
	Control	300 mgL^{-1}	320 mgL^{-1}	340 $mg.L^{-1}$	360 $mg.L^{-1}$	380 $mg.L^{-1}$
24 h	5.33 ± 0.88^{a} (4-7)	0.67 ± 0.33^{b} (0-1)	0.67 ± 0.67^{b} (0-2)	0.67 ± 0.33^{b} (0-1)	1 ± 0.58^{b} (0-2)	1.33 ± 0.33^{b} (1-2)
48 h	5.67 ± 1.45^{a} (3-8)	0.67 ± 0.67^{b} (0-2)	1 ± 0.58^{b} (0-2)	0.67 ± 0.67^{b} (0-2)	0.33 ± 0.33^{b} (0-1)	1 ± 1^{b} (0-2)

Different superscript letters in a row denote significant differences (P<0.05) indicated by one-way ANOVA followed by Tukey's pairwise comparison.

According to histopathological observations, cavitation due to the removal of metacercariae was observed in the primary gill filaments of treated fish (Fig. 5). The number of fish deaths in five treatment aquaria was significantly lower than that of control one (P<0.05). The mortalities of fish between 24 and 48 h were not significantly different in both treatment and control aquaria (P>0.05) (Table 3).

Fish mortalities during the recovery period: During the recovery period, the skin pigmentation, swimming pattern, swimming position, activity and opercular rate of fish treated with plant extract were normal, while control fish showed symptoms of parasitic infection. Fish mortalities in treated aquaria ranged from 1-2, while more than 50% of fish died in control aquaria.

Water quality in the experimental tanks: Temperature, DO, conductivity and TDS did not vary significantly between the control and the treatment tanks (P>0.05). However, pH values were significantly lower in the treatment tanks than in the control tanks (P<0.05) during the experimental period (Table 4).

Discussion

All the ethanol extracts of *A. occidentale* apple used in the study effectively reduced metacercariae infection in *X. hellerii*. The observation of dead metacercariae and cavities of cysts produced from the gill tissues of the treated fish showed the impact of plant extract on the metacercariae of *C. formosanus*. The parasitic intensity of fish treated with 300-380 mgL^{-1} plant extract was significantly lower than in the control fish within the experimental period. The low parasitic intensity may have increased the chance of survival of treated fish, decreasing their mortality. The toxic constituents of *A. occidentale* apple extract may have inactivated and killed the metacercariae in treated fish. According to Dao et al. (2021), fresh *A. occidentale*

Table 4. Mean±SE of Physico-chemical parameters of water in aquaria treated with ethanol (95%) extract of *Anacardium occidentale* and control aquaria during the experiment.

	Control	300 mg.L^{-1}	320 mg.L^{-1}	340 mg.L^{-1}	360 mg.L^{-1}	380 mg.L^{-1}
pH	7.21±0.00^{a}	7.17±0.3^{a}	7.00±0.01^{b}	6.97±0.04^{b}	6.99±0.01^{b}	6.96±0.02^{b}
Temperature (C°)	24.54±0.00	24.47±0.02	24.45±0.02	24.52±0.05	24.56±0.01	24.73±0.01
Dissolved Oxygen (mgl^{-1})	7.04±0.00	7.05±0.06	6.90±0.06	6.84±0.10	6.99±0.05	7.00±0.09
Conductivity (µcm^{-1})	846.27±0.03	835.87±2.26	840.20±3.24	843.13±5.02	834.53±6.11	827.47±3.41
TDS (mgl^{-1})	410.63±0.03	409.40±1.15	411.53±1.45	413.07±2.77	408.87±3.11	406.87±1.27

Different superscript letters in a row denote significance difference (P<0.05) indicated by one-way ANOVA followed by Tukey's pairwise comparison.

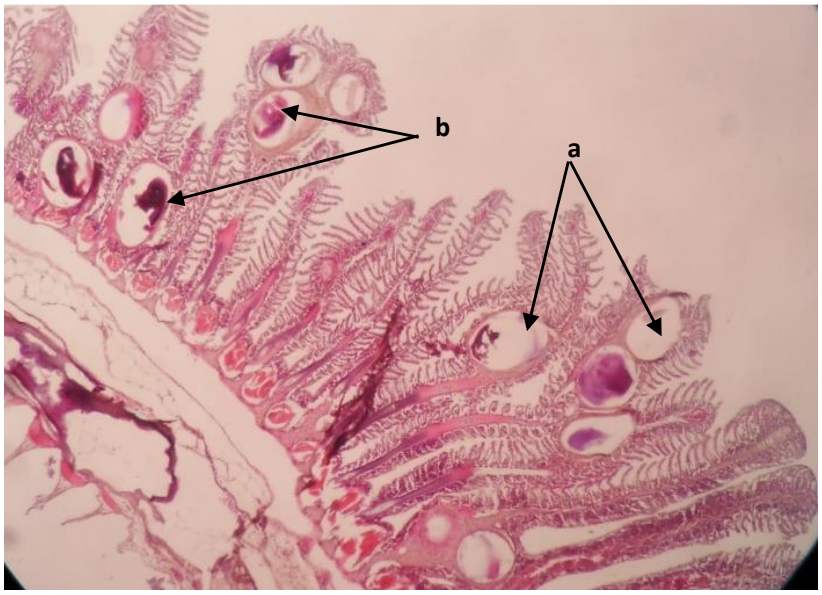

Figure 5. Gills of *Xiphophorus hellerii* after the treatment with the ethanol extract of *Anacardium occidentale* (360 mgL^{-1}) a. Cavitation in the primary gill filaments due to removal of the metacercariae, and b. Dead metacercariae (100x H&E).

apple contains 2 mg.g^{-1} tannin and 76 mg.g^{-1} total phenolic substances. The anti-parasitic properties of tannins, especially on nematodes, have been previously shown in several in vitro and in vivo studies (Hoste et al., 2006; Molan et al., 2002; Paolini et al., 2002). Bath treatment of 340 mg.L^{-1} of plant extract reduced more than 70% of metacercarial cysts from infected gills of *X. hellerii* within 24 h. Due to this high efficacy of plant extract in removing metacercariae, a 100% reduction of parasites may be achieved by extending immersion time or repeating the same treatment. The mean parasitic intensity of treated fish decreased with the increasing concentration of *A. occidentale* apple extract. A similar observation was made by Buchmann et al. (2003) when garlic extract, *Allium sativum,* was tested in controlling *Ichthyophthirius multifiliis* infestation in fish.

Though the metacercariae infected to the gills of the control fish were active throughout the experimental period, the parasitic intensity in the same fish did not significantly vary during the experimental period. It indicates that the intensity of infection in experimental fish did not change within the experimental period. Therefore, the rapid reduction of mean parasitic intensity with increasing exposure time (24 and 48 h) in the treated fish might be attributed to the toxic effects of different concentrations of ethanol (95%) extract of *A. occidentale*. The results of the present study are comparable with the findings of Ekanem et al. (2004), who observed a 90% reduction in *I. multifiliis* infection in fish after treatment in baths of *Mucuna pruriens* and *Carica papaya* plant

extracts (200 $mg.L^{-1}$) within 72 h and 96 h, respectively compared to untreated controls.

Centrocestus formosanus metacercariae infection damaged the primary gill lamellae and respiratory epithelium of infected *X. hellerii*. The gills of severely infected fish were swollen and paled in coloration, and hemorrhagic areas on the opercula were also observed. Hypoxia due to these symptoms (Mitchell et al., 2000; Scholz and Salgado-Maldonado, 2000; Mitchell et al., 2005) could be the reason for the significantly higher number of fish deaths observed in the control aquaria. Gill arches of infected *X. hellerii* contained various developmental stages of larvae indicating continual infection.

The reduction of water pH in the aquaria treated with plant extract could be attributed to ascorbic, gallic, and tannic acids present in the apple of *A. occidentale* (Gordon et al., 2011; Queiroz et al., 2011). Ripe apples of *A. occidentale* were used in the experiment, and they contain more ascorbic acid (17.31 $mg.g^{-1}$) than unripe apples (Gordon et al., 2011). Though organic acid can strongly influence the pH in water (Munson and Gherini, 1993), the narrow pH variation (6.9-7.1) observed in the experimental aquaria may not severely impact fish health or parasitic activity.

The 96 h LC50 of ethanol extract of *A. occidentale* on *X. hellerii* (387.28 $mg.L^{-1}$) was significantly higher than that of the 96 h LC_{50} of *Moringa oleifera* seed extract (124.0 mgL^{-1}) on *Cyprinus carpio* (Kavitha et al., 2012), *Nerium Indicum* leaf extract (96 $mg.L^{-1}$) on *Channa punctatus* (Sudhanshu and Singh, 2004), and *Tephrosia candida* leaf extract (6.43 $mg.L^{-1}$) on *Oreochromis niloticus* (Mohotti and Epa, 2016). Toxicity symptoms of fish treated with plant extract observed in the present study were similar to those of *O. niloticus* exposed to *Derris* powder extracts (Akinbulumo et al., 2005).

During the recovery period, the skin pigmentation, swimming pattern, swimming position, activity and opercular rate of treated fish were normal. Therefore, ethanol extraction of *A. occidentale* apple used in the study may not have any severe adverse effect on the behavior or health of *X. hellerii* due to its comparatively lower fish toxicity. It is necessary to conduct further experiments with the purified effective substances of *A. Occidentale* in the vicinity of the lowest dose (340 mgL^{-1}; 24 h) to establish the therapeutic amount needed for a good parasiticidal effect.

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