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Evaluating the use of garlic (*Allium sativum*) for the remedy of *Cryptocaryon irritans* in guppies (*Poecilia reticulata*)

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Abstract

Garlic (*Allium sativum*) has been shown to possess antimicrobial properties against a range of disease-causing agents, including fish parasites. Our study aimed to investigate the potential use of garlic as a treatment against *Cryptocaryon irritans* infection, using guppies (*Poecilia reticulata*) as the fish model. Garlic was freeze-dried, powdered, and used as it is or as an aqueous extract. The content of allicin, its main active ingredient, was 1.25 mg/g in garlic powder and 0.82 mg/ml in the aqueous extract, as determined by HPLC analysis. Aqueous garlic extract fully immobilized *C. irritans* theronts and protomonts within 20 and 40 min, respectively, following exposure to 5 µl/ml. Treatment trials were performed, in which infected fish were fed with garlic powder-supplemented feeds (5%, 10%, or 20% supplementation), bathed in garlic aqueous extract (250 or 500 µl/L) and subjected to a combined treatment which included both feeding and bathing. Results revealed that the tested treatments failed to cure the infection, though reduction in infection intensity on the caudal fin, but not on the gills, was evident following dietary supplementation with 20% of powdered garlic in one of the trials.

KEYWORDS
Cryptocaryon irritans, feeding, garlic, guppies, immersion, treatment

1 | INTRODUCTION

The ciliate parasite *Cryptocaryon irritans* Brown (1951) infects a wide range of fish species in temperate and tropical seas and is the cause of the "marine white spot disease" (Colorni & Burgess, 1997). By invading the epithelium of the skin and gills and disrupting osmotic balance (Colorni, 1985), this parasite is responsible for high mortality rates and losses of fish worldwide, resulting in substantial financial damage due to fish morbidity and mortality and the cost of control measures and treatments (Colorni & Burgess, 1997; Dickerson, 2006; Wang et al., 2018; Watanabe, How, Zenke, Itoh, & Yoshinaga, 2018). The life cycle of *C. irritans* includes four stages: theront, trophont, protomont, and tomont. The ciliated theront is the infective stage, where the parasite penetrates the gill and skin epithelium; the trophont is the feeding stage, where the parasite ingests debris and body fluids from the fish; the mature trophont then leaves the fish, sheds its cilia, and transforms into a protomont, which adheres to a solid substrate and encysts, thus developing into the reproductive tomont stage that produces the infective daughter cells (Colorni & Burgess, 1997). The unprotected, water-associated stages of the parasite include the theront and protomont, which are potentially susceptible to anti-parasitic therapeutants.

The control of *C. irritans* relies on breaking its lifecycle, namely, by separating the fish from the encysting tomonts, which is feasible only in small scale systems (Colorni, 1987), or by applying a chemical treatment that kills the unprotected, water-associated theronts and protomonts (Dickerson, 2006). Commonly used chemicals for this purpose include malachite green, formalin, and copper sulfate (Dickerson, 2006), but these have various limitations. For example, the use of malachite green is now prohibited due to its carcinogenicity (Schnick & Meyer, 1978); formalin, despite its wide use in aquaculture (Bowker & Trushenski, 2016), is increasingly restricted due to

its carcinogenic effect (National Toxicology Program, 2010); and copper sulfate, which is considered the most effective treatment against *C. irritans* (Yanong, 2009), is often toxic to fish at therapeutic concentrations (0.5–1.0 mg/L; Colorni, 1987) and is not permitted for food fish in many countries. In addition, the development of resistance to treatment is a routinely encountered problem.

The use of plants for the treatment of aquaculture diseases has raised much interest in recent years (Reverter, Bontemps, Lecchini, Banaigs, & Sasal, 2014; Valladão, Gallani, & Pilarski, 2015). Although the effect of many plant treatments on various parasites has been demonstrated, only a few studies examined the effect of plant treatments on *C. irritans*. More specifically, Picón-Camacho, Ruiz de Ybáñez, Holzer, Arizcun Arizcun, and Muñoz (2011) demonstrated that epigallocatechin gallate, a flavonoid, polyphenolic compound found in green tea (*Camellia sinensis* L.), and L-DOPA found in the velvet bean (*Mucuna pruriens* L.), exert a significant anti-parasitic effect on *C. irritans* theronts in vitro. In addition, Goto, Hirazawa, Takaishi, and Kashiwada (2015) demonstrated that dietary supplementation with matrine—the active ingredient of the *Sophora flavescens* root—significantly reduced, although did not completely cure, *C. irritans* infection in red sea bream (*Pagrus major*).

The objective of the current study was to explore the application of garlic (*Allium sativum*) as a natural treatment against *C. irritans* and to evaluate its potential as an alternative to the conventionally used chemical therapeutants. Garlic has been used as a medicinal plant for thousands of years, and its main active ingredient, allicin (diallyl thiosulfinate), has been shown to possess remarkable antimicrobial effects (Cavallito & Bailey, 1944). Indeed, garlic has been shown to be effective against cardiovascular diseases, possess antitumor properties, and antimicrobial effects against a wide range of microorganisms, including bacteria, fungi, protozoa, and viruses (Bayan, Koulivand, & Gorji, 2014). As a potential therapeutant for aquaculture diseases, garlic has been reported to eradicate parasites of the phylum *Ciliophora* (to which *C. irritans* belongs), including *Trichodina jadranica* (Abd El-Galil & Aboelhadid, 2012; Madsen, Buchmann, & Mellergaard, 2000) and *Ichthyophthirius multifiliis* (Buchmann, Jensen, & Kruse, 2003). Hence, we hypothesized that garlic may be affective against *C. irritans* infection in fish. As a model organism, we selected the guppy (*Poecilia reticulata*), as its small size facilitates laboratory-based trials and its euryhalinity enables its use in seawater (SW). We used SW-adapted barramundi (*Lates calcarifer*) to obtain large numbers of *C. irritans* parasites and tested the effect of garlic against these parasites in vitro, followed by in vivo trials using immersion and oral application of garlic.

2 | MATERIALS AND METHODS

2.1 | Preparation of garlic powder and aqueous extracts

Fresh garlic (*A. sativum* L.) cloves from Southern Arava R&D (Yotvata, Israel) were peeled, washed, sliced to 0.5–1 cm thick slices, and then either snap-frozen in liquid nitrogen or placed in −80°C, followed by freeze-drying and storage at −80°C. The freeze-dried garlic was then ground to powder using a stainless steel laboratory blender and kept at −80°C. The freeze-dried garlic powder was extracted with double-distilled water (DDW) at a ratio of 1:10 g/ml. The mixture was vortexed twice for 1 min at 5 min intervals and kept on ice between vortexes. For in vitro trials, the extract was centrifuged (Labofuge 400R) at 3,939 *g* for 20 min at 4°C, followed by filtration through 0.45 μm and 0.22 μm Millex-GV filter. For in vivo trials, the extract was either centrifuged three times at 3,939 *g* for 20 min at 4°C or centrifuged at 3,939 *g* followed by 20,817 *g* for 20 min at 4°C. Finally, the extract (considered as the 100% stock solution) was divided into aliquots and stored at −80°C.

2.2 | Analysis of allicin content in garlic powder and extract

Allicin content in the aqueous extract and the freeze-dried powder were determined based on the standardized INA (Institute for Nutraceutical Advancement) (2004) procedure 110.001 with some modifications. The garlic extract was filtered through a 0.45 μm GF nylon filter and analysed with a previously described HPLC system (Borochov-Neori et al., 2013). Isocratic elution was carried out with a solution of 40% acetonitrile in water, delivered at a flow rate of 1.2 ml/min. An allicin standard was freshly prepared from a water extract of freeze-dried garlic (200 mg garlic powder in 10 ml DDW) using solid-phase extraction. Standard purity was verified spectroscopically, and chromatographic purity >88% was established by HPLC. Allicin concentration was calculated using the published extinction coefficient value (Lawson, Wood, & Hughes, 1991), and a calibration curve was generated from the chromatograms of the standard dilutions in the range of 10–120 μg/ml.

2.3 | Fish maintenance and adaptation to SW

Adult guppies and ~2-month-old juvenile barramundi (weighing ~50 g) were obtained from local commercial aquaculture farms and maintained in 100 L containers in freshwater until used for experimentation. Adaptation to SW was carried out prior to exposure to *C. irritans* through a gradual acclimation using artificial SW (Red Sea Fish Pharm Ltd., Eilat, Israel). Fish were gradually adapted to increasing salinities in 30 L aquaria over 20 days (moved to increasing salinities of 10, 15, and 20 g/L). Mineral concentration in the 20 g/L SW was Ca^{2+}, 236–248 mg/L; Mg^{2+}, 704–739 mg/L; K^{+}, 214–226 mg/L. All aquaria and containers were supplied with aeration and submerged biofilters. Fish were fed daily with commercial guppy food at about 2% of their bodyweight. Siphoning was applied daily, followed by about 10%–25% water change. Water quality parameters were monitored weekly and maintained as follows: temperature 25 ± 2°C, ammonia and nitrite <0.5 mg/L, dissolved oxygen >80% saturation.

2.4 | Propagation of *C. irritans*

Cryptocaryon irritans were kindly provided by Dr. Angelo Colorni and Dr. Galit Sharon from the Israeli Oceanographic and

Limnological Research (IOLR), Eilat, and maintained in a small-scale laboratory system with the SW-adapted guppies (Fridman & Zilberg, 2014).

Protomonts were collected from infected moribund or freshly dead guppies from the infection aquaria. The fish were placed in Petri dishes filled with 25 g/L SW for 1–5 hr, such that the mature trophonts left the fish, sank to the bottom of the dish, and were either immediately used for analysis or allowed to attach to the plastic and develop into tomonts for later analysis.

To obtain large numbers of theronts for infection trials, infected barramundi were placed in aquaria, the bottom of which was covered with square plastic plates. Parasites that left the fish sank to the bottom, attached to the plates, and encysted. The cysts (tomonts) were carefully collected by brushing them off the plates with a fine paintbrush and transferred to 6-well plates, where they were washed with clean 25 g/L SW followed by two or three washes with 500 µl/L formalin in 25 g/L SW for disinfection and, finally, incubated at 30°C in clean SW. SW was replaced daily. Once the cysts hatched, SW was collected, centrifuged (3,939 *g* for 10 min at 10°C), and the theronts were collected from the bottom of the tube by carefully removing most of the overlaying SW. A sample from a homogenous suspension was mixed with 350 µl/L formalin (in 25 g/L SW) at a ratio of 1:1 and the number of theronts was counted in 10 µl drops of the suspension under a light microscope (×100 magnification, using a ×10 objective).

2.5 | Analysing the effect of garlic extract on *C. irritans* in vitro

The effect of garlic extracts was tested against *C. irritans* protomonts and theronts. A total of 10 protomonts and 50 theronts in 25 g/L SW were placed in each well of 24- and 96-well plates, respectively, and a garlic aqueous extract was added at concentrations of 5, 10, 20, or 40 µl/ml, in four replicates. For a positive control, 50 µl/L formalin was used; for a negative control, 25 g/L SW was used. Parasites were observed under an inverted microscope over 120 min, and the time of the halt in motility was recorded. At this stage of no motility, the parasites were considered as dead.

2.6 | Fish treatment trials

2.6.1 | Trial 1: Immersion treatment

In a preliminary toxicity test, a garlic aqueous extract (250 or 500 µl/L) was added daily for 28 days and did not induce evident toxicity to the fish (data not shown). Application of a garlic extract at 500 µl/L was selected for a subsequent treatment trial. The experiment was conducted in 10 L aquaria with 10 fish per aquarium. Fifty *C. irritans* tomonts were added to each aquarium on day 0 and the 500 µl/L garlic extract was added every other day from day 1, for 30 days (n = 3). For a positive control, 25 µl/L of formalin was similarly added (n = 2), and for a negative control, no treatment was applied (n = 1). Mortality was recorded every day and dead fish were examined for the presence of *C. irritans*. Infection was confirmed microscopically in wet mounts from the skin and fins of freshly dead fish.

2.6.2 | Trial 2: Combined immersion and feeding treatment

The trial was conducted in 10 L aquaria stocked with 10 fish per aquarium, in three replicates. A preliminary palatability test with 10% and 20% garlic-supplemented feed revealed minor to no negative effect on food acceptance and no evident effect on fish behavior. Treatments included daily immersion in 500 µl/L garlic extracts, with the addition of feeding with 10% garlic-supplemented feed. Fish were fed at 2% of their body weight per day, in two separate feed applications. No immersion treatment occurred and a non-supplemented feed was used in control aquaria. Fish were challenged by the addition of tomonts (total of 120 tomonts per aquarium) on days 0 (70 tomonts) and 4 (50 tomonts). Mortality was recorded every day and dead fish were examined for the presence of *C. irritans*.

2.6.3 | Trial 3: Dietary supplementation with garlic for the prevention of *C. irritans* infection (three separate experiments)

Experiments were conducted in 10 and 30 L aquaria, stocked with 20 and 50 fish respectively. The concentration of theronts for challenge and fish organs for analysis of infection levels were selected based on preliminary experiments (J. H. Kim unpubl. results). The effect of dietary garlic application prior to infection, as a preventative treatment, was tested. In three separate feeding trials, guppies were fed with 5%, 10%, or 20% garlic-supplemented feeds, or with non-supplemented feed as a control, for 14–32 days, followed by challenge with *C. irritans* (Table 1). Challenge exposure was carried out in gently aerated 1 L plastic containers filled with 300 ml of 20 g/L SW, to which 9,000 theronts (30 theronts/ml) were added. Fish from each aquarium were exposed in a separate 1 L container for 4 hr and then transferred, together with the SW and theronts, back to the original aquarium. Infection levels on the gills and caudal fin were examined 3 days post challenge in 10 fish from each aquarium by examining wet mounts of the respective organs under a light microscope. The caudal fin surface area of photographed fish tail images (by scanning) was determined by IMAGE-J software (imagej.nih.gov/ij/), the number of trophonts per centimeter was calculated, and the infection prevalence and intensity were evaluated. For the gill's analysis, all the gills from both sides of the fish (right and eft gill sets) were collected and the total number of trophonts was recorded.

2.6.4 | Garlic-supplemented feed preparation

Commercial fish feed (Mem Ornamental, BernAqua, Belgium) was supplemented with 5%, 10%, or 20% garlic powder and used for the

TABLE 1 Feeding trials: the effect of garlic-supplemented feed on infection prevalence (%) and mean intensity (±*SEM*; calculated considering infected fish only) with *Cryptocaryon irritans*. *n* = 1 for experiments 1 and 2; *n* = 2 for each challenge in experiment 3; 10 fish per replicate aquarium

		Infection prevalence (%)		Infection intensity		
					Caudal fin	
Treatment	Feeding period (days)	Gills*	Caudal fin	Gills*	Total trophonts	Trophonts/cm^2
Trial 1	14					
Control		90	100	8 ± 2	13 ± 3	No data
Garlic 5%		90	100	9 ± 2	10 ± 2	No data
Garlic 10%		90	100	6 ± 2	10 ± 3	No data
Trial 2	14					
Control		100	100	18 ± 6	105 ± 13^a	96 ± 12^a
Garlic 10%		100	100	15 ± 5	143 ± 13^a	140 ± 13^a
Garlic 20%		100	100	21 ± 5	26 ± 9^b	28 ± 7^b
Trial 3: challenge 1	28					
Control		45	65	1 ± 0	2 ± 0	2 ± 0
Garlic 10%		35	50	2 ± 0	2 ± 0	2 ± 0
Garlic 20%		5	30	2 ± 0	2 ± 0	2 ± 0
Trial 3: challenge 2	32					
Control		55	85	2 ± 0.3^a	5 ± 1^a	5 ± 1^a
Garlic 10%		90	100	7 ± 1^b	10 ± 1^b	10 ± 1^b
Garlic 20%		85	90	6 ± 1^{ab}	8 ± 2^{ab}	7 ± 1^{ab}

Note. a,b, different letters denote significant differences between guppies fed different supplemented diets within a feeding trial; $p < 0.05$.
*Total number of trophonts on all the gills from both sides of each fish (right and left gill sets) were considered.

different feeding trials. Incorporation of garlic into the food differed between trials, as described below.

Trial 2: Cold-powdered garlic and fish food were mixed, followed by the addition of egg white to glue them together and prepare small balls at the equivalent weight of a single feed application.

Trial 3, experiment 1: Garlic powder was coated on fish food at a concentration of 5% or 10%, followed by spraying with a 2.5% gelatin solution (2.5 g gelatin powder in 100 ml DDW) to attach the powder onto the food pellets, followed by freeze-drying.

Trial 3, experiments 2 and 3: Cold-powdered garlic was added to cold-powdered fish food at a concentration of 10% or 20%. Cold DDW was then added at about half the food volume, the mixture was flattened on a tray and freeze-dried, and then broken into small pieces, which were passed through 1,000 and 500 µm laboratory mesh sieves. Control feed was similarly prepared, but without the addition of garlic, for each experiment. The experimental feeds were stored at –20°C and used within 1 month.

2.7 | Statistical analysis

Statistical analysis was conducted using one-way ANOVA followed by Tukey's post hoc test or Holm–Sidak method. Differences were considered significant at $p < 0.05$. All statistical analyses were performed using SigmaPlot version 13.0 (San Jose, CA).

3 | RESULTS

3.1 | Allicin content in garlic aqueous extract and freeze-dried garlic powder

Allicin content in the garlic aqueous extract used for in vitro experiments was 0.82 mg/ml, and allicin content in the freeze-dried garlic powder for experimental food was 1.25 mg/g.

3.2 | The effect of garlic extract on *C. irritans* in vitro

Garlic aqueous extract immobilized and then killed *C. irritans*, revealing a dose-dependent effect. At 5 µl/ml, a complete immobilization of theronts and protomonts was evident within 20 and 40 min respectively (Figure 1), revealing the higher vulnerability of theronts to the treatment. In the control treatment, protomonts went through an encystment process, while the garlic-exposed protomonts halted their movement and then started to disintegrate.

3.3 | In vivo treatments

3.3.1 | Immersion and/or dietary supplementation

Mortality following immersion treatment with garlic aqueous extract at 500 µl/L did not differ from the control treatment groups

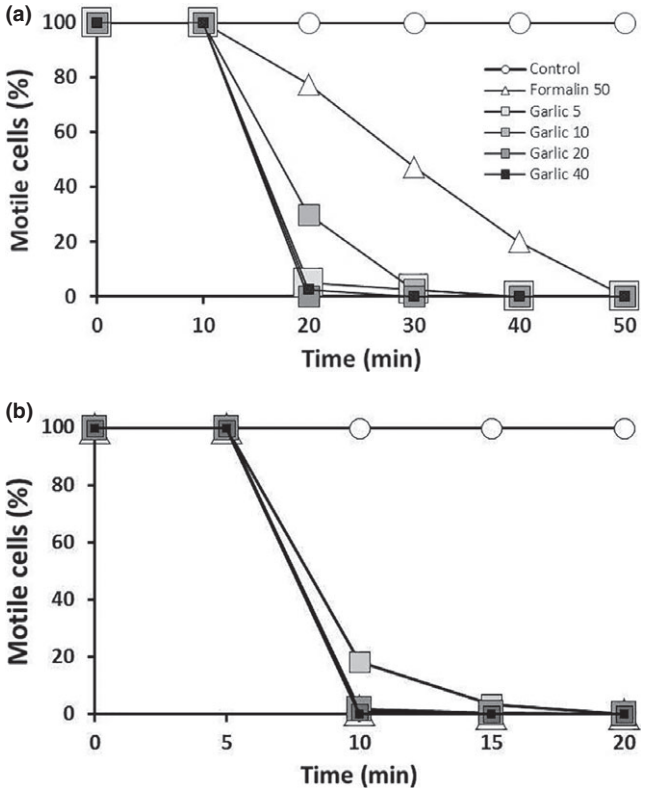

FIGURE 1 Effect of garlic aqueous extracts on the motility of *Cryptocaryon irritans* protomonts (a) and theronts (b). Formalin was used at 50 µl/L and garlic concentrations were 5, 10, 20 and 40 µl/mL. Data represent the percent of motile cells, as observed visually under a light microscope. n = 4 replicate wells; 10 protomonts or 50 theronts per replicate

(Figure 2). Infection with *C. irritans* was confirmed by direct microscopic observation of skin and gill wet mounts (data not shown). Reduction in mortality was recorded in the formalin-treated group (Figure 2). Garlic was dietary supplemented at a level of 10%, a dose which was previously shown to be effective against a skin-associated parasite (Fridman, Sinai, & Zilberg, 2014). The result of this experiment revealed that the combined treatment, which included daily immersion with 500 µl/L of garlic extract and feeding with 10% of garlic supplemented feed, also failed to control *C. irritans*. Infection was confirmed in all treatment groups and the mortality of infected fish was similar to the control group (Figure 3).

3.3.2 | Prevention treatment trials with garlic-supplemented feed

Feeding trials were conducted with different garlic supplementation levels, fed to fish for 2 or 4 weeks, followed by a controlled challenge exposure to *C. irritans* theronts. High variability in infection was evident between trials, as presented in Table 1. The highest variability was noted in infection intensity, reaching levels of a hundred trophonts on the caudal fin in trial 2 and only 2–10 trophonts in the other trials. The effect of garlic supplementation on infection levels and prevalence varied between trials. In fish fed with the 20% supplemented garlic, infection intensity was significantly reduced in experiment 2, as was the infection prevalence in experiment 3, challenge 1. In the other trials, there was no difference between treatment and control groups.

4 | DISCUSSION

Cryptocaryon irritans, the causative agent of "marine white spot diseases," is a cosmopolitan parasite, infecting a wide range of fish species and causing significant losses in the marine aquaculture industry. Treatments of choice include copper sulfate and formalin, both banned in most European countries. The current study aimed to investigate the use of garlic against *C. irritans*, as a potential natural solution that could be permitted and effective as a bath treatment and/or an in-feed treatment.

Garlic is well known to possess therapeutic properties (Bayan et al., 2014) and has been used in traditional medicine for centuries. Buchmann et al. (2003) previously demonstrated that garlic extracts

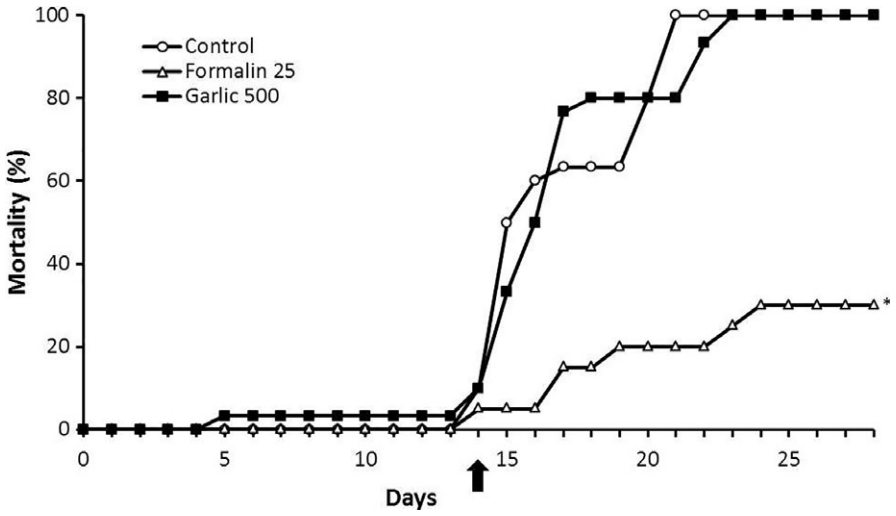

FIGURE 2 Cumulative mortality (%) in immersion treatment trials against *Cryptocaryon irritans*. Fish were infected by the addition of tomonts on day 0, and treatment began on the next day. Treatments included immersion in 500 µl/L of a garlic aqueous extract, applied on alternate days. Formalin was used at 25 µl/L as a positive control and no treatment was applied to the negative control group. On day 14, infection was confirmed microscopically (arrow). n = 3 aquaria in control and 500 µl/L garlic treatment, n = 2 aquaria in formalin treatment; 10 fish per replicate aquarium. *$p < 0.05$

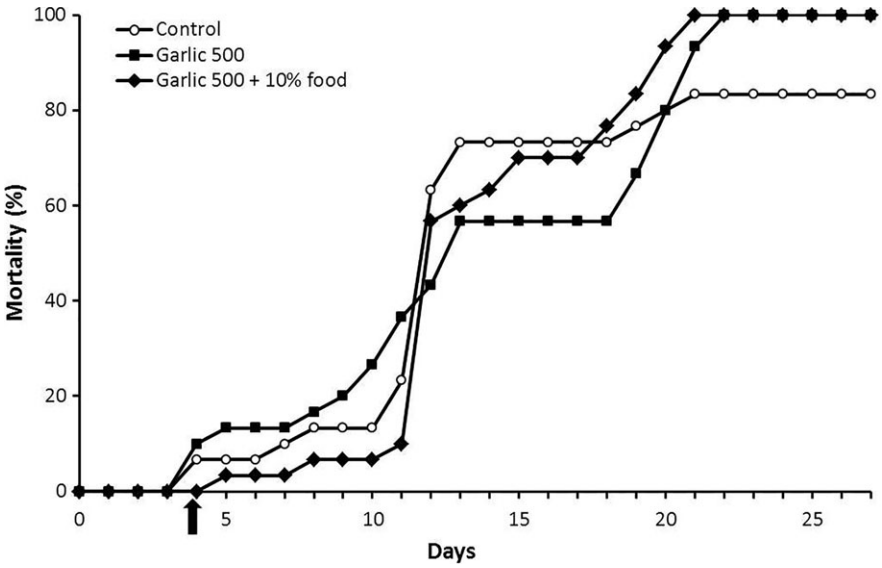

FIGURE 3 Cumulative mortality (%) in fish infected with *Cryptocaryon irritans* (at day 0) and treated with garlic by immersion in 500 µl/L of garlic aqueous extract, which was applied daily, with and without 10% dietary garlic supplementation. On day 4, the infection was confirmed by microscopic examination (arrow). No treatment was applied to the negative control group. *n* = 3 aquaria; 10 fish per aquarium

effectively killed *I. multifiliis* (a freshwater parasite with a similar life cycle to *C. irritans*) in a dose-dependent manner. Similarly, in our study, in vitro trials clearly demonstrated a time- and dose-dependent effect of garlic aqueous extract against *C. irritans* protomonts and theronts (Figure 1), causing immobilization followed by the death of the parasite. The main active compound in garlic, allicin, has been comprehensively studied, and its pharmacological effects are well characterized (Ilic et al., 2011). Allicin is membrane permeable (Miron, Rabinkov, Mirelman, Wilchek, & Weiner, 2000), enters cells easily, and reacts with thiol groups, which are protein bound and ubiquitously present in all living cells, including key enzymes such as glutathione and thioredoxin reductases, acting to maintain the redox potential of the parasite (Ankri & Mirelman, 1999; Rabinkov et al., 2000). Allicin acts as a dose-dependent biocide, disrupting enzymatic activity (Gruhlke, Nicco, Batteux, & Slusarenko, 2016). To achieve this effect, allicin must reach the target cell. Since the parasitic life stages of trophont and tomont are protected by the fish epithelium and the cyst, only the theront and protomont stages are likely to be susceptible to treatment. Thus, repeated treatment application was necessary to target these susceptible life stages. Garlic extract was initially applied every 48 hr as an immersion treatment. The selected application schedule was based on previous results showing that both the levels and the biological activity of allicin are maintained at room temperature, with a small gradual decrease of 14.7% and 3.4%, respectively, over 48 hr (J. H. Kim unpubl. results). However, results revealed that such immersion treatment was not effective against *C. irritans* infection (Figure 2). As application of the immersion treatment on alternate days failed to control the infection, the frequency of the immersion treatment was increased to a daily application. We assumed that, despite the above-mentioned findings (J. H. Kim unpubl. results), the reduction in activity may have hampered the treatment efficacy; thus maintaining higher levels by using a more frequent application was examined. However, this attempt was ineffective, and even the addition of dietary supplementation failed to effectively control *C. irritans* infection in fish (Figure 3). The potential of garlic for immersion treatment against parasitic infection was addressed in several studies. Madsen et al. (2000) demonstrated that bathing European eel (*Anguilla anguilla*) in freshly squeezed garlic at 200 µl/L for 24 hr was effective in treating trichodiniasis and was non-toxic to fish. (Abd El-Galil & Aboelhadid, 2012) Abd El-Galil and Aboelhadid (2012) showed that immersion in garlic aqueous extract and garlic oil dramatically reduced infection rates with trichodinids and gyrodactylids in juvenile tilapia (*Oreochromis niloticus*). In a study previously done in our laboratory, bathing in garlic extract was effective against *Gyrodactylus turnbulli* infection in guppies (Fridman et al., 2014). However, in all cases, very high levels of garlic and/or its extract were required, with effective levels ranging from 200 µl/L to about 10 ml/L. Militz, Southgate, Carton, and Hutson (2014) previously demonstrated the potential of garlic against *Neobenedenia* sp., a monogenean parasite. When applied by immersion, it was lethal to the parasite's larval life stage and reduced its hatching success, but once attached to the fish host, the parasite was unaffected. A similar problem was encountered in our infection model, as *C. irritans* is unaffected by treatment once parasitizing the fish. The preventative effect of garlic-supplemented feed against *Neobenedenia* sp. was demonstrated by Militz, Southgate, Carton, and Hutson (2013), in which barramundi were fed with 50 or 150 ml garlic extract per kg of food for 30 days. Dietary supplementation is the preferred route for application of therapeutants, as it is usually non-stressful to the fish and often requires lower amounts of the active material and less labor; thus, dietary supplementation was also examined in the present study. However, the results did not reveal a clear preventative effect against *C. irritans* from the garlic-supplemented feed. The dietary supplementation reduced infection rates only in one out of the three performed feeding trials.

Surprisingly, despite the similar application of challenge procedures in the three separate feeding trials conducted in this study, high variability was evident (as presented in Table 1). The highest variability was noted in infection intensity, reaching levels of 100

trophonts on the caudal fin in trial 2 and only 2–10 trophonts in the other trials. This variability might have arisen from differences between fish stocks and variability in theront virulence. Matthews and Burgess (1995) reported a wide range of infection levels in *C. irritans*-infected mullet [*Chelon labrosus* (Risso)], even under confined experimental conditions, and suggested that genetic variation within a fish stock may account for differences in their susceptibility. Another explanation for the change in infectivity could be the pathogenicity of the parasite; *C. irritans* theronts emerge 3 hr after the onset of darkness and continue until the end of the dark period (Burgess & Matthews, 1994), and their infectivity decreases with time post-excystment (Yoshinaga & Dickerson, 1994). Theronts were similarly managed in all trials, being collected in the morning and used for challenge within 2 hr, but different batches of tomonts were used, which may explain the variability.

5 | CONCLUSION

This is the first study to examine the effect of garlic against *C. irritans*. Our study demonstrated that garlic was effective against *C. irritans* protomonts and theronts in vitro. However, in vivo trials with garlic applied orally, by immersion, or both, failed to effectively cure or prevent *C. irritans* infection in fish. The parasite selected for this study, *C. irritans*, is challenging to treat due to its life cycle, which involves fish-associated and cyst stages, both of which are resistant to treatment. Thus, a continuous application of the treatment is required to maintain levels that are toxic to the parasite. To do this, continuous monitoring of the applied therapeutant is necessary, which cannot be achieved with garlic as its monitoring requires specialized tools. Allicin, the main active ingredient in garlic, is known to be relatively short-lived, yet its positive effects on a range of medical conditions have been proven (Ilic et al., 2011). This study highlights the importance of in vivo trials to confirm the efficacy of therapeutants that appear to be effective in vitro.

INFORMED CONSENT

Informed consent was obtained from all individual participants included in the study.

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ETHICAL APPROVAL

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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REFERENCES

Abd El-Galil, M. A. A., & Aboelhadid, S. M. (2012). Trials for the control of trichodinosis and gyrodactylosis in hatchery reared *Oreochromis niloticus* fries by using garlic. *Veterinary Parasitology*, *185*, 57–63. https://doi.org/10.1016/j.vetpar.2011.10.035

Ankri, S., & Mirelman, D. (1999). Antimicrobial properties of allicin from garlic. *Microbes and Infection*, *2*, 125–129. https://doi.org/10.1016/S1286-4579(99)80003-3

Bayan, L., Koulivand, P. H., & Gorji, A. (2014). Garlic: A review of potential therapeutic effects. *Avicenna Journal of Phytomedicine*, *4*(1), 1–14.

Borochov-Neori, H., Judeinstein, S., Greenberg, A., Volkova, N., Rosenblat, M., & Aviram, M. (2013). Date (*Phoenix dactylifera* L.) fruit soluble phenolics composition and anti-atherogenic properties in nine Israeli varieties. *Journal of Agricultural and Food Chemistry*, *61*(18), 4278–4286. https://doi.org/10.1021/jf400782v

Bowker, J., & Trushenski, J. (2016). *Guide to using drugs, biologics, and other chemicals in aquaculture*. American Fisheries Society Fish Culture Section. https://drive.google.com/file/d/0B43dblZIJqD3Q2NqQkhfeV84emc/view

Buchmann, K., Jensen, P. B., Kruse, K. D., & (2003). Effects of sodium percarbonate and garlic extract on Ichthyophthirius multifiliis theronts and tomocysts: In vitro experiments. *North American Journal of Aquaculture*, *65*(1), 21–24. https://doi.org/10.1577/1548-8454(2003)065<0021:Eospag>2.0.Co;2

Burgess, P. J., & Matthews, R. A. (1994). *Cryptocaryon irritans* (Ciliophora)—Photoperiod and transmission in marine fish. *Journal of the Marine Biological Association of the United Kingdom*, *74*(3), 535–542.

Cavallito, C., & Bailey, J. (1944). Allicin, the antibacterial principle of *Allium sativum*. I. isolation, physical properties and antibacterial action. *Journal of the American Chemical Society*, *66*(11), 1950–1951. https://doi.org/10.1021/ja01239a048

Colorni, A. (1985). Aspects of the biology of *Cryptocaryon irritans*, and hyposalinity as a control measure in cultured gilt-head sea bream *Sparus aurata*. *Diseases of Aquatic Organisms*, *1*, 19–22. https://doi.org/10.3354/dao001019

Colorni, A. (1987). Biology of *Cryptocaryon irritans* and strategies for its control. *Aquaculture*, *67*, 236–237.

Colorni, A., & Burgess, P. (1997). *Cryptocaryon irritans* Brown 1951, the cause of "white spot disease" in marine fish: An update. *Aquarium Sciences and Conservation*, *1*, 217–238. https://doi.org/10.1023/A:1018360323287

Dickerson, H. W. (2006). Ichthyophthirius multifiliis and Cryptocaryon irritans (phylum Ciliophora). In: P. K. T. Woo (Eds.), *Fish diseases and disorders vol 1. protozoan and metazoan infections* (3nd ed., pp. 116–153). Wallingford, CT: CAB International.

Fridman, S., Sinai, T., & Zilberg, D. (2014). Efficacy of garlic based treatments against monogenean parasites infecting the guppy (*Poecilia reticulata* (Peters)). *Veterinary Parasitology*, *203*(1–2), 51–58. https://doi.org/10.1016/j.vetpar.2014.02.002

Fridman, S., & Zilberg, D. (2014). The development of a small-scale laboratory system to study *Cryptocaryon irritans* infection in seawater-adapted guppies, *Poecilia reticulata*. *The Israeli Journal of Aquaculture – Bamidgeh*, *66*, 1–5.

Goto, T., Hirazawa, N., Takaishi, Y., & Kashiwada, Y. (2015). Antiparasitic effect of matrine and oxymatrine (quinolizidine alkaloids) on the ciliate *Cryptocaryon irritans* in the red sea bream *Pagrus major*. *Aquaculture*, *437*, 339–343. https://doi.org/10.1016/j.aquaculture.2014.12.026

Gruhlke, M., Nicco, C., Batteux, F., & Slusarenko, A. (2016). The effects of allicin, a reactive sulfur species from garlic, on a selection of mammalian cell lines. *Antioxidants*, *6*, 1–16. https://doi.org/10.3390/antiox6010001

Ilic, D., Nikolic, V., Nikolic, L., Stankovic, M., Stanojevic, L., & Cakic, M. (2011). Allicin and related compounds: Biosynthesis, synthesis and pharmacological activity. *Facta Universitatis – Series: Physics, Chemistry and Technology*, *9*(1), 9–20. https://doi.org/10.2298/FUPCT1101009I

INA (Institute for Nutraceutical Advancement). (2004). Determination of allicin in garlic by high performance liquid chromatography. Retrieved from https://doi.org/https://publications.europa.eu/en/publication-detail/-/publication/230610b3-d43f-44f1-97c9-5c77b69b43c6/language-en

Lawson, L. D., Wood, S. G., & Hughes, B. G. (1991). HPLC analysis of allicin and other thiosulfinates in garlic clove homogenates. *Planta Medica*, *57*(3), 263–270. https://doi.org/10.1055/s-2006-960087

Madsen, H. C. K., Buchmann, K., & Mellergaard, S. (2000). Treatment of trichodiniasis in eel (*Anguilla anguilla*) reared in recirculation systems in Denmark: Alternatives to formaldehyde. *Aquaculture*, *186*, 221–231. https://doi.org/10.1016/S0044-8486(99)00379-8

Matthews, R. A., & Burgess, P. J. (1995). *Cryptocaryon irritans* (Ciliophora): Primary infection in thick-lipped mullet, *Chelon labrosus* (Risso). *Journal of Fish Diseases*, *18*, 329–333. https://doi.org/10.1111/j.1365-2761.1995.tb00309.x

Militz, T. A., Southgate, P. C., Carton, A. G., & Hutson, K. S. (2013). Dietary supplementation of garlic (*Allium sativum*) to prevent monogenean infection in aquaculture. *Aquaculture*, *408–409*, 95–99. https://doi.org/10.1016/j.aquaculture.2013.05.027

Militz, T. A., Southgate, P. C., Carton, A. G., & Hutson, K. S. (2014). Efficacy of garlic (*Allium sativum*) extract applied as a therapeutic immersion treatment for *Neobenedenia* sp. management in aquaculture. *Journal of Fish Diseases*, *37*(5), 451–461. https://doi.org/10.1111/jfd.12129

Miron, T., Rabinkov, A., Mirelman, D., Wilchek, M., & Weiner, L. (2000). The mode of action of allicin: Its ready permeability through phospholipid membranes may contribute to its biological activity. *Biochimica Et Biophysica Acta – Biomembranes*, *1463*, 20–30. https://doi.org/10.1016/S0005-2736(99)00174-1

Picón-Camacho, S. M., Ruiz de Ybáñez, M. R., Holzer, A. S., Arizcun Arizcun, M., & Muñoz, P. (2011). *In vitro* treatments for the theront stage of the ciliate protozoan *Cryptocaryon irritans*. *Diseases of Aquatic Organisms*, *94*, 167–172. https://doi.org/10.3354/dao02315

National Toxicology Program. (2010). Report on Carcinogens - Background Document for Formaldehyde, U.S. Department on Health and Human Services, Public Health Service, National Toxicology Program, Research Triangle Park, NC.

Rabinkov, A., Miron, T., Mirelman, D., Wilchek, M., Glozman, S., Yavin, E., & Weiner, L. (2000). *S*-Allylmercaptoglutathione: The reaction product of allicin with glutathione possesses SH-modifying and antioxidant properties. *Biochimica Et Biophysica Acta – Molecular Cell Research*, *1499*, 144–153. https://doi.org/10.1016/S0167-4889(00)00119-1

Reverter, M., Bontemps, N., Lecchini, D., Banaigs, B., & Sasal, P. (2014). Use of plant extracts in fish aquaculture as an alternative to chemotherapy: Current status and future perspectives. *Aquaculture*, *433*, 50–61. https://doi.org/10.1016/j.aquaculture.2014.05.048

Schnick, R. A., & Meyer, F. P. (1978). Registration of thirty-three fishery chemicals: Status of research and estimated costs of required contract studies. In *Investigations in fish control 86. U.S. Department of the Interior, Fish and Wildlife Service* (pp. 8–9). Washington, DC: Fish and Wildlife Services.

Valladão, G. M. R., Gallani, S. U., & Pilarski, F. (2015). Phytotherapy as an alternative for treating fish disease. *Journal of Veterinary Pharmacology and Therapeutics*, *38*, 417–428. https://doi.org/10.1111/jvp.12202

Wang, J. L., Lao, G. F., Li, Y. W., Yang, M., Mo, Z. Q., & Dan, X. M. (2018). Effects of temperature and host species on the life cycle of *Cryptocaryon irritans*. *Aquaculture*, *485*, 49–52. https://doi.org/10.1016/j.aquaculture.2017.11.024

Watanabe, Y., How, K. H., Zenke, K., Itoh, N., & Yoshinaga, T. (2018). Dormancy induced by a hypoxic environment in tomonts of *Cryptocaryon irritans*, a parasitic ciliate of marine teleosts. *Aquaculture*, *485*, 131–139. https://doi.org/10.1016/j.aquaculture.2017.11.026

Yanong, R. P. E. (2009). Cryptocaryon irritans infections (marine white spot disease) in fish. FA164, One of a Series of the Fisheries and Aquatic. Sciences Department, UF/IFAS, Extension, pp. 1–9.

Yoshinaga, T., & Dickerson, H. W. (1994). Laboratory propagation of *Cryptocaryon irritans* on a saltwater-adapted *Poecilia* hybrid, the black molly. *Journal of Aquatic Animal Health*, *6*, 197–201. https://doi.org/10.1577/1548-8667(1994) 006<0197:LPOCIO>2.3.CO;2

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