
Volume 198, issues 1–2 15 November 2013
ISSN 0304-4017
ELSEVIER
veterinary parasitology
An International Scientific Journal
Official Organ of the American Association of Veterinary Parasitologists (A.A.V.P.), the European Veterinary Parasitology College (E.V.P.C.) and the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.)

Veterinary Parasitology 198 (2013) 96–101

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

In vitro and *in vivo* efficacy of garlic compounds against *Gyrodactylus turnbulli* infecting the guppy (*Poecilia reticulata*)

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ARTICLE INFO

Article history:
Received 15 April 2013
Received in revised form 27 August 2013
Accepted 30 August 2013

Keywords:
Garlic
Guppy
Gyrodactylosis
Monogenean
Poecilia reticulata
Treatment

ABSTRACT

Traditional compounds used to treat fish diseases in aquaculture and the ornamental fish industry (such as formalin and malachite green) can be more toxic to the hosts than their parasites. With the reviviscence in the use of herbal products, various botanicals have been heralded as cures for particular pathogens, but the efficacy of these compounds for parasitic worms is questionable. Here, we tested a range of garlic (*Allium sativum*) products against a major aquarium pathogen, *Gyrodactylus turnbulli*, infecting the guppy (*Poecilia reticulata*). All garlic products significantly reduced parasite mean survival time *in vitro*, from 13 h to <1 h. In fully randomised trials, the number of parasites was also significantly reduced on infected fish exposed to garlic from different sources. Two garlic treatments (minced and granule forms) reduced worm burdens by 66% and 75% after three doses, whereas Chinese freeze-dried garlic and allyl disulphide were 95% effective after a single application. In fact, Chinese freeze dried garlic was equally effective as Levamisole, a licensed livestock dewormer that is highly effective against *G. turnbulli* but not routinely prescribed for use in fish; hence, garlic may be a potential alternative treatment for gyrodactylosis.

1. Introduction

Ornamental fish aquaculture accounted for US$238 and 283 million in global exports and imports, respectively, in 2005 alone and the trade is increasing (European Commission, 2008). As with food fish, ornamental aquaculture is greatly affected by infectious disease, the containment of which relies heavily on broad spectrum anti-parasitic compounds (Srivastava et al., 2004; Wooster et al., 2005). Due to their toxicity, use of these anti-parasitic compounds is being restricted. Malachite green was banned in the UK for use on food fish in 2002, as it is a known carcinogen (Srivastava et al., 2004). Legal application of formalin is expected to be reduced in the near future, too, for both environmental and human health reasons (Wooster et al., 2005; Cabello, 2006; Commission of the European Communities, 2009). No effective alternatives to malachite green and formalin are available, but they are urgently needed to counteract increasing pathogen resistance to commonly used drugs, and to improve fish welfare, conservation and aquaculture economy (Cabello, 2006; Ashley, 2007).

Traditional herbal remedies such as garlic (*Allium sativum*) offer potentially new treatments for a range of parasitic infections (Anthony et al., 2005). Garlic and its metabolites have antibacterial, antiprotozoal, antifungal and antiviral properties (reviewed by Ankri and Mirelman, 1999; Harris et al., 2001; Williams and Lloyd, 2012). The evidence for garlic efficacy against helminths, however, is ambiguous (positive effect: Riad et al., 2009; Strickland et al., 2009; Masamha et al., 2010; Abd El-Galil and Aboelhadid, 2012; Militz et al., 2013; no effect: Burke et al., 2009; Worku et al., 2009). In aquaculture and the hobbyist

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http://dx.doi.org/10.1016/j.vetpar.2013.08.027

market, the use of garlic is generally recommended as a broad spectrum prophylactic anti-parasitic treatment and appetite stimulant due to its long history of use on Asian fish farms and recommendations in fish keeping magazines (Cortes-Jorge, 2000; Bartelme, 2003; Haskell et al., 2004; Rodgers and Furones, 2009; Jegede, 2012). Indeed, garlic has been shown to eradicate a range of fish pathogens *in vitro* (Wei and Musa, 2008; Peyghan et al., 2008) and *in vivo* (Ankri et al., 1997; Wattley, 1999; Buchmann et al., 2003; Soko and Barker, 2005; Chitmanat et al., 2005; Nya et al., 2010). There is some evidence that garlic is effective against monogenean parasites reducing *Anacanthorus penilabiatus* infections in cultivated pacu (*Piaractus mesopotamicus*; see Martins et al., 2002), *Neobenedenia* sp. in barramundi (*Lates calcarifer*, see Militz et al., 2013) and gyrodactylosis in *Oreochromis niloticus* fry (Abd El-Galil and Aboelhadid, 2012).

Gyrodactylids are ubiquitous, monogenean ectoparasites of teleost fish which have the potential for explosive population growth due to their short generation time (24 h doubling time at 25 °C; Scott, 1982), hyper-viviparity (Cable and Harris, 2002) and multiple reproductive modes (Schelkle et al., 2012). Containment of infection is difficult as potential treatments are not 100% effective (Bakke et al., 2007; Schelkle et al., 2009, 2010; Bowker et al., 2012), with exception of Levamisole which is not routinely prescribed for fish in the UK (William H Wildgoose, personal communication) and requires very controlled application (Schelkle et al., 2009). Hence, this study compares the efficacy of four garlic products and two garlic components as alternative treatments against *Gyrodactylus turnbulli* infections on guppies, *Poecilia reticulata*, with the aim of determining whether any have the potential to be used commercially.

2. Materials and methods

2.1. Source of animals and herbal compounds

The current study utilised juveniles or young adults (mean standard length ± SE: 11 ± 0.3 mm; less than one year old) from an inbred ornamental stock of guppies (*P. reticulata*) and a pet shop strain of *G. turnbulli* (*Gt3*). An additional isogenic parasite strain, *Gt1*, originally isolated from guppies obtained from the Lower Aripo River, Trinidad, in November 2001 served as an additional negative control for *in vitro* experiments. All experiments were conducted at 25 ± 0.5 °C with a 12 h light:12 h dark cycle and fish were fed twice daily on Aquarian®tropical fish flakes.

Six garlic treatments including two garlic components were tested: Chinese freeze dried garlic powder and freeze dried garlic flakes (Cultech, Port Talbot); minced garlic and garlic granules (ASDA Stores Ltd.); allyl alcohol and allyl disulphide (Sigma Aldrich, the latter dissolved with 0.01% Tween 20). *In vitro* garlic concentrations were established using preliminary trials on a small number of parasites to ensure doses were safe for use in fish. Dosing levels were in line with those shown to be non-toxic in other fish species (Nile tilapia fry) (Abd El-Galil and Aboelhadid, 2012). *In vivo* garlic concentrations were chosen based on the most effective *in vitro* concentrations, but may have needed further adjustment (typically doubling or halving the final *in vitro* concentration) to maintain fish health and welfare or to increase treatment efficacy if it was safe for fish to do so. This selection process led to a range of concentrations being used which was not consistent between treatments (Tables 1 and 2), but which lie within the range of those used in other studies both *in vitro* and *in vivo* (range: 0.08–500 mg/ml; *e.g.* Wei and Musa, 2008; Millet et al., 2010; Nya et al., 2010; Abd El-Galil and Aboelhadid, 2012). All treatments were prepared as concentrated stock solutions and stored in sealed containers overnight at 4 °C. Positive (Levamisole: Levacide 7.5% solution, Norbrook®, UK) and negative (dechlorinated water) controls were used, in addition to an emulsifier control for allyl disulphide, 0.01% Tween 20, for both *in vitro* and *in vivo* experiments.

2.2. *In vitro*

The effects of the six different garlic treatments were tested on individuals of *G. turnbulli* ($n \pm SE = 35 \pm 5.6$ per treatment) isolated from fin clips or scales of naturally infected *P. reticulata* donors, which were held as stock fish within the parasitology laboratory. Parasites were dislodged from host tissue using fine insect pins and transferred individually to wells of 96 well microtitre plates in 20 μl of dechlorinated water using a micropipette (following the methods of Cable et al., 2002a). Any worms that died or showed any abnormal behaviour (see Cable et al., 2002a, b) within 1 h were discarded from the trial. Thereafter, 180 μl of treatment was added to the wells containing a healthy worm leading to a final concentration of between 0.01 and 18 mg/ml (Table 1). To the controls, the same volume of water, Levamisole or Tween 20 was added. The parasites were observed immediately after application of the treatment, 5 min later and thereafter every hour with their status recorded until they died. Parasites were classified as alive and moving, moribund (movement after insect pin has been passed near non moving parasite) or dead as described in Cable et al. (2002a).

2.3. *In vivo*

Fish acquired parasites naturally from other guppies through exposure to conspecifics or were utilised following completion of behavioural experiments conducted in the laboratory by other researchers (mean parasite load ± SE: 35 ± 2, range: 2–285). Recipient fish ($n \pm SE = 29 \pm 4$ per treatment) were observed with a low power stereo-microscope (Nikon C-DSLS) and fibre optic illumination and the number of attached parasites counted. Infected fish were isolated for use in trials and subsequently transferred to 1 ml of dechlorinated water in a glass dish, which is sufficient to fully immerse the juvenile fish in water, but confinement allows the fish to be continuously viewed with a stereo-microscope. Immediately after transfer, 100 μl of the treatment was applied (initial high dose). After 5 min fish were screened again, then transferred with the initial treatment solution to a larger

Table 1
In vitro effects of garlic compounds at various concentrations on *Gyrodactylus turnbulli* with sample sizes (N), mean maximum survival (in h), standard error (SE) and minimum and maximum survival times (range in h).

Treatments *in vitro*	Conc. (mg/mL)	*N*	Mean survival	SE Mean	Range of survival
Water *Gt1* strain (control)	0	59	11.39	0.43	6–21
Water *Gt3* strain (control)	0	209	13.72	0.57	2–31
Levamisole (control)	18	50	0.18	0.04	0.08–1
	6	30	0.36	0.10	0.08–2
Tween 20 (control)	0.1	20	1.1	0.07	1–2
Allyl alcohol	10	30	0.08	0	0.08
	7.5	30	0.42	0.08	0.08–1
	5	30	1.07	0.07	0.08–2
	3.75	20	1.33	0.22	0.08–3
Allyl disulphide with 0.01% Tween 20	0.5	20	0.08	0	0.08
	0.4	20	0.08	0	0.08
	0.1	20	0.27	0.08	0.08–1
	0.05	20	0.59	0.11	0.08–1
Chinese freeze dried garlic powder	18	31	0.21	0.09	0.08–2
	9	30	0.08	0	0.08
	6	30	0.68	0.12	0.08–2
	0.09	38	15.46	1.31	0.08–27
Freeze dried garlic flakes	18	32	0.08	0	0.08–0.08
	10	30	0.08	0	0.08–0.08
	6.67	29	0.08	0	0.08–0.08
	6	30	0.74	0.14	0.08–2
	1	30	0.36	0.08	0.08–1
	0.75	30	0.64	0.11	0.08–2
	0.5	30	0.45	0.08	0.08–1
	0.07	32	0.76	0.13	0.08–2
	0.01	38	16.09	1.34	0.08–27
Garlic granules	18	30	0.11	0.03	0.08–1
	6	33	0.08	0	0.08
	2.25	30	0.08	0	0.08
Minced garlic	18	36	0.31	0.07	0.08–1
	6	35	0.45	0.08	0.08–1

container with 99 ml of water (subsequent low dose). Two further observations of the fish under anaesthetic (0.02% MS222) were carried out at 1 h and 24 h post-treatment to again count parasites. If fish were still infected 24 h after initial treatment, they received up to two subsequent treatments with a 24 h interval between each treatment which affected over a third of the replicates ($n = 133$).

2.4. *Ethical note*

This work was conducted under UK Home Office licence (PPL 30/2357) regulations with approval by the Cardiff University Ethics Committee. All fish utilised in these experiments were infected with *G. turnbulli* at levels well tolerated by guppies and were closely monitored throughout the trials. If the parasite burden appeared

Table 2
In vivo effects of garlic treatments at various concentrations on *Gyrodactylus turnbulli* infected guppies (*Poecilia reticulata*) with their samples size (*N*), mean maximum efficacy and standard error (SE) of the mean. Efficacies ranged for all treatments between 0 and 1, with 0 being not efficacious and 1 having 100% efficacy.

Treatment	Conc. (mg/ml)	*N*	Mean max. efficacy	SE mean
Water (control)	0	60	0.23	0.05
Levamisole (control)	0.2	48	0.94	0.04
Tween 20 (control)	0.1	20	0.33	0.10
Allyl alcohol	10	23	0.61	0.10
Allyl disulphide + Tween 20	0.5	20	0.95	0.05
Chinese freeze dried garlic powder	0.03	20	0.95	0.05
Freeze dried garlic flakes	1	28	0.96	0.04
	0.5	20	0.76	0.09
	0.07	10	0.3	0.15
Minced garlic	0.07	30	0.78	0.07
Garlic granules	0.07	40	0.78	0.07
	0.03	30	0.59	0.09

to be influencing fish behaviour and welfare, hosts were removed from trials and treated immediately with Levamisole to clear them from parasite infection. Fish completely cleared of *G. turnbulli* received three separate screenings to confirm that they were clear of parasites before being returned into aquaria housing other stock fish (Schelkle et al., 2009).

2.5. Statistical analyses

In vitro data were analysed with a non-parametric Cox proportional hazard model with an average hazard, time-to-death as independent variable and treatment as dependent variable. For this analysis, which does not allow for nesting groups, treatments at different concentrations were tested independently.

Efficacy of *in vivo* treatments was expressed as $\Delta E_t = (L_0 - L_t)/L_0$, for $L_t < L_0$, and $\Delta E_t = 0$ when $L_t > L_0$ according to Schelkle et al. (2010) to account for the initial variation in parasite load where E = treatment efficacy; L_0 = pre-treatment parasite load; and L_t = post-treatment parasite load. Differences in efficacy were assessed with a Kruskal–Wallis test with treatment as independent variables and *post hoc* differences between groups assessed using Mann Whitney tests. Multiple testing was accounted for by Bonferroni corrections after Benjamini and Yekutieli, 2001(see also Narum, 2006) leading to an α-level of $p = 0.010472$. In the *in vivo* experiment, a Mann Whitney test investigated differences in efficacy between the allyl disulphide treated and control fish. All analyses were performed in R v.2.10.0 (R Development Core Team, 2010).

3. Results

Chinese freeze dried garlic and allyl disulphide (the latter applied with 0.01% Tween 20) were the most effective treatments both *in vitro* and *in vivo*, clearing gyrodactylid infections on fish in a single application. Freeze dried garlic was similarly effective, but required three treatment applications to reach the same efficacy (Tables 1 and 2).

3.1. *In vitro*

In untreated controls (water), parasites of the *Gt1* and *Gt3* strains survived an average of 11.4 h and 13.7 h, respectively, with a maximum of 21 h and 31 h, comparable with previous experiments conducted at 25 °C on *G. turnbulli* (see Schelkle et al., 2010). Most garlic treatments were as effective as Levamisole in reducing parasite survival to an hour or less (Cox proportional hazard survival analysis: likelihood ratio test = 1525, d.f. = 32, $p < 0.001$; Table 1, Supplementary Material S1), apart from freeze dried garlic flakes at a concentration of 0.01 mg/ml and Chinese freeze dried garlic powder at 0.09 mg/ml, both of which actually increased average survival time to over 15 h.

3.2. *In vivo*

When infected fish were treated with an initial high dose and subsequent low dose of Chinese freeze dried garlic flakes (0.033 mg/ml), freeze dried garlic flakes (1 mg/ml) and allyl disulphide (0.5 mg/ml), parasite survival was significantly reduced compared to the control with all three treatments equally effective (Kruskal–Wallis: $\chi^2 = 132.34$, d.f. = 11, $p < 0.001$) (Table 2, Supplementary Material S2). Efficacy was increased for all three treatments (mean efficacies of 0.964, 0.95 and 0.95, respectively) compared to the negative control (mean efficacy: 0.227) and was similar to the positive control Levamisole (mean efficacy: 0.938). However, garlic induced slight damage to the fin edges, particularly at higher doses, although fish recovered well post-treatment in aquarium water. Any fin damage quickly healed within weeks of the trial being completed.

4. Discussion

The current study shows unambiguously that different forms of garlic can significantly reduce the survival of gyrodactylids. Generally, treatments were more effective *in vitro* and may be due to worms not being protected by the host's mucus layer, which provides a barrier to water borne compounds (reviewed by Shephard, 1994; Burka et al., 1997; Bakke et al., 2007). Nevertheless, the *in vitro* tests were useful in providing a general indication of the suitability of a compound as a treatment and its effective concentration: ineffective *in vitro* botanicals therefore did not require *in vivo* testing, so reducing the number of animals used for the trials.

The most potent treatments against *G. turnbulli* were Chinese freeze dried garlic powder, freeze dried garlic flakes and allyl disulphide. The high efficacy of whole garlic preparations is potentially due to synergistic effects of garlic's constituents (Kyung, 2012; Williams and Lloyd, 2012) or result from the preservation process which alters bioactivity (*i.e.* freeze drying, mincing, *etc.*; Lemar et al., 2002). For instance, one of the main active compounds of garlic to become concentrated during the freeze drying process is allicin (Ratti et al., 2007), which degrades rapidly and therefore might not be very efficacious (see Williams and Lloyd, 2012). The lowered effectiveness may, however, be counter-acted by one of allicin's breakdown products, allyl disulphide, which when tested at 0.5 mg/ml was as effective as Levamisole against gyrodactylids.

The current study builds upon experiments by Abd El-Galil and Aboelhadid (2012) which indicate that garlic oil administered over 24 h may be effective against gyrodactylids infecting *O. niloticus* fry in a time and dose dependent manner, and that prolonged exposure may slow the spread of disease. This previous study and our data suggest that gyrodactylids are directly affected by the botanical rather than indirectly *via* enhanced host immune function, which in fish may take several weeks to develop (Martins et al., 2002; Sahu et al., 2007).

Garlic efficacy against gyrodactylids *in vitro* is dose dependant. High doses of and/or prolonged treatment with garlic may impair fish health and behaviour highlighting the importance of determining appropriate dosage requirements for the particular host–parasite system and the method of application (Chitmanat et al., 2005; Schelkle et al., 2010). With exception of allyl alcohol application at 10 mg/ml, all treatments in the current study were used at 1 mg/ml or less which is 100× less the lethal limit established by Abd El-Galil and Aboelhadid (2012) and Chitmanat et al. (2005). Such comparatively low doses also allow concentration adjustments which may help to counteract natural variation in garlic efficacy arising from climate during plant growth and geographical origin (Burt, 2004). However, even minor concentration changes have to be monitored carefully and hobbyists who currently use garlic *ad hoc*, should be made aware of the potential harm to their fish by using high garlic doses.

In the UK, garlic treatments are already commercially available, in both feed and water additive formats; however, there is no published data on the efficacy of these products on helminth infection in fish. This study supports the growing evidence of garlic's general anti-helminthic properties indicating that conserved garlic preparations and the garlic compound allyl disulphide can effectively reduce and, in some cases, eliminate infections of the aquarium pathogen *G. turnbulli*.

Acknowledgements

We thank Linda Erlandsson, Tracey A. King, Loys Richards and Claire Yarwood for practical help. This work was funded by a Biotechnology and Biological Sciences Research Council (BB/D526137/1) CASE studentship with WALTHAM Centre for Pet Nutrition to BS.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2013.08.027.

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