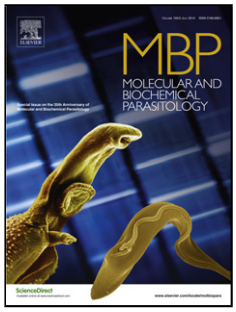


Title: The redox-active drug metronidazole and thiol-depleting garlic compounds act synergistically in the protist parasite *Spironucleus vortens*

Author: C.F. Williams A.R. Vacca L. Dunham D. Lloyd M.P. Coogan G. Evans M. Graz J. Cable

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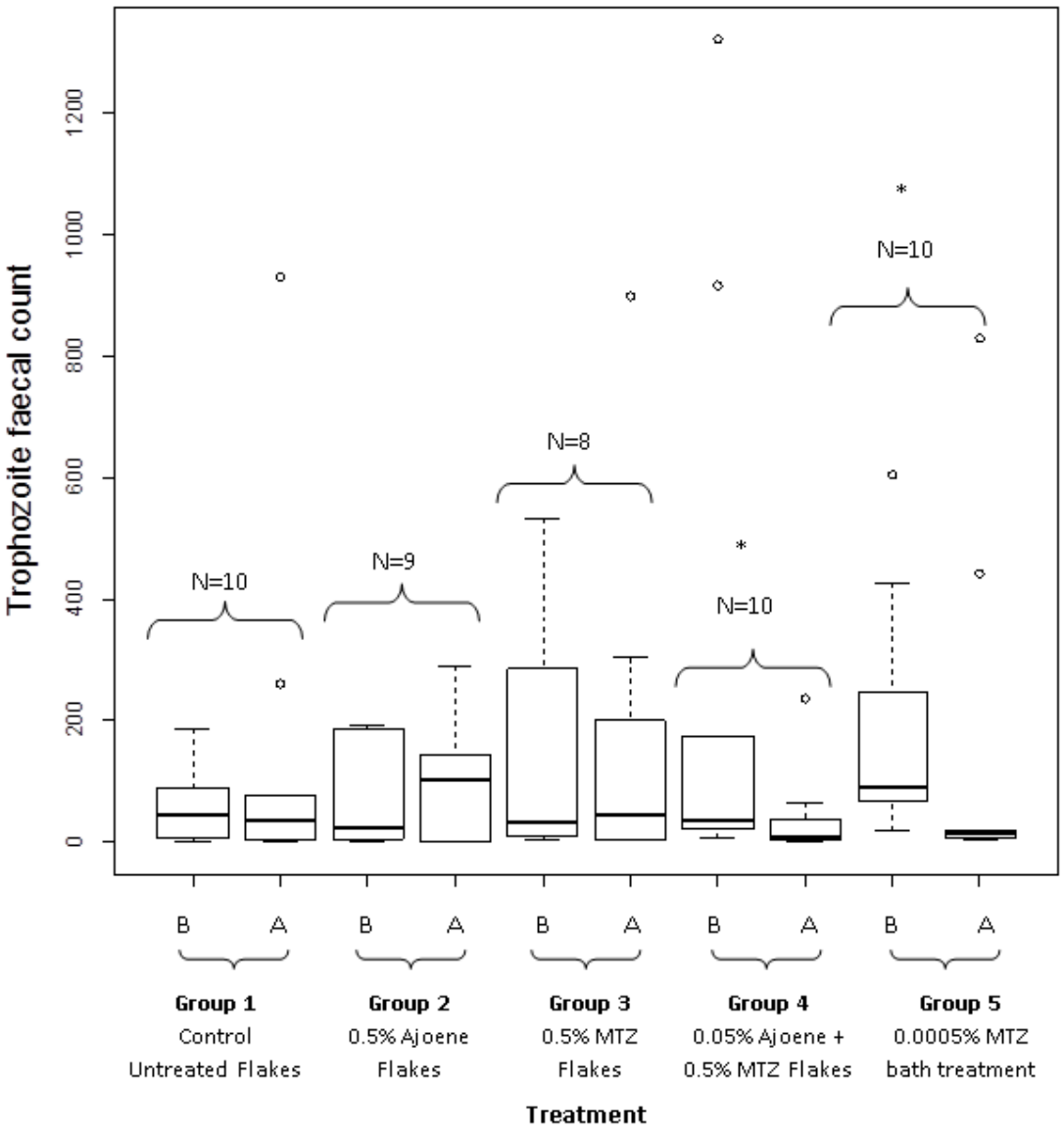
**HIGHLIGHTS**

- Synergism between ajoene and metronidazole is effective *in vitro* and *in vivo*
- Ajoene reduces the minimum inhibitory concentration of metronidazole by 16-fold
- The efficiency of the ajoene + metronidazole treatment was 64.5% in fish

**GRAPHICAL ABSTRACT:**




**Summary:**

Treatment of *Pterophyllum scalare* (angelfish) with a combination of ajoene and MTZ-supplemented diet significantly reduces *Spironucleus vortens* faecal shedding in a synergistic manner.

# The redox-active drug metronidazole and thiol-depleting garlic compounds act synergistically in the protist parasite *Spironucleus vortens*

Williams CF[1*], Vacca AR[1], Dunham L[1], Lloyd D[1], Coogan MP[2], Evans G[3], Graz M[3] & Cable J[1]

[1]Cardiff University, School of Biosciences, Museum Avenue, Cardiff, CF10 3AT, UK
[2]Department of Chemistry, Lancaster University, Lancaster, LA1 4YB, UK
[3]Neem Biotech Ltd, Units G and H, Roseheyworth Business Park, Roseheyworth, Abertillery, Blaenau Gwent, NP13 1SX, UK

*Corresponding author. Email: williamscf@cf.ac.uk

**Keywords:** Ajoene; 5-nitroimidazole; angelfish; *in vivo*; *in vitro*

**Abbreviations:**

Allyl alcohol, AA; allyl disulphide, AD; allyl methyl disulphide, AMD; allyl methyl sulphide, AMS; allyl sulphide, AS; diallyl disulphide, DADS; diallyl trisulphide, DATS; fractional inhibitory concentration, FIC; methyl disulphide, MD; metronidazole, MTZ; minimum inhibitory concentration (MIC); propyl disulphide, PD; propyl sulphide, PS.

**ABSTRACT**

*Spironucleus vortens* is a protozoan parasite associated with significant mortalities in the freshwater angelfish, *Pterophyllum scalare*. Control of this parasite is especially problematic due to restrictions on the use of the drug of choice, metronidazole (MTZ), on fish farms. Use of garlic (*Allium sativum*) is undergoing a renaissance following experimental validations of its antimicrobial efficiency. Ajoene ((E,Z)-4,5,9-trithiadodeca-1,6,11-triene 9-oxide), is a stable transformation product of allicin, the primary biologically active component of garlic. In the current study, an ajoene oil crude extract had a minimum inhibitory concentration (MIC) of 40 µg/ml against *S. vortens*. GC-MS and NMR spectroscopy revealed this ajoene extract contained a mixture of the (E) and (Z)-ajoene isomers along with diallyl disulphide (DADS) and diallyl trisulphide (DATS). The only component of the ajoene crude oil found to substantially inhibit *S. vortens* growth by optical density monitoring (Bioscreen C Reader) was (Z)-ajoene (MIC 16 µg/ml). Ajoene oil acted in synergy with MTZ *in vitro*, reducing the individual MIC of this drug (4 µg/ml) by 16-fold, and that of ajoene oil by 200-fold with a fractional inhibitory concentration (FIC) index of 0.263. This synergistic interaction was confirmed *in vivo*. *S. vortens*-infected *P. scalare* angelfish dosed orally with 0.5% (v/w) MTZ combined with 0.05% (v/w) ajoene displayed a significant reduction in faecal trophozoite count, whilst those fed on 0.5% MTZ flakes (half the recommended oral dose) alone did not. This study demonstrates for the first time the synergistic interaction between the synthetic drug MTZ and natural ajoene oil both *in vitro* and *in vivo*. Future work should evaluate the potential synergy of ajoene and MTZ against MTZ-resistant bacteria and protists.

## 1. INTRODUCTION

Disease control is a major limiting factor of fish production in aquaculture. Treatments such as malachite green, antibiotics and other drugs have been banned from use on food fish in Europe and North America due their toxicity and carcinogenicity in fish and humans (see reviews by Schelkle et al. 2009; Williams et al. 2011). Hence tonnes of fish are lost annually due to disease outbreaks and ineffective infection control (FAO 2012). This is especially true in the case of Spironucleosis, an intestinal disease caused by the protozoan parasite *Spironucleus*. Several outbreaks of Spironucleosis have been documented on fish farms (Mo et al. 1990; Poppe et al. 1992; Poppe & Mo 1993; Sterud et al. 1998), with the only known effective treatment being the 5-nitroimidazole, metronidazole (MTZ), which has been banned from use on European and American food fish due to its carcinogenic propensities in humans (Commission Regulation No. 613/98 1998; FARAD 2010). MTZ is still available for treatment of *Spironucleus vortens* in ornamental fish, including *Pterophyllum scalare* (angelfish) in contained aquaria. However this synthetic drug has been documented as cytotoxic and genotoxic in fish and requires veterinary prescription, and so is only used as a last resort (Cavas & Ergene-Gozukara 2005; Khalil et al. 2007). *S. vortens* is an opportunistic pathogen and has a direct life cycle, infecting the freshwater fish host via the faecal-oral route, its primary habitat being the hind-gut (Williams et al. 2011). Infections may become systemic in immunosuppressed hosts, leading to a characteristic condition known as hole-in-the-head disease, and possibly death (Paull & Matthews 2001).

The antimicrobial effects of *Allium sativum* (garlic)-derived compounds have been extensively documented and validated *in vitro* (reviewed by Williams & Lloyd 2012). Despite this, little in the way of pharmacological development of *Allium*-based drugs has taken place (Martin & Ernst 2003). Garlic extracts are commonly used as fish food additives in aquaculture as a result of their ability to improve the growth, immune response and survival of fish (Palavesam et al. 2006; Sahu et al. 2007; Immanuel et al. 2009; Millet et al. 2011a). Garlic has mainly been tested as a preventative agent in aquaculture, for example in the prevention of *Lates calcarifer* (barramundi) infection with the monogenean parasite *Neobenedenia* sp. (Militz et al. 2013), the prevention of *Aeromonas hydrophila* in *Oncorhynchus mykiss* (rainbow trout, Nya et al. 2009; 2010), and increased disease resistance of *Lates calcarifer* (Asian sea bass) against *Vibrio harveyi* infection (Talpur & Ikhwanuddin 2012). Garlic has also been shown to be an effective therapeutic agent against *Neobenedenia* sp. in barramundi (Militz et al. 2014). Another promising application of garlic in veterinary and human medicine is as a synergistic partner to commonly prescribed antibiotics (see

review by Sivam 2001). In this context, its predicted roles include retardation of the development of antibiotic resistance (Nnamchi & Nwosa 2013) and increased antibiotic efficiency via reduction of the minimum inhibitory concentration (MIC) of the partner antibiotic against antibiotic resistant bacteria (Pillai et al. 2013; Singh et al. 2001; Sohn et al. 2009). Synergistic activity of the garlic-derived compound allicin with streptomycin has been demonstrated to have an immunosuppressive effect in *Dicentrarchus labrax* (sea bass) infected with *Mycobacterium marinum* (see Colorni et al. 1998). However the synergistic role of such a treatment has yet to be proven as an effective antimicrobial agent in aquaculture.

Most studies investigating the antimicrobial effects of garlic use allicin (diallyl thiosulfinate, Ankri & Mirelman 1999). However, due to the unstable nature of allicin, it is likely that many of the antimicrobial effects of garlic can be attributed to the reaction products of this highly reactive garlic component, and not always directly linked to the action of allicin itself. On decomposition, allicin yields hundreds of reaction products, many of which are antimicrobial (Block 2010). Of particular note is ajoene ((*E*, *Z*)-4, 5, 9-trithiadodeca-1, 6, 11-triene 9-oxide), which is extracted from allicin under heating or methanolic conditions (Block et al. 1984; Naznin et al. 2008). Ajoene has a comparable antimicrobial activity to that of allicin; however its stability is much greater after exposure to elevated temperature and UV light, and following long term storage (Naznin et al. 2008).

We have previously shown that *Allium*-derived compounds, including ajoene oil, are effective against *Spironucleus vortens*, the putative causative agent of hole-in-the-head disease in *P. scalare* (see Paull & Matthews 2001), *in vitro* (Millet et al. 2011a). In a subsequent study (Williams et al. 2012) we revealed the mode of action of ajoene oil and MTZ in *S. vortens*, implicating redox imbalance as the mechanism of action of both treatments. As a result, the current study aimed to evaluate the combinatorial effect of MTZ with ajoene oil *in vitro* and *in vivo*, using *P. scalare* naturally infected with *S. vortens*.

## 2. MATERIALS AND METHODS

### 2.1. Organism and culture

Trophozoites of two *Spironucleus vortens* strains, ATCC 50386 and *Sv1* (freshly isolated from juvenile English-bred *Pterophyllum scalare*, described in Williams et al. 2013), were maintained separately in Keister's modified TYI-S-33 medium using a modified method employed by Millet et al. (2011b), but without bile supplementation. This medium contained per 900 ml distilled $H_2O$: 20 g pancreatic digest of casein (Oxoid); 10 g yeast extract (Oxoid); 10 g glucose (Acros Organics); 2 g NaCl (Fisher Scientific); 2 g L-cysteine HCl (Sigma-

Aldrich); 0.2 g ascorbic acid (Sigma-Aldrich); 1 g $K_2HPO_4$ (Fisher Scientific); 0.6 g $KH_2PO_4$ (Fisher Scientific) and 22.8 mg ferric ammonium citrate (Sigma-Aldrich). On dissolving these constituents, the pH of the medium was adjusted to 6.8 using NaOH (Fisher Scientific) and autoclaved, before adding 100 ml sterile, heat-inactivated new-born calf serum (Gibco). Trophozoites were routinely subcultured aseptically after 48 h incubation, by transferring 500 µl of a homogenous log-phase culture into 10 ml of culture medium, leaving a 5 ml head space in 15 ml screw capped Falcon tubes (Greiner Bio-one). When accurate cell counts were necessary, trophozoites were fixed using 1.5% (v/v) formaldehyde and counted using an Improved Neubauer haemocytometer (Weber Scientific International). To ensure axenic maintenance, cultures were regularly checked for microbial contamination by plating-out 100 µl of a log-phase culture on nutrient agar and incubating under the above conditions for 5 d. The identity of the new *S. vortens* Sv1 isolate was confirmed via 16S rDNA PCR, as described in Williams et al. (2013).

### 2.2. Inhibitors

The garlic-derived allicin (2% v/v), ajoene oil (18% v/v), (*Z*)-ajoene (98% v/v), (*E*)-ajoene (98% v/v) and dithiin (un-quantified dithiin-rich fraction) extracts were provided by NEEM Biotech Ltd. All other garlic-derived compounds, allyl alcohol (AA), allyl sulphide (AS), allyl disulphide (AD), diallyl disulphide (DADS), diallyl trisulphide (DATS), methyl disulphide (MD), allyl methyl sulphide (AMS), allyl methyl disulphide (AMD), propyl sulphide (PS) and propyl disulphide (PD) were obtained from Lancaster Synthesis Ltd. The 5-nitroimidazole, metronidazole (MTZ) was sourced form Sigma-Aldrich.

### 2.3. Gas chromatography mass spectrometry (GC-MS)

In order to assess the impurities present in the ajoene oil (18% (v/v)) preparation, samples were subjected to GC-MS analysis. The instrument used was an Agilent 6890 GC with a 5973N MS and a 7683 AS. Sample separation was achieved using an Agilent HP-5MS non-polar column with a 30 m x 0.25 mm internal diameter and a 0.25 µm phase thickness. An on-column injection method was employed with an injection port temperature of 36°C, and an injection volume of 0.2 µl. The initial oven temperature was 35°C, with an initial ramp of 5°C/min to 240°C, where it was held for 3 min, and a second ramp of 10°C/min to a final temperature of 300°C, held for 2 min. The total run time was 55 min. The MS source temperature was 230°C, with scanning between 35-250 amu ($EI^+$ 70eV). Samples were analysed after a 10 min solvent delay and identified by the MS library software (Agilent

ChemStation). A positive peak ID was only confirmed with >90% accuracy (R match) from the library.

### 2.4. Nuclear magnetic resonance (NMR) spectroscopy

The presence of (*E*) and/or (*Z*)-ajoene isomers in the ajoene oil extract was determined by $^{1}$H NMR spectroscopy. Ajoene oil was diluted 1:100 in $CDCl_3$ (Sigma-Aldrich) and its $^{1}$H NMR spectrum was recorded according to Millet et al. (2011b) using a Bruker Avance DPX400 spectrometer with a 5 mm $^{1}$H probe at 400 MHz and 300 K. The acquisition time of spectra was 2.56 s using 90° pulses and a relaxation delay time of 1 s over a spectral width of 11 ppm over 16 scans. $CDCl_3$ solvent was used as an internal lock and chemical shifts are expressed as parts per million (ppm). Compounds were identified by comparison with spectra of standard solutions according to Naznin et al. (2008).

### 2.5. Preparation of inhibitors

The inhibitory effects of a number of *Allium*-derived compounds were quantified against *S. vortens* (ATCC strain) *in vitro*, these were: allicin, ajoene oil, (*E*)-ajoene, (*Z*)-ajoene, AA, AS, AD, DADS, DATS, MD, AMS, AMD, PS, PD and dithiins (concentration range: 100, 50, 25, 12.5, 6, 3 and 1, 0.5 and 0.25 µg/ml and 1:100, 1:200, 1:400, 1:800, 1:1600 for the dithiin-rich extract). MTZ was used as a positive control (concentration range: 160, 80, 40, 20, 10, 5, 2.5, 1, 0.5 and 0.25 µg/ml). Various combinations of ajoene oil and MTZ were also employed using the following concentration ranges: 8, 4, 2, 1, 0.5, 0.25 µg/ml ajoene oil and 4, 2, 1, 0.5 and 0.25 µg/ml MTZ (the MIC of ajoene oil and MTZ being 40 and 4.5 µg/ml, see results). All concentrations of ajoene oil were combined with one of the MTZ concentrations in turn. The inhibitory effect of MTZ, ajoene oil, AA and DADS was also tested against the freshly isolated *S. vortens Sv1* strain, as described above. All compounds were diluted in DMSO apart from allicin and AA, which were diluted in sterile distilled water.

### 2.6. *In vitro* experiments: growth curves

A Bioscreen C optical density reader (Labsystems, Finland) was employed to monitor the growth of *S. vortens* trophozoites in 100 well honeycomb plates (Thermo-Fisher), according to Millet et al. (2011a). Each well of the honeycomb plate contained 290 µl Keister's modified TYI-S-33 medium, 10 µl log-phase cell suspension, at a final density of $10^4$ cells/ well, and 3 µl of the required inhibitor (in the combination experiment, 1.5 µl of each compound was loaded) or supplement concentration (as specified below). Positive (cells with

no inhibitors), negative (no cells or inhibitors) and, if applicable, solvent (cells with 3 µl DMSO, Sigma-Aldrich) controls were also prepared and analysed. All treatments were performed in triplicate. Optical density (OD) values were recorded every 15 min on a vertical light path using a wideband filter (420-580 nm) at 24°C, with plates shaken at low amplitude for 10 s before each reading to ensure mixing of well contents. Logarithmic growth rates (µ) and final growth yields were calculated as follows from the resulting growth curves:

$$\mu=(OD_{t2}-OD_{t1})/(t_2-t_1)$$

$$\text{Final yield}= OD_{tf}- OD_{t0}$$

Where, $OD_{t1}$ is the optical density at the beginning of log phase growth, $OD_{t2}$ is the optical density at the end of log phase growth, $OD_{t0}$ is the initial optical density at the beginning of the growth curve and $OD_{tf}$ is the final optical density after entering the stationary phase.

### 2.7. Determining minimum inhibitory concentrations (MIC) and synergy

Final growth yields were plotted against inhibitor concentration in order to determine the MIC of compounds, with MIC defined as the minimum inhibitor concentration(s) to completely inhibit *S. vortens* growth. For the combination experiment, the fractional inhibitory concentration (FIC), or the lowest concentration of each ajoene + MTZ combination to completely inhibit growth, was derived for each component, as follows:

$$\text{FIC(component A)} = MIC_{(A)1} / MIC_{(A)2}$$

Where $MIC_{(A)1}$ corresponds to the MIC of component A when tested in the most efficient combination of A and B, and $MIC_{(A)2}$ is the MIC of component A when tested alone. An FIC index for each ajoene oil + MTZ combination was calculated to assess the synergistic, additive or antagonistic effects of the various combinations, as follows:

$$\text{FIC Index} = \Sigma \text{ FIC A} + \text{FIC B}$$

Guidelines proposed by Odds (2003) were used to determine synergy, no interaction (indifference) or antagonism between inhibitor concentrations, as follows: synergy = FIC<0.5, indifference = FIC 0.5-4.0 and antagonism = FIC>4.0. Normally distributed FIC indexes of equal variance, derived from the combinatorial effect of ajoene oil and MTZ (N=3

for each combination), were analysed by one-sample T-test using Minitab software (Version 16). Each FIC index was analysed for significant difference from 0.5, the FIC index value that indicates synergistic interaction between inhibitors according to Odds (2003).

### 2.8. *In vivo* treatments

#### 2.8.1. Fish origin and maintenance

Juvenile *Pterophyllum scalare* (angelfish, N=50) were obtained from J&K Aquatics (Taunton, UK). Fish were randomly allocated individually into 12 L plastic tanks (i.e. one fish per tank), each filled with dechlorinated water, an air supply and a plastic tank decoration for cover. Fish were maintained at 24±1°C with a 12h: 12h light: dark cycle and fed on a flake food (TetraMin), described further below. Chemical water properties were measured using Sera® colorimetric test kits: pH=7, general water hardness (dGH) = 6, carbonate water hardness (dKH) = 5, [ammonia] <0.5 mg/l, [nitrite] <0.5 mg/l and [nitrate] < 25 mg/l). During the experiment, fish were fed daily with the allocated flake treatment (approx. 3% body weight) for 5 d, with daily 25% water changes of aquaria in order to dilute the levels of accumulated ajoene and MTZ (Whaley & Francis-Floyd 1991). On Day 5 post-treatment, 100% water changes took place for all treatment groups. Thus, the only variable during this experiment was composition of the feed/ bath to reflect the 5 different treatment groups (described below).

#### 2.8.2. Preparation of fish food treatments

All treatments were first dissolved in 1 ml ethanol (Fisher Scientific) and then re-suspended in 100 ml $dH_2O$ to the desired concentration. Fish flake food (TetraMin, 2 g for each treatment) was soaked in each treatment at the desired concentration overnight at room temperature. Excess moisture was removed from the flake treatments by freeze drying. The resulting ajoene content of dried flakes was quantified by high performance liquid chromatography (HPLC). HPLC analysis was carried out on an Agilent 1100 series HPLC system using an ODS 4.6 x 250 mm with ODS guard cartridge. All HPLC solvents were purchased from Rathburn Chemicals (Scotland). Samples were dissolved in methanol and eluted with an aqueous acetonitrile mixture using a gradient method as follows: 45% acetonitrile up to 13 min, after which the concentration was increased up to 80% before decreasing again to 45% at 18 min. The flow rate was 1 ml/min with a pressure of 400 bar and detection at 254 nm. The recommended MTZ dose for treatment of Spironucleosis is 10

mg/g fish food (Francis-Floyd & Reed 1994). This concentration is approximately 1000-fold greater than the MIC of this drug *in vitro*. Hence a higher concentration of ajoene, compared to its *in vitro* MIC was prepared. Preliminary investigations were conducted to define the concentration of flake-soaked ajoene that was palatable to angelfish. Fish (N=2 for each concentration) were fed with 0, 5, 10 and 50 µl ajoene/g flakes (0, 0.5, 1 and 5% v/w, respectively) at regular intervals over the course of 1 d. The only ajoene concentration readily consumed by fish was the 0.5% preparation. Fish from the 0.5% ajoene flakes preliminary treatment group were then continuously fed on this preparation for 5 d; they displayed no behavioural changes (i.e. inappetite or erratic behaviour). Subsequently, the concentration of each treatment employed in the main experiment was as follows: 0.5% ajoene oil, 0.5% (v/w, 5 mg/g) MTZ, 0.05% (0.5 µl/g) ajoene oil and 0.5% MTZ combination. The chosen concentration of MTZ is half the recommended dose in ornamental fish (Francis-Floyd & Reed 1994) in order to assess possible synergistic interactions between lower concentrations of this drug and ajoene oil. Control flakes were treated with 2 ml ethanol in 100 ml $dH_2O$ without inhibitors before freeze drying.

### 2.8.3. Treatment groups

Treatment groups were as follows: Group 1 (N=10): control flakes, Group 2 (N=9): 0.5% ajoene flakes, Group 3 (N=8): 0.5% MTZ flakes, Group 4 (N=10): 0.05% ajoene and 0.5% MTZ combination flakes and, as an added control, Group 5 (N=10): 0.0005% (w/v, 5 mg/L) MTZ bath. The reduction in sample sizes from N=10 to 9 and 8 for the ajoene oil and MTZ treatment groups respectively is due to difficulties in obtaining faecal samples from some fish during the experiment (described below). The concentration of MTZ employed in the bath treatments was as recommended by Tojo & Santamarina (1998) as a positive control. The concentration of ajoene employed was the highest concentration tested that was palatable to fish (as described below).

### 2.8.4. Quantification of faecal trophozoite counts

Naturally occurring *S. vortens* infections in angelfish were quantified using faecal trophozoite counts (described in Williams et al. 2013). Briefly, faecal pellets were collected immediately after defecation and mounted as a squash preparation between a glass slide and coverslip. Squash preparations were viewed microscopically (400x magnification) for 15 min, analysing *ca.* 50 different fields of view, for the presence of *S. vortens* trophozoites displaying characteristic pyriform cell shape (10 µl in length) with rapid motility. The average

trophozoite counts from two faecal samples were quantified in order to gain a more accurate estimation of the degree of infection due to differential host parasite shedding over time. *Spironucleus vortens* infections were quantified in this way at Day 0 (pre-treatment) and Day 5 (post-treatment) for the flake food treatment (Groups 1-4). For the MTZ bath treatment (Group 5), pre-treatment tropohozoite faecal counts were conducted on Day 0, after which fish were incubated for 1 h in a 1 L plastic pot containing 0.0005% MTZ on Day 1, Day 3 and Day 5. Post-treatment trophozoite faecal counts were conducted on Day 6.

### 2.8.5. Data analysis

To identify potential significant differences, angelfish faecal trophozoite counts from each (Group 1 = control, Group 2 = ajoene oil, Group 3 = MTZ, Group 4 = ajoene oil + MTZ combination and Group 5 = MTZ bath treatment), were independently analysed before and after treatment using a one-tailed Mann-Whitney non-parametric test. The pre-treatment faecal counts were hypothesized to be significantly greater than the post-treatment faecal counts with an α value of 0.05. Efficacy of treatments ($E_t$) were calculated according to Schelkle et al. (2011) as follows:

$$\textbf{If } L_t < L_0\textbf{, then } E_t = (L_0 - L_t) / L_0$$

$$\textbf{If } L_t \geq L_0\textbf{, then } \Delta E_t = 0$$

Where $L_0$ = faecal trophozoite count before treatment and $L_t$ = faecal trophozoite count after treatment.

## 3. RESULTS

Ajoene oil and MTZ had a synergistic effect against *Spironucleus vortens* growth *in vitro* and *in vivo*. The only component of the ajoene oil found to have antiparasitic activity against *S. vortens in vitro* was (*Z*)-ajoene.

### 3.1. Analysis of garlic-derived compounds present in ajoene oil

GC-MS chromatograms revealed a number of garlic-derived components present in the crude ajoene oil extract. These were DADS (14.8 min), 3-vinyl-4H-1,3-dithiin (17.8 min), 2-vinyl-4H-1,2-dithiin (18.7 min) and DATS (21.2 min), see Figure 1. No ajoene was detected by this method; however a mixture of (*E*) and (*Z*)-ajoene isomers were identified in NMR spectra of the ajoene oil (Table 1), which account for the 18% ajoene proportion of the crude oil extract.

### 3.2. *In vitro* inhibitory effect of *Allium*-derived compounds and MTZ against *S. vortens*

The MIC and $IC_{50}$ values of MTZ and all *Allium*-derived compounds against *S. vortens* (ATCC strain) are summarised in Table 2. The MICs of ajoene oil (50 µg/ml), AA (6 µg/ml) and DADS (>160 µg/ml) against the *S. vortens Sv1* strain was similar to that of the ATCC strain. Interestingly, however, the MTZ MIC values for the two strains varied greatly, with the ATCC strain being more susceptible to MTZ (MIC = 4.5 µg/ml, $IC_{50}$ = 1.6 µg/ml, Fig 2g) than the more recently isolated *Sv1* strain (MIC undetermined). The $IC_{50}$ of MTZ against the *Sv1* strain was calculated as ~0.5 µg/ml, however none of the concentrations tested (≤160 µg/ml) completely inhibited the growth of this strain, demonstrated in Figure 3 by increased lag-phases.

### 3.3. *In vitro* synergistic activity of ajoene oil and MTZ against *S. vortens* growth

A combination of 0.25 µg/ml MTZ and 0.2 µg/ml ajoene oil had a significant synergistic effect against *S. vortens* growth. This combination had a calculated FIC index of 0.263 (±0 SD), which is significantly lower than Odds' (2003) threshold value of 0.5 (T-test, $p<0.05$), indicating considerable synergistic activity between these combinations. The FIC index for all ajoene oil and MTZ combinations tested are summarized in Table 3.

### 3.4. *In vivo* combinations of ajoene oil and MTZ significantly reduce *S. vortens* infections

The effects of administration of flakes containing (1) 0.5% ajoene oil, (2) 0.5% MTZ, (3) 0.05% ajoene oil + 0.5% MTZ in combination as well as a 0.0005% MTZ bath treatment on *S. vortens* faecal counts in angelfish are summarized in Figure 4. The only treatments that significantly reduced *S. vortens* faecal counts of angelfish were the flakes containing a combination of ajoene oil (0.05%) and MTZ (0.5%), and the MTZ bath treatment (0.0005%, MW, $p<0.05$ for both). These treatments had relatively high $E_t$ values of 64.5% and 68.2%, respectively, whist $E_t$ values for all other treatments was <50% (Table 4).

## 4. DISCUSSION

The antimicrobial effects of ajoene, a stable garlic derivative, against *Spironucleus vortens* was evaluated alone and in combination with the 5-nitroimidazole MTZ. Synergistic activity

between MTZ and ajoene oil were observed against *S. vortens in vitro* and *in vivo*, in the freshwater angelfish, *Pterophyllum scalare*.

The ajoene oil crude extract employed during this study contained 18% ajoene as a mixture of (*E*) and (*Z*)-ajoene isomers. *In vitro* analyses showed that ajoene oil inhibited *S. vortens* growth with an MIC of 40 µg/ml. This MIC was 10-fold greater than that of MTZ (4 µg/ml), the drug of choice for treatment of *S. vortens* infections. In order to assess whether the ajoene component of the crude oil extract was responsible for the observed growth inhibition of *S. vortens*, pure preparations of (*E*) and (*Z*)-ajoene as well as the potential polysulphide and dithiin contaminants of the oil: DADS, DATS and a dithiin-rich preparation, were also assayed. The only component of the ajoene oil that was found to have a significant inhibitory effect against *S. vortens in vitro* growth was (*Z*)-ajoene, MIC = 16 µg/ml. This individual MIC is approximately 2.5-fold less than that of the ajoene oil, which is reflected in the lower ajoene content found in this crude extract (18%). The antimicrobial activity of the (*Z*)-ajoene isomer has previously been documented to exceed that of the (*E*)-isomer (see review by Williams & Lloyd 2012).

The MIC value for the majority of the remaining compounds examined was greater than the highest concentration tested. However AA and AMD had substantially low MIC values of 10 and 20 µg/ml, respectively. The MIC of AA against *S. vortens* is comparable with that of *Giardia intestinalis* and *Trichomonas vaginalis* (see review by Williams & Lloyd 2012). However, this compound is classed as being hazardous to the aquatic environment, making it impractical for use in an aquaculture setting. The antimicrobial effect of AMD has not been well-documented; however it would seem that the presence of two S-atoms in this methylated compound greatly contributes to its biological activity, as AMS did not inhibit parasite growth at the concentrations tested. Allicin, the primary biologically active component of the garlic clove, was less potent than ajoene, AA and AMD, having a comparably high MIC value of 1240 µg/ml against *S. vortens* growth. This value is comparable to that documented previously by Millet et al. (2011a, >160 µg/ml). Thus, as well as having increased stability, ajoene oil also out-performs allicin in terms of inhibiting *S. vortens* growth, making this ajoene extract an ideal candidate in the treatment of Spironucleosis in fish, as was tested in our *in vivo* experiments.

Significant synergy between MTZ and ajoene oil was obtained using concentrations of 0.25 and 0.2 µg/ml, respectively. This resulted in a 16-fold reduction from the individual MIC of MTZ, and a 200-fold decrease in the *in vitro* MIC of ajoene oil. The efficiency of this ajoene-MTZ combination against *S. vortens* infections in angelfish was confirmed *in vivo*.

Treated flake food containing 0.5% ajoene oil or 0.5% MTZ individually did not significantly reduce *S. vortens* infections in angelfish. For the MTZ food treatment, this was not surprising as the recommended dose for ornamental fish is higher than the concentration employed here (10 mg/g flakes or 1%, Francis-Floyd & Reed 1994). However, when this 0.5% MTZ dose was combined with just 0.05% ajoene oil in flake food, a significant reduction in *S. vortens* infection was observed.

The observed synergy between ajoene oil and MTZ may be explained by the complementary modes of action of these compounds. We have previously demonstrated that ajoene oil depletes intracellular thiols and inhibits the thioredoxin reductase (TrxR) of *S. vortens* at concentrations close to the *in vitro* MIC and $IC_{50}$, respectively (Williams et al. 2012). TrxR and other non-protein thiols are important cellular antioxidants, which play a key role in detoxification of reactive oxygen species within cells (see review by Müller et al. 2003). MTZ had a similar effect to ajoene, but was also shown to form covalent adducts with several redox-related proteins (Williams et al. 2012). Hence, treatment of *S. vortens* with a combination of ajoene and MTZ leads to redox imbalance through the synergistic action of these inhibitors on important redox-related proteins, leading to severe oxidative stress and eventually cell death. The bulk of this activity is likely to come from (Z)-ajoene, which was found in this study to be the main antiparasitic component of the ajoene oil. Thus the pure (Z)-ajoene extract alone is likely to have a comparable, if not enhanced, synergistic activity with MTZ, based on the lower *in vitro* MIC. However, (Z)-ajoene has a number of disadvantages over ajoene oil. In particular, the purification process is currently very laborious and expensive, meaning that commercialisation would be difficult. Furthermore, ajoene oil contains a mixture of (E) and (Z)-ajoene, polysulphides and vinyl dithiins (see Table 1 and Fig 1), all of which have been shown previously to have antimicrobial activity and may act synergistically with one another. Thus, in terms of restricting the probability of future development of antimicrobial resistance to one of garlic's active components (i.e. (Z)-ajoene) it would be safer to administer a combination of active ingredients, as found in the ajoene oil.

The MTZ bath treatment, consisting of 0.0005% MTZ (the recommended dose in fish, Tojo & Santamarina 1998), also significantly reduced *S. vortens* infections in angelfish, further validating this dose for successful treatment of ornamental fish. However, there are inherent problems with the use of bath treatment for disease management, particularly where the ornamental fish studied are reared in earthen ponds (e.g. S.E. Asia). In these situations, antibiotics can leach into the surrounding environment thus contributing to the development

of antibiotic resistance in environmental bacteria. Thus, oral delivery of synergistic treatments, such as the ajoene oil-MTZ combination proposed during this study, has the benefit of being delivered directly to the fish and immediately removed from the environment via ingestion. Furthermore, the fact that a lower MTZ dose is required in combination with ajoene oil means reduced cytotoxicity/ genotoxicity as well as a reduced usage of the drug.

A final interesting observation to come from this study was that the MIC of MTZ could not be determined against the new *S. vortens* Sv1 isolate. However, the *in vitro* MIC values of ajoene oil, AA and DADS against the Sv1 strain (*ca.* 3 months in culture) were comparable to that of the ATCC strain (*ca.* 22 years in culture). This suggests that the Sv1 strain is less susceptible to MTZ than the ATCC strain and may be the result of increased drug pressure in the ornamental aquaculture industry. This highlights the need for more in depth investigation into the prevalence and mechanism of MTZ resistance in aquaculture, and emphasizes the importance of identification and development of alternative treatments for effective parasite control.

The study demonstrates, for the first time, the synergistic activity of ajoene oil with MTZ and provides an in-depth account of the inhibitory effects of a wide range of garlic derivatives against *S. vortens* growth. As well as having increased efficacy, ajoene also offers the advantage of having increased stability over allicin, the primary biologically active component of the garlic clove. Furthermore, the ajoene oil concentration required to synergistically enhance the efficacy of MTZ is palatable to the *P. scalare* angelfish utilized in this study, thus reducing the MTZ concentration required to treat Spironucleosis and therefore the cytotoxicity/ genotoxicity associated with MTZ treatment. This study highlights ajoene oil as a viable candidate for commercialisation as an additive to fish food to control Spironucleosis in the ornamental aquaculture industry. Future work should be directed towards determining the efficiency of ajoene oil against other commercially important *Spironucleus* spp., e.g. *Spironucleus salmonicida* and *S. barkhanus*, which infect food fish such as salmon and trout. The synergy between ajoene oil and metronidazole should be further investigated in other host-parasite systems, e.g. *Giardia intestinalis* and *Trichomonas vaginalis*, where metronidazole remains the drug of choice. Furthermore, synergistic activity between ajoene oil and other antibiotics should be investigated as a possible means of reviving the efficacy of redundant antibiotics against antibiotic resistant organisms in other host-parasite systems.

## 5. ACKNOWLEDGEMENTS

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**TABLES AND FIGURES**

**Table 1.** $^{1}$H chemical shifts (δ) of ajoene isomers derived from NMR spectra of ajoene oil crude extract. (*E*) and (*Z*)-ajoene spectra were consistent with that described previously by Naznin et al. (2008).

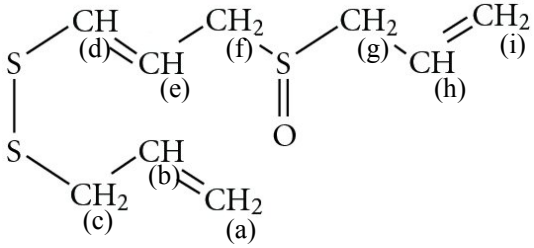
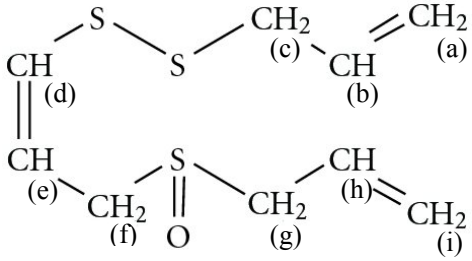
| Compound | H atom group | δ (ppm) |
|---|---|---|
|  <br>(E)-Ajoene | (a) $\underline{CH_2}$=CH-$CH_2$ | 5.20 |
| | (b) $CH_2$=$\underline{CH}$-$CH_2$-S-, m | 5.20 |
| | (c) –S-S-$CH_2$-, d | 3.36 |
| | (d) =CH-S-S-, d | 6.38 |
| | (e) =$\underline{CH}$-$CH_2$-,m | 5.90 |
| | (f, g) -$\underline{CH_2}$-SO-$\underline{CH_2}$-, m | 3.50 |
| | (h) $CH_2$=$\underline{CH}$-$CH_2$-SO-, m | 5.40 |
| | (i) $\underline{CH_2}$=CH-$CH_2$- | 5.40 |
|  <br>(Z)-Ajoene | (a) $\underline{CH_2}$=CH-$CH_2$- | 5.20 |
| | (b) $CH_2$=$\underline{CH}$-$CH_2$-S-, m | 5.20 |
| | (c) –S-S-$CH_2$-, d | 3.38 |
| | (d) =CH-S-S-, d | 6.56 |
| | (e) =$\underline{CH}$-$CH_2$-,m | 5.80 |
| | (f, g) -$\underline{CH_2}$-SO-$\underline{CH_2}$-, m | 3.50 |
| | (h) $CH_2$=$\underline{CH}$-$CH_2$-SO-, m | 5.40 |
| | (i) $\underline{CH_2}$=CH-$CH_2$ | 5.40 |

**Table 2.** Minimum inhibitory concentrations (MICs) and 50% inhibitory concentrations ($IC_{50}$) of all inhibitors tested against *Spironucleus vortens* (ATCC 50386). Concentrations are given in μg/ ml and μM units. Where the MIC and/ or $IC_{50}$ values were >160 μg/ ml or >1:100 dilution of the dithiin-rich extract i.e. (the maximum concentrations tested), inhibitors are designated 'ND' (not determined).

| **Inhibitor** | **MIC** | | **$IC_{50}$** | |
|---|---|---|---|---|
| | **μg/ ml** | **μM** | **μg/ ml** | **μM** |
| MTZ | 4.5 | 26.29 | 1.6 | 9.35 |
| Ajoene Oil | 40 | 30.72* | 5 | 3.84* |
| (*Z*)-Ajoene | 16 | 68.26 | 10 | 42.66 |
| AA | 10 | 172.18 | 0.33 | 5.68 |
| AMD | 20 | 166.33 | 6 | 49.90 |
| DADS | ND | ND | 7 | 47.85 |
| Allicin | 1240 | 152.84* | 880 | 108.46* |
| (*E*)-Ajoene<br>AS<br>AD<br>DADS<br>DATS<br>MD<br>AMS<br>PS<br>PD<br>Dithiins | ND | ND | ND | ND |

*Molarities are based on the percentage of pure ajoene or allicin found in the preparations, 18 and 2% (v/v).

**Table 3.** *In vitro* fractional inhibitory concentrations (FICs) of different combinations of metronidazole (MTZ) and ajoene against *Spironuleus vortens* (ATCC strain) growth. The FIC index, with ± standard deviation (N=3), represents the sum of the MTZ and ajoene FIC values for each combination. IND and SYN indicate indifferent and synergistic interactions, respectively. (*) Asterisks indicate significant difference from 0.5, the threshold value of synergy (T-test, $p<0.05$ *, $p<0.0001$ **).

| **MTZ + Ajoene combination (µg/ml)** | **FIC MTZ** | **FIC Ajoene** | **FIC Index (±SD)** |
|---|---|---|---|
| 4 + 0.1 | 1 | 0.204 | 1.204 (±0.261) IND |
| 2 + 0.1 | 0.5 | 0.1 | 0.600 (±0) IND |
| 1 + 0.025 | 0.25 | 0.083 | 0.333 (±0.101) IND |
| 0.5 + 0.2 | 0.125 | 0.175 | 0.300 (±0.035) SYN* |
| 0.25 + 0.2 | 0.0625 | 0.2 | 0.263 (±0) SYN** |

**Table 4.** Efficacy of different treatments ($E_t$) in reducing *Spironucleus vortens* faecal counts of infected angelfish. $E_t$ is calculated as follows: (trophozoite faecal count (TFC) before treatment - TFC after treatment) / TFC after treatment. SD = standard deviation.

| **Treatment Group** | **N** | **$E_t$ (±SD)** |
|---|---|---|
| Control flakes | 10 | 0.395 (±0.471) |
| Ajoene flakes | 9 | 0.305 (±0.359) |
| MTZ flakes | 8 | 0.480 (±0.419) |
| Ajoene +MTZ flakes | 10 | 0.645 (±0.397) |
| MTZ bath | 10 | 0.682 (±0.388) |

**Figure legends**

**Fig. 1.** GC-MS chromatogram of sulphonated compounds present in ajoene oil crude extract. (a) diallyl disulphide (DADS), (b) 3-vinyl-4H-1,3-dithiin, (c) 2-vinyl-4H-1,2-dithiin and (d) diallyl trisuphide (DATS).

**Fig. 2.** Minimum inhibitory concentrations (MIC) of garlic derivatives against *Spironucleus vortens* (ATCC strain). Ajoene oil (a), (*Z*)-ajoene (b), allyl alcohol (AA) (c), allyl methyl disulphide (AMD) (d), diallyl disulphide (DADS) (e), allicin (f) and metronidazole (MTZ) (g) against *S. vortens* growth by optical density (OD) monitoring at 420-580 nm. Final yield was derived from the highest cell density (or OD) obtained from growth curves under incubation with various concentrations of ajoene oil. $IC_{50}$ is defined as the ajoene oil concentration at which final yield was reduced by 50% (indicated by the red dashed line). Error bars represent ± standard deviation of 3 replicates.

**Fig. 3.** *Spironucleus vortens Sv1* growth over time under incubation with various concentrations of metronidazole (MTZ) by optical cell density monitoring at 420-580 nm. A colour version of this figure is available on the web.

**Fig. 4.** Boxplots of *Spironucleus vortens* faecal trophozoite counts in angelfish before (B) and after (A) treatment as follows: (a) untreated flakes (Group 1), (b) 0.5% (v/w) ajoene flakes (Group 2), (c) 0.5% (v/w) metronidazole (MTZ) flakes (Group 3), (d) combination of 0.05% ajoene and 0.5% MTZ (v/w) flakes (Group 4) and (e) 0.0005% (w/v) MTZ bath treatment (Group 5). Circles indicate outliers in the data set, bold horizontal lines represent the median of the dataset, whiskers represent the maximum and minimum values (excluding outliers) and boxes represent the upper and lower quartiles. Asterisks indicate treatments where trophozoite faecal counts were significantly lower after treatment as compared to before treatment.

Figure 1

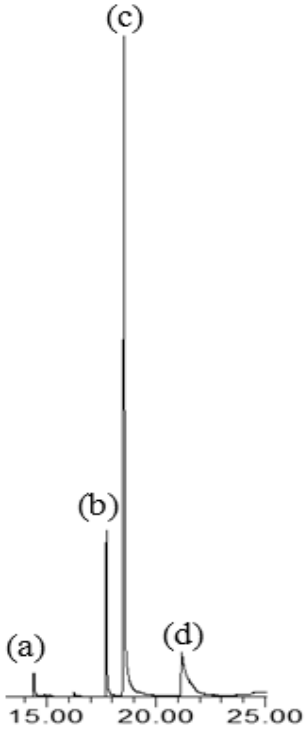


Figure 2

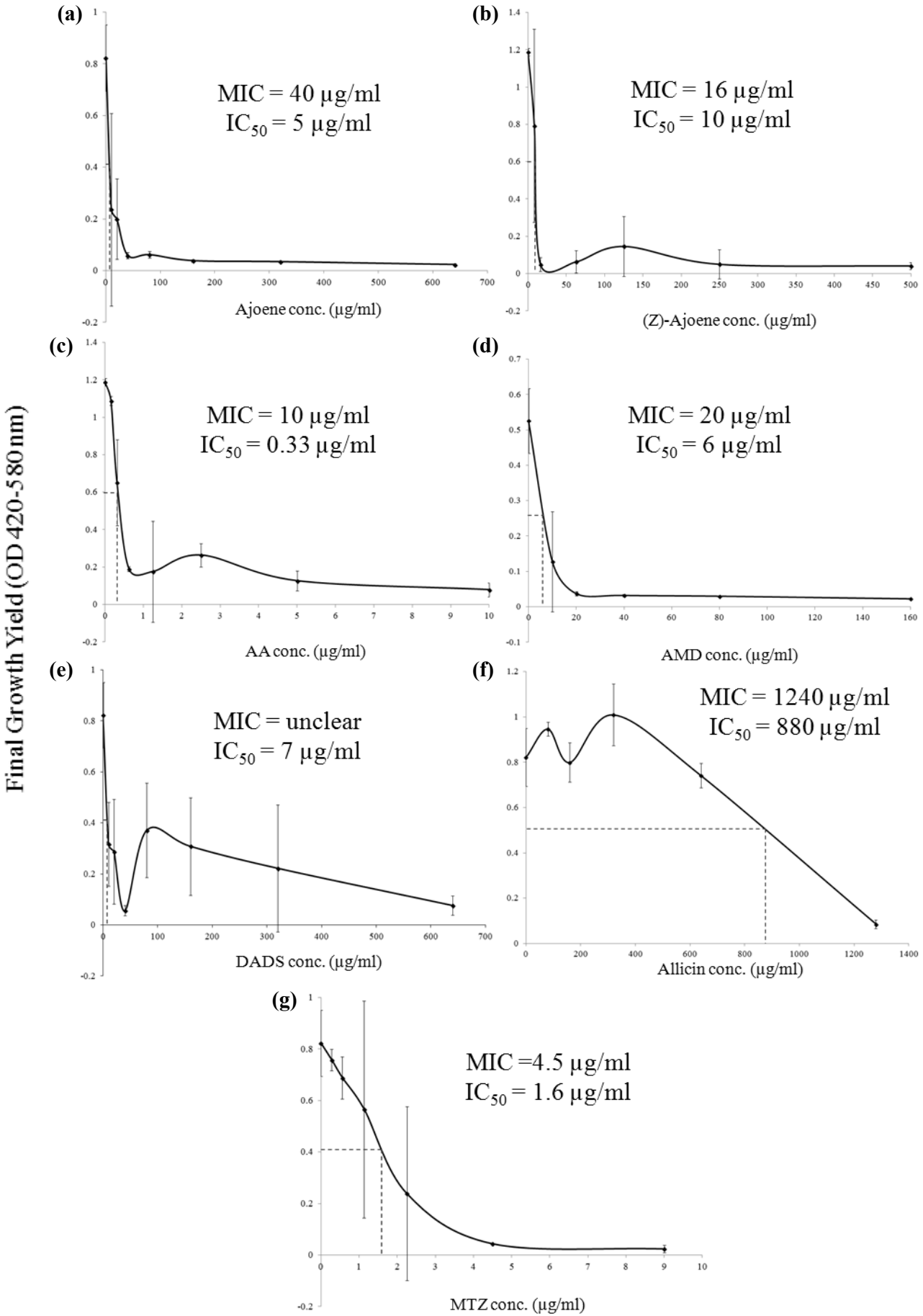


Figure 3

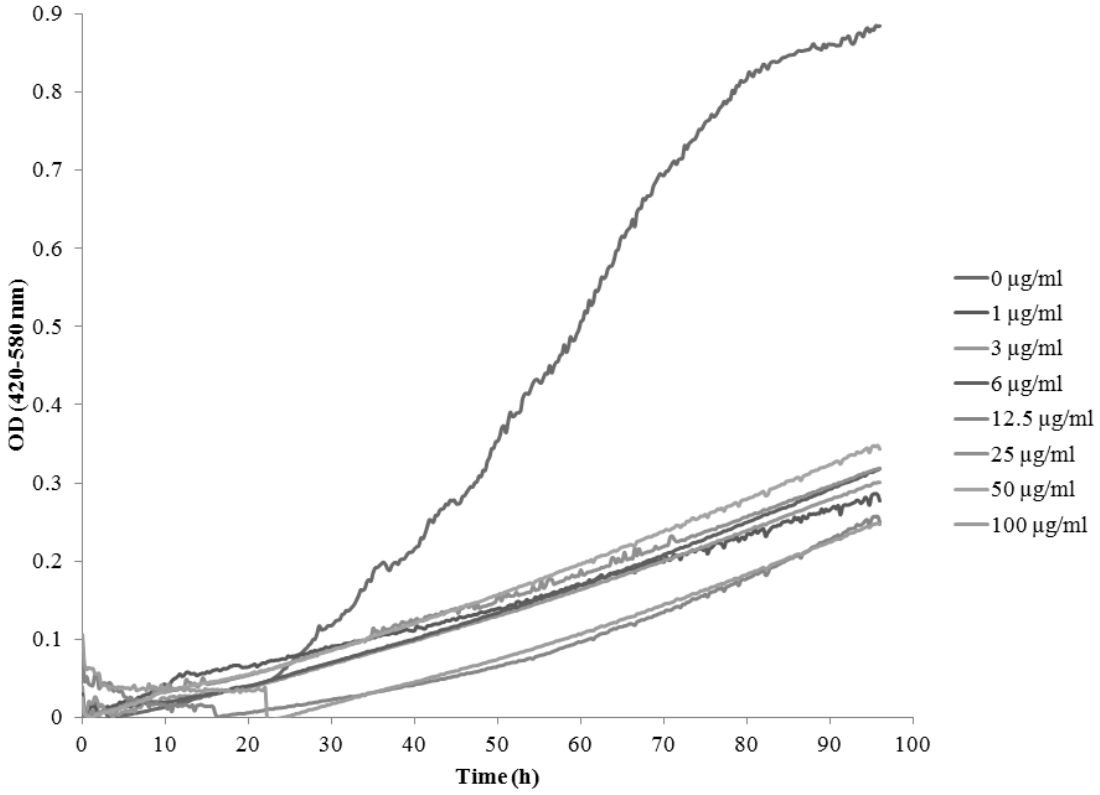


Figure 3

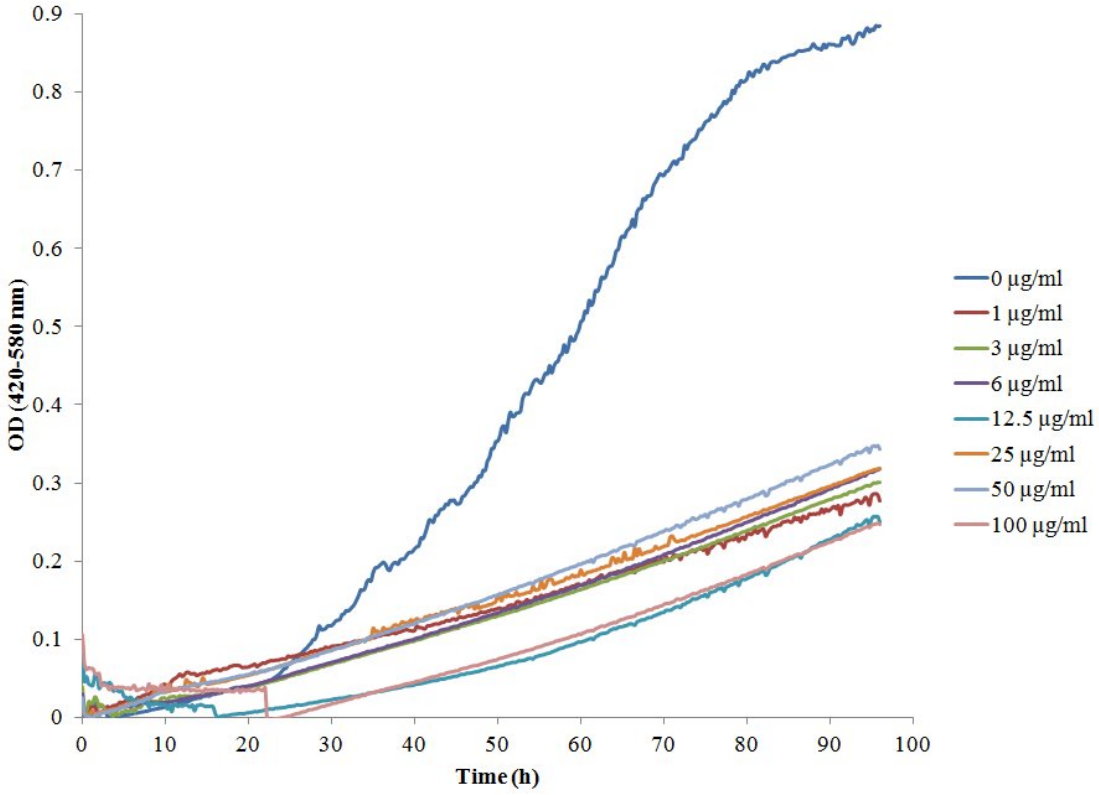


Figure 4