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Efficacy of commercially available products against *Gyrodactylus turnbulli* infections on guppies *Poecilia reticulata*

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ABSTRACT: The demand for ornamental fish has led to a steep rise in aquaculture for the hobbyist trade, promoting the emergence, persistence and spread of various infectious diseases. Complete control of disease outbreaks with antibiotics and chemical-based medicines is rare, but plant compounds may herald potential alternatives effective against a range of pathogens. Melafix® and Pimafix® are formulated with the essential oils cajuput (*Melaleuca cajuputi*) and West Indian bay (*Pimenta racemosa*) and are marketed against bacterial and fungal infections, respectively. Previous experiments showed high efficacy of emulsified cajuput oil against gyrodactylids; the current study tested Melafix® and Pimafix® and their individual compounds against *Gyrodactylus turnbulli* infecting the guppies *Poecilia reticulata*. In particular, a combination treatment of Melafix® and Pimafix® was highly effective at reducing *in vitro* survival of parasites from 15 to 2 h and eradicating 95% of gyrodactylids *in vivo*. The unexpected high efficacy of this combination treatment is likely explained by the high content of terpenes and phenol propanoids in the cajuput and West Indian bay oils, as well as the anti-helminthic properties of the emulsifier Crovol PK 70. Hence, Melafix® and Pimafix® effectively reduce gyrodactylid burdens on fish, increasing the chances of efficient disease control in ornamental fish.

KEY WORDS: Gyrodactylosis · Treatment · Helminth infection · Gas chromatography-mass spectrometry · Levamisole · Guppy

INTRODUCTION

In both the human food chain and ornamental trade, the demand for fish is predicted to increase from 154 to 186 million tons between 2011 and 2030 to satisfy the growing market which can only be met by expansion of aquaculture (Tlusty 2002, Cressey 2009, World Bank 2013). However, the high stocking density of fish in aquaculture imposes additional stressors, leading to increased risk and susceptibility to disease (Harris et al. 2000, Bondad-Reantaso et al. 2005, Ashley 2007). Development of drug resistance (e.g. Verner-Jeffreys et al. 2009), virulence evolution (Mennerat et al. 2010, Pulkkinen et al. 2010), emerging disease (Murray & Peeler 2005), climate change (Karvonen et al. 2010) and the ban of broad anti-parasitic compounds, such as malachite green and formalin (European Union Biocide Product Directive 98/8/EC, European Council Regulation 2377/90) further intensify the impact of parasites on aquaculture (e.g. Scholz 1999, Kim et al. 2002, Thilakaratne et al. 2003). Hence, there is an urgent need for alternative treatments that are effective and safe for fish, humans and the environment (Citarasu 2010, Chakraborty & Hancz 2011).

The screening of plant extracts has become increasingly important in the search for viable alternatives, with the expectation that individual compounds may have anti-parasitic properties that, when combined, act synergistically and to which parasites are hyper-susceptible (Anthony et al. 2005, Athanasiadou et al. 2007, Hu et al. 2010). Essential oils pro-

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duced by aromatic plants and their secondary metabolites have become popular research subjects against a whole range of diseases in many animal species, with a strong focus on bacterial disease caused by *Escherichia coli*, *Candida albicans*, *Staphylococcus aureus* and *Pseudomonas* spp. (see Bakkali et al. 2008, Hammer & Carson 2011).

Two examples of oils effective against fungi, bacteria, protozoa and macroparasites in a range of host animals are cajuput (*Melaleuca cajuputi*) and West Indian bay (*Pimenta racemosa*) (e.g. De Colmenares et al. 1998, Hammer et al. 1999, Valdés et al. 2008, George et al. 2010). The efficacy of cajuput is not surprising as it is closely related to tea tree (*M. alternifolia*) oil which has well studied anti-microbial properties (Carson et al. 2006, Hammer & Carson 2011, Zhu et al. 2011) and is also effective against monogenean infections (Steverding et al. 2005). West Indian bay oil also has a wide range of anti-viral and anti-parasitic activity, including anti-helminthic and larvicidal properties (Lee 2006, Yousif et al. 2007, Meneses et al. 2009, Si et al. 2009). Both oils show strain-specific efficacy against microbes (Burt & Reinders 2003, Saenz et al. 2004) and may reduce the transmission of vector-borne diseases (George et al. 2010, Greive et al. 2010); however, such effects may be dependent on seasonal and geographical variation in essential oil constituents (Burt 2004). Cajuput, for instance, varies significantly in its chemotype depending on its origin, but overall is dominated by terpenes (Cuong et al. 1994, De Colmenares et al. 1998, Farag et al. 2004, Piño et al. 2010). West Indian bay, on the other hand, appears to be dominated by a mixture of phenols and some terpenes (Nadal et al. 1973, McHale et al. 1977), with both chemical groups generally showing anti-microbial efficacy (Gershenzon & Dudareva 2007, Hammer & Carson 2011).

Cajuput and West Indian bay oils are the essential ingredients in 2 commercially available treatments marketed for their anti-bacterial and anti-fungal properties, Melafix® and Pimafix®, respectively, in the aquarium industry. Research on related botanical extracts or various host–parasite combinations indicates that particularly emulsified cajuput oil may be effective against parasitic worms (Steverding et al. 2005, Yousif et al. 2007, Schelkle et al. 2015); however, neither Melafix® nor Pimafix® have been tested against helminths in an aquatic environment. In this study the efficacy of Melafix® and Pimafix® and their individual essential oil components was tested *in vitro* and *in vivo* against gyrodactylids. Gyrodactylids are ectoparasitic helminths that are ubiquitous on teleost fish and the cause of considerable economic damage in infected fish populations (Bakke et al. 2007). There is no 100% effective treatment available against gyrodactylids (Schelkle et al. 2009, 2010, 2013, 2015, Bowker et al. 2012); a model system using *Gyrodactylus turnbulli* infecting guppies (*Poecilia reticulata*) under controlled conditions was employed to test commercial products alongside gas chromatography-mass spectrometry to identify and measure the relative abundance of individual compounds in cajuput and West Indian bay essential oils.

MATERIALS AND METHODS

Sources of animals and compounds

In vivo trials were conducted using an inbred strain of ornamental guppies (*Poecilia reticulata*) maintained at 25 ± 1°C, a 12 h light:12 h dark cycle and fed twice a day with Aquarian® tropical fish flakes. Additional, weekly feeds included live *Daphnia* spp. and frozen *Tubifex*. Parasites were of the *Gt3* strain of *Gyrodactylus turnbulli*, which had been isolated from pet shop guppies in 1997.

Two commercially available treatments were tested *in vivo*: Melafix® and Pimafix® (both Aquarium Pharmaceuticals™, MARS Petcare). Separately, the essential oils from cajuput and West Indian bay (Berje) were tested in combination with the emulsifier Crovol™ PK 70 (Croda International Plc.); in addition, both oils and the emulsifier were used individually as controls. Water and the anti-helminthic levamisole (Levacide) were used as negative and positive controls. Both Melafix® and Pimafix® were tested at the concentrations recommended by the manufacturer (see Table 1), the individual and combination treatments were administered to reflect the concentration in the products and levamisole was used at the known effective concentration of 1 ml l^{-1} (Schelkle et al. 2009, 2015).

Samples of cajuput and West Indian bay oil (Berje) were analysed by gas chromatography-mass spectrometry (GC-MS; see 'Results'). Chemical standards for reference were sourced from Sigma Aldrich, with the exception of α-thujene, which was obtained from Rose Chemicals.

In vitro efficacy against *G. turnbulli*

All compounds were applied to individual *G. turnbulli* worms (n = 297; see Table 1) that had been

removed using an insect pin from fin clips of infected donor fish following light anaesthesia (MS222 at a concentration of 0.02%). Following removal from the host, the parasites were transferred individually in 10 µl of water into wells of a 96-well microtitre plate using a micropipette. Treatments (90 µl) were applied 1 h after the worms were isolated and observed for abnormal behaviour (immobile, moribund) or death before application (Cable et al. 2002).

In vivo efficacy against *G. turnbulli*

Guppies (n = 176, standard length = 6.1 to 27 mm; see Table 1) were infected with parasites by exposure over 3 to 4 d to conspecifics (to mimic natural transmission) that carried the *Gt3* strain of *G. turnbulli*. Screening for parasites before (Day 1) and after treatment (Day 8) followed the protocol of Schelkle et al. (2009), whereby fish were lightly anaesthetized with 0.02% MS222 before their parasites were counted using a dissection microscope and optic fibre illumination. Fish with a parasite load of between 3 and 200 parasites were isolated and individually maintained in 1 l pots for the duration of the experiment, receiving daily water changes followed by treatment applications at the same concentrations as *in vitro* (see Table 1). A positive control *in vivo* using levamisole was not included, as a 7 d exposure to levamisole would have been detrimental to fish health. Instead, individual, uninfected fish (n = 14) not receiving any treatment were maintained alongside the experimental fish to act as controls.

Essential oil characterization and quantification

The essential oil samples were prepared for GC-MS analysis by diluting 0.2 g in 100 ml diethyl ether followed by addition of 200 µl of 2.5% (m/v) 5-methylhexan-2-one. GC-MS was performed using an Agilent 7890 GC coupled to an Agilent 5975C MS. The samples (1 µl) were applied directly to the GC column by cold-on-column injection, using an Agilent 7693 autosampler at 35°C. The GC was equipped with 2 columns (each 30 m in length and 0.25 mm in diameter, with 0.25 µm film thickness): a phenomenex non-polar DB5 (5% diphenyl/95% diemethyl siloxane) column and a phenomenex polar FFAP (nitroterphthalic acid–modified polyethylene glycol) column. Both columns were connected to an Agilent Deans switch which enabled the MS to automatically connect to the required column. Helium was used as a carrier gas.

The GC injector and oven temperature were initially held at 35°C for 1 min, then increased to 240°C at 3°C min^{-1} and held for 5 min with a total run time of 77 min. The transfer line to the MS was heated to 220°C. The MS ionisation was carried out by electron impact mode at 70 eV; source temperature was 230°C. The mass scan range was 40 to 550 atomic mass units, with a scan speed of 2.86 scans s^{-1}. Blanks were run between each sample to ensure complete clean out of the column before each component analysis.

Chromatographs were analysed using the Enhanced Agilent MSD ChemStation software, with tentative identification of mass spectra peaks through comparison to Wiley and NIST 2.0 libraries. Identification of the peaks was completed by comparison of the linear retention index (LRI) and mass spectra to authentic standards. The LRI for each compound was calculated on both polar and non-polar columns from the retention times of n-alkanes (C_7 to C_{26}) by linear interpolation (Kováts 1958). The relative abundance of each compound was calculated by comparison of peak area to the internal standard, 5-methylhexan-2-one, providing us with a rough guide to the relative amount of compounds within a single batch. Quantification based on relative abundance should not be taken for absolute and should be treated with caution, as each compound will produce a different response at the detector (Bicchi et al. 2008, Rubiolo et al. 2010, IOFI Working Group on Methods of Analysis 2011). Hence, for this study, the constituents are presented ranked, rather than according to their relative abundance within the compound.

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 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 3 separate screenings to confirm that they were clear of parasites before being returned into aquaria housing other stock fish (Schelkle et al. 2009).

Statistical analysis

In vitro data were analysed using a Cox proportional hazard survival analysis, with maximum survival of individual worms (n = 297) as the dependent variable (Therneau 2014). Crovol applications at different concentrations were treated as independent treatments rather than nested within an overall emulsifier group, as statistical results already showed clear differentiation of efficacy between treatment groups and nesting would not have changed the parameter estimates substantially.

Efficacy of *in vivo* treatments was calculated based on the initial (L_0) and final (L_t) parasite burdens of infected fish to give an efficacy value (E_t) of between 0 (not effective) and 1 (effective). Specifically, $E_t = (L_0 - L_t)/L_0$ for $L_t < L_0$ and $\Delta E_t = 0$ for $L_t \geq L_0$ (see Schelkle et al. 2010). As the data did not meet many of the assumptions of standard modelling approaches, the impact of host size and sex were not analysed in the current experiment. Hence, a non-parametric Kruskal-Wallis test established differences between treatment groups which were followed up with individual Mann-Whitney tests. Multiple testing was controlled for with a modification to the Bonferroni procedure after Benjamini & Yekutieli (2001, see also Narum 2006), leading to a new α level of p = 0.017 for the *in vitro* and p = 0.011 for the *in vivo* statistical tests. All analyses were performed in R 2.13.2 (R Development Core Team 2012).

RESULTS

Both *in vitro* and *in vivo* combination treatments of Melafix® and Pimafix® were highly effective against gyrodactylids, reducing *Gyrodactylus turnbulli* survival to approximately 2 h *in vitro* and being 95% efficacious *in vivo* (Table 1, Figs. 1 & 2). The emulsifier Crovol, which is used in both products, also exhibited anti-helminthic properties.

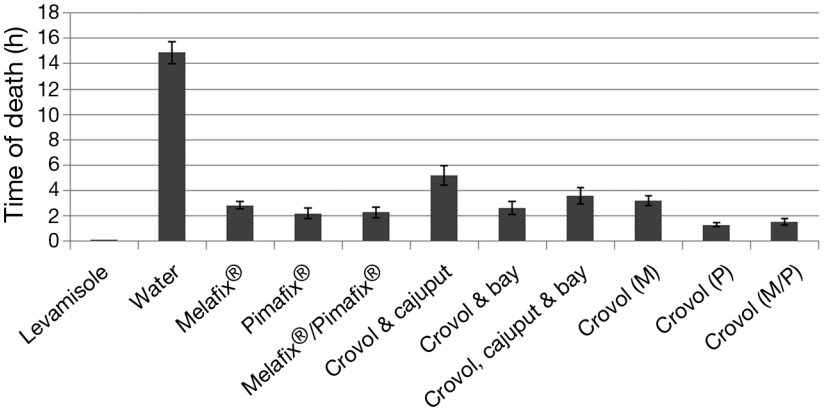

Fig. 1. *In vitro* time of death of *Gyrodactylus turnbulli* (± standard error of the mean). Crovol was used relative to its amount in Melafix® (M), Pimafix® (P) and the combination treatment (M/P)

Table 1. Concentration of treatments used plus sample size and descriptive statistics for survival of gyrodactylids *Gyrodactylus turnbulli* tested *in vitro*, and efficacy tested *in vivo* on guppies *Poecilia reticulata* (minimum and maximum efficacy ranged from 0 to 1, except for Crovol (M) which was 0 to 0.79). Conc.: treatment concentration (in µl l^{-1}); N: number of replicates (for survival, N = no. of gyrodactylids; for efficacy, N = no. of infected fish); SE: standard error; Min.: minimum; Max.: maximum. Crovol was used relative to its amount in Melafix® (M), Pimafix® (P) and the combination treatment (M/P)

Treatment	Conc.	Survival (h)				Efficacy		
		N	Mean	SE	Range	N	Mean	SE
Levamisole (positive control)	100	43	0.023	0.023	0 – 1	–	–	–
Water (negative control)	–	56	14.875	0.863	3 – 28	31	0.226	0.069
Melafix®	132	23	2.826	0.306	1 – 7	20	0.652	0.102
Pimafix®	132	22	2.182	0.398	0 – 7	20	0.320	0.088
Melafix®/Pimafix®	132 (each)	19	2.263	0.404	1 – 7	21	0.950	0.048
Crovol & cajuput oil	4.8	23	5.174	0.794	1 – 15	12	0.401	0.131
Crovol & bay oil	6.9	22	2.591	0.537	0 – 8	13	0.217	0.115
Crovol & cajuput oil plus crovol & bay oil	4.8 plus 6.9	23	3.565	0.662	1 – 12	18	0.422	0.097
Crovol (M)	3.5	25	3.160	0.382	1 – 6	12	0.200	0.090
Crovol (P)	5.6	18	1.278	0.158	0 – 3	13	0.205	0.097
Crovol (M/P)	9.1	23	1.522	0.242	0 – 6	12	0.416	0.129

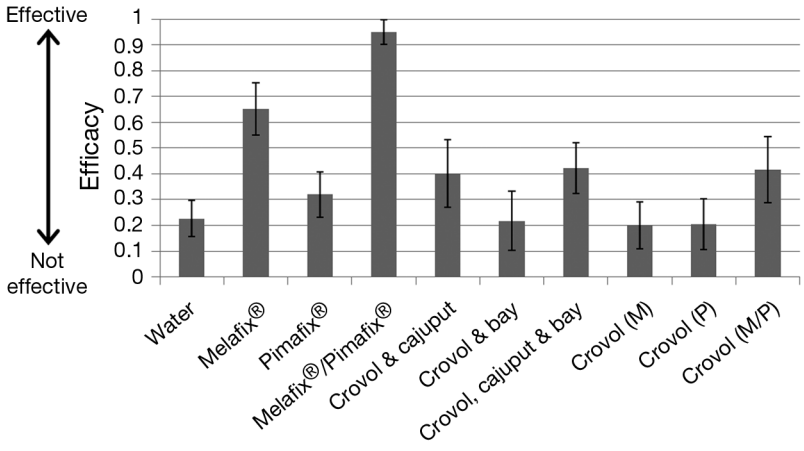

Fig. 2. Mean *in vivo* efficacy (± standard error of the mean; 0 = not effective, 1 = effective) of treatments tested *in vivo* against *Gyrodactylus turnbulli* infecting guppies

In vitro efficacy

Off the host, parasites survived up to 28 h in aquarium water (mean survival: 14.9 h) but were killed almost instantly when exposed to levamisole (mean survival: 0.02 h). Overall, there was a significant difference in survival between treatments (survival analysis: likelihood ratio test = 213.2, df = 10, $p < 0.001$). All treatments, including Crovol at all concentrations tested, significantly reduced survival time of worms when compared with aquarium water ($z \geq 4.217$, $p > 0.001$), and all were as effective as levamisole ($z \leq 1.926$, $p \geq 0.05$), except the slightly less effective Crovol with cajuput oil ($z = 2.436$, $p = 0.015$; Table S1 in the Supplement at www.int-res.com/articles/suppl/d115p129_supp.pdf). Crovol efficacy appears to be dose dependent (Table 1).

In vivo efficacy

Melafix® and the combination treatment Melafix®/Pimafix® are highly efficacious compared to the control (Kruskal-Wallis: $\chi^2 = 50.9$, df = 9, $p < 0.001$; Mann-Whitney tests: $W = 167$, $p = 0.002$ and $W = 64.5$, $p < 0.001$, respectively). No other treatments were significantly different from the negative control (Tables S1 & S2 in the Supplement at www.int-res.com/articles/suppl/d115p129_supp.pdf).

GC-MS

Characteristics and quantities of oils are summarised in Table 2. The chemical composition of cajuput oil was dominated by various terpenes consisting largely of 1,8-cineole and limonene, whereas West Indian bay contained mainly a mixture of the phenols eugenol and chavicol and the monoterpene myrcene.

DISCUSSION

Both Melafix® and Pimafix® were 95% effective against *Gyrodactylus turnbulli in vitro* and *in vivo*. The chemical composition of cajuput oil, the essential ingredient of Melafix®, was dominated by various terpenes consisting largely of 1,8-cineole and limonene, whereas West Indian bay, the base ingredient for Pimafix®, contained mainly a mixture of eugenol, myrcene and chavicol.

Previous studies on the survival of *G. turnbulli in vitro* reported mean survival times of between 25 and 30 h and indicated that treatments such as garlic and salt are similarly or even more effective than the botanicals in the current study *in vitro* and *in vivo* when tested against the same host–parasite combination (Schelkle et al. 2010, 2013). Indeed, cajuput applied with Tween 20 was nearly as effective as the Melafix®/Pimafix® combination with approximately 90% efficacy using *G. turnbulli* infecting guppies (*Poecilia reticulata*) (see Schelkle et al. 2015). The longer duration of exposure and a lower dose of the cajuput treatment in the current study increased the efficacy to 95%. In the long term, however, repeated cycles of partially effective or prematurely terminated treatments may lead to higher virulence of and drug resistance in the parasite (Gandon et al. 2001, Verner-Jeffreys et al. 2009, Pulkkinen et al. 2010). Generally, it is desirable to have 100% efficacy as just a single reproducing parasite remaining can lead to a new epidemic (Cable & Harris 2002); hence, a treatment derived from pitch (Schelkle et al. 2012) or salt (Schelkle et al. 2011) may be more efficacious, but it would also require the extra labour associated with preparing treatment solutions and treating individual fish. The ease of application of the commercialized products to a whole aquarium of fish makes the Melafix®/Pimafix® combination a viable, convenient and attractive alternative to hobbyists.

The GC-MS results confirm previous studies on cajuput and West Indian bay oils, confirming the quality of these oils to declared specifications (e.g.

Table 2. Compounds identified in (A) cajuput and (B) West Indian bay oils and presented with their linear retention indices (LRI) for the non-polar (DB5) and polar (FFAP) gas chromatography-mass spectrometry columns, peak area on the FFAP column and ranked, relative abundance of the compound. Ranks for the DB5 column are not presented due to the co-elution of 1,8-cineole and limonene for cajuput oil. Identification by comparison of the mass spectrometry result with NIST or Wiley libraries (MS), direct comparison with pure standard (STD), comparison to previously published LRIs (LRI). ND: not detected; NA: not applicable, as compound was not detected on FFAP column

Component	DB5 LRI	LRI	FFAP Area	Rank	Identification
(A) Cajuput oil					
1,8-Cineole	1029[a]	1190	5731.71	1	MS, STD, LRI
Limonene	1029[a]	1181	968.86	2	MS, STD, LRI
Cymene[b]	1021	1253	586.18	3	MS, STD, LRI
γ-Terpinen	1055	1228	290.29	4	MS, STD, LRI
Sabinene	968	1105	230.88	5	MS, STD, LRI
4-Terpineol	1177	1587–1588	226.29	6	MS, STD, LRI
α-Terpineol	1192	1686	197.75	7	MS, STD, LRI
β-Pinene	972	1086–1087	165.09	8	MS, LRI
α-Pinene	928	1007	149.82	9	MS, STD, LRI
Myrcene	987/988	1153	109.82	10	MS, STD, LRI
α-Terpinen	1013	1160–1161	67.42	11	MS, STD, LRI
Terpinolen	1081	1263	61.08	12	MS, STD, LRI
Camphor	1142	1488	57.14	13	MS, STD, LRI
Phellandrene	1002	1146	56.38	14	MS, STD, LRI
α-Thujene	922	1013	10.94	15	MS, STD, LRI
Camphene	944	1044	Trace	16	MS, STD, LRI
(B) West Indian Bay oil					
α-Farnesene	1512/1513	ND	NA	NA	MS, LRI
α-Thujene	928	ND	NA	NA	MS, STD, LRI
Eugenol	1349/1350	2158–2159	8634.09	1	MS, STD, LRI
Myrcene	988	1153–1154	1435.75	2	MS, STD, LRI
Chavicol	1253/1254	2333	1421.99	3	MS, LRI
Limonene	1025	1179	168.83	4	MS, STD, LRI
Linalool	1098/1099	1544	166.09	5	MS, STD, LRI
Methyleugenol	1397	2010	150.83	6	MS, STD, LRI
trans-Caryophyllene[b]	1412/1413	1565	140.12	7	MS, LRI
Ocimene	1044/1045	1240	51.03	8	MS, STD, LRI
3-Octanone	984	1244	46.85	9	MS, STD, LRI
1-Octen-3-ol	979/980	1446–1447	43.25	10	MS, STD, LRI
Cymene	1021	1252–1253	41.50	11	MS, STD, LRI
Unknown (possibly 1,8-cineole + another monoterpene)	ND	1187	37.95	12	
4-Terpineol	1176/1177	1587	34.39	13	MS, STD, LRI
3-Octanol	ND	1390	21.05	14	MS, STD, LRI
Phellandrene	1002	1146–1145	19.75	15	MS, STD, LRI
δ-Cadinene[b]	ND	1730	17.73	16	MS, LRI
α-Pinene	ND	1007	13.50	17	MS, STD, LRI

[a]Co-eluted; [b]tentative identification

Farag et al. 2004, Kim et al. 2008) and also explain their anti-parasitic activity. Both terpenes and phenols are generally dominant in essential oils and are well known for their anti-microbial properties (Gershenzon & Dudareva 2007, Bakkali et al. 2008, Hammer & Carson 2011). Cajuput was dominated by 1,8-cineole (Farag et al. 2004, Piño et al. 2010). Importantly, in cajuput oil 1,8-cineole co-occurs with limonene: the combination of these constituents may cause antagonistic, synergistic, or additive interactions against microbes depending on the ratio and the limonene entantiomer present (van Vuuren & Viljoen 2007). Both the ratio of 1,8-cineole to limonene and the presence of limonene entantiomers can vary seasonally and geographically with cajuput (Burt 2004), but also depends on which *Melaleuca* sp. is used for extraction, as several species of the same genus are referred to as cajuput (Craven 1999). With regard to the current study, the commercial supplier of the botanical oils used produces a raw material to

meet certain specifications all year round to ensure the products give repeatable results.

West Indian bay was dominated by eugenol, myrcene and the tentatively identified chavicol. The same constituents occur in bay oil (Nadal et al. 1973, McHale et al. 1977). Eugenol and chavicol are anti-microbial, but there is little evidence of anti-microbial activity from myrcene (e.g. Caccioni & Guizzardi 1994, Blaszyk & Holley 1998, Walsh et al. 2003, Chang et al. 2008), and no study has previously investigated the anti-parasitic effects of these 3 compounds together. Based on using the whole plant oil against *G. turnbulli* in this study, none of the 3 constituents have anti-parasitic properties, at least not at the concentrations used, or they may act antagonistically.

Interestingly, but not surprisingly, there were discrepancies in the current study between the *in vitro* and *in vivo* findings. Such discrepancies highlight that *in vitro* studies may give us an indication that certain treatments are potentially anti-parasitic, but that conditions *in vivo* are confounding and can have a significant effect on the perceived efficacy of drugs after *in vitro* tests. For research on fish, these confounding factors affecting treatment may include parasite load (although not supported by our data), host mucus layer, immune response, potential absorption of the compound and compound intake by the host affecting systemic activity. In the current study, one particular discrepancy in efficacy has been observed for Crovol which appears to be more effective than both the individual essential oils *in vitro*, whereas *in vivo* results clearly show the emulsifier to have very low efficacy. Further, both essential oils were not as effective anti-helminthics *in vivo* as the *in vitro* trials indicated. We are unsure why the combination treatment of Melafix® and Pimafix® was more effective than the combination of West Indian bay, cajuput and Crovol; however, we know that the production process does not expose any of the products to light or air. By preparing the mixture in the laboratory, the compounds may have been affected by light degradation or oxidation processes which, in turn, may have affected treatment efficacy. Finally, seeing that cajuput has previously been shown to be highly anti-parasitic (Schelkle et al. 2015), a double dose of Melafix® by itself may be equally as effective as the combination treatment used in the current study; however, such speculation would have to be confirmed through further testing.

Overall, Melafix® and Pimafix® are effective as a combination treatment against gyrodactylids. For a recommended 7 d treatment, it is reasonable to suggest that the dose may need to be increased slightly in order to attain 100% efficacy. This would require confirmation by further research, but, if validated, could resolve the problem of gyrodactylid infections in ornamental fish.

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