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In vitro and *in vivo* antiparasitic action of essential oils of *Lippia* spp. in Koi Carp (*Cyprinus carpio*) fed supplemented diets

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Abstract

This study evaluated the *in vitro* antiparasitic activity of the essential oils of *Lippia alba*, *L. origanoides* and *L. sidoides* against monogenean parasites of koi carp *Cyprinus carpio* and its zootechnical performance in net cages. The oils were obtained from the leaves by hydro distillation, and the chemical composition was evaluated via gas chromatography. *In vitro* assays were performed with each essential oil separately and combined in binary (1:1) and tertiary (1:1:1) mixtures with the *Lippia* species at 10, 20, 40, 60, 80 and 100 mg L^{-1} and two control groups (grain alcohol and tank water). To determine zootechnical performance, *L. sidoides* oil was added to the feed at 0.00 (control), 0.25, 0.50, 0.75 and 1%, in triplicate and with 20 fish per net cage fed for 60 days. The best results *in vitro* against monogeneans were observed for *L. sidoides* (40 mg L^{-1} in 8 min), followed by *L. origanoides* (40 mg L^{-1} in 25 min) and *L. alba* (40 mg L^{-1} in 4 h). Reductions in weight gain, protein efficiency rate and specific growth rate were observed in diets containing 0.75% of *L. sidoides* oil in comparison to the control and the 0.25% diet. There were no significant differences in growth, individual mean feed intake, apparent feed conversion and parasitological indices. Based on our results, 0.25% *L. sidoides* oil showed the best zootechnical performance, but was not effective against koi carp parasites *in vivo*.

Key words: fish farming, phytotherapics, efficacy, ectoparasites.

Introduction

Koi carp (*Cyprinus carpio*) is the most widely cultured ornamental fish species in the world and the second best-selling exotic species in Brazil after the goldfish (*Carassius auratus*). The southeastern and eastern regions have the highest farming volumes of koi carp, and along with the marketing of other ornamental fish species, they move more than R$ 700 million per year (50, 74). Despite the economic importance of koi carp, studies on the parasitic issues and the use of essential oils in the diet as antiparasitic treatment are still scarce. Fish health is a

constant concern of ornamental fish farmers, and the Ministry of Fisheries has launched manuals of good management practices, as chronic stress and fish losses in the farms occur mainly in intensive systems with high stocking densities (88, 13). Among intensive culture systems in net tanks, floating structures have an advantage regarding the easy catching of the fish and the possibility of growing several species at different stocking densities. However, intensive systems with high stocking densities can cause stress to animals, because the closer physical contact among fish can facilitate the proliferation of diseases caused by parasites such as monogeneans, cestodes and trichodinids (68, 56). The most common monogeneans found in the gills and skin of koi carp is Dactylogyrids (12). In the state of Santa Catarina, Brazil, two species have been identified, *Dactylogyrus extensus* Mueller et Van Cleave, 1932 and *Dactylogyrus minutus* Kulwiec, 1927 (71). Trichodinids are globally distributed and mainly parasitize the body surface and gills of fish. Some species can be endoparasites and cause serious lesions that can serve as a gateway for other pathogenic agents such as bacteria (42, 84). Regarding the trichodinid species reported in koi carp, *Trichodina mutabilis* Kazubski and Migala, 1968 (46) and *Trichodina* sp. (71) have already been identified in the state of Santa Catarina. These parasites reproduce rapidly under conditions such as poor water quality, high stocking density and immunosuppressed hosts, which are most susceptible to severe infestations, and can cause mortality in all fish life stages (27), necessitating strict control in farming systems.

Fish farmers control parasites by using chemicals such as praziquantel, mebendazole and levamisole (55), but the continuous and inadequate use of these products can result in serious environmental contamination, pathological changes in fish, risks to human health and resistance of the pathogens to the active principles of chemotherapeutic agents (64). These factors have stimulated the search for alternatives to minimise these negative effects. Within this context, certain plant-based products are effective in the prevention, control and treatment of bacterial and parasitic diseases in fish (44, 38). Studies have shown that some plants have antiparasitic properties for fish and other animals (87, 82).

Among the plant species studied for therapeutics are those from the family Verbenaceae, with emphasis on the genus *Lippia*, which presents diverse biological activities and is used for the treatment of respiratory diseases, menstrual disorders and as analgesics and sedative in humans (40, 39, 18). This genus is native to Brazil and can tolerate various types of environments, grows rapidly and is rich in essential oils, with yields varying from 2.44 to 4.4% (92, 89). Among the species with therapeutic importance are bushy matgrass (*Lippia alba*), oregano (*Lippia origanoides)* and pepper rosemary (*Lippia sidoides*), whose essential oils have antimicrobial, antitumor, antifungal, antiparasitic and anaesthetic activities (57, 7, 53, 38).

Lippia alba has antimicrobial activity against *Aeromonas hydrophila* (79), fungicidal action against *Trichophyton rubrum*, *Epidermophyton floccosum* and *Microsporum gypseum* (19), besides acting as stress reducer in the transport of silver catfish *Rhamdia quelen* juveniles (7) and as an anaesthetic for *R. quelen* (33, 80, 78). *Lippia origanoides* has antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella choleraesuis* (5) and *Aeromonas hydrophila* (51), fungicidal action against *Aspergillus fumigatus* (10) and antiparasitic effect against monogeneans from tambaqui *Colossoma macropomum* (77).

Lippia sidoides presents fungicidal action against *Candida* spp. (30), antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* (17) and *Aeromonas hydrophila* (51) and antiparasitic activity against monogeneans from tambaqui *C. macropomum* and tilapia *Oreochromis niloticus* (38, 77). In view of the positive results regarding the use of these essential oils, this study aimed to evaluate the efficacy of the essential oils from *L. alba*, *L. origanoides* and *L. sidoides* against monogenean and trichodinid parasites in koi carp. We evaluated both in vitro and in vivo effects after dietary supplementation, verifying their impacts on zootechnical performance.

Materials and methods

Biological material

Koi carp (*Cyprinus carpio*) used for *in vitro* and *in vivo* assays were obtained from "Vale dos Bettas" fish farm located in the municipality of Biguaçu, Santa Catarina State, Southern Brazil. *In vitro* and *in vivo* assays were performed in this farm. For *in vitro* assays, parasites were collected from carps with mean weight of 3.61±0.56 g and standard length 4.71±0.54 cm. The *in vivo* assay was performed in 1.0 x 1.0 x 1.30 m net tanks with 0.5 cm mesh opening, installed in a pond with approximately 1 hectare of total area. All animal procedures were approved by the Ethic Committee on Animal Use of Federal University of Santa Catarina (CEUA/UFSC 1440100217).

Chemical composition of the essential oils

The essential oils were obtained from the leaves of plants cultivated and processed in the Medicinal Plants and Phytochemistry Laboratory of EMBRAPA Western Amazon, Manaus- AM, Brazil. After the leaves were collected in the morning, they were dried in a continuous circulation oven at 45°C for 48 h and then the extraction of the essential oils was performed by hydro distillation process in a Clevenger type apparatus (56, 70).

The chemical composition of the oils from *L. alba*, *L. origanoides* and *L. sidoides* was determined by gas chromatographic method with Agilent 6890 equipment and selective mass detector Agilent 5973N. The separation of

the components was performed on a capillary column HP5-MS (30 m x 0.25 mm x 0.25 µm) with temperature of 60ºC to 240ºC and variation of 3ºC m $^{-1}$. 1.0 microliter of a solution containing 1% of each oil was injected into the flux splitter in the ratio of 1: 100 and maintained at 250°C. The relative quantification (%) of the components of the oils was carried out in gas chromatograph Agilent 6890N, which was equipped with a flame ionization detector, maintained at 280°C, and by an HP5 capillary column (30 m x 0.32 mm x 0.25 µm) with the use of hydrogen (1.5 mL min-1) as carrier gas. The identification of the constituents of each oil was performed by comparing the mass spectra obtained by Wiley 6th edition spectra library and by comparing the calculated retention index of each component with literature data. The calculation of the index was performed by injecting a series of n-alkanes in the same analytical conditions used for the other oils (3).

In vitro test of the essential oils of Lippia alba, Lippia origanoides and Lippia sidoides separately and in combination

Initially, the three oils were tested *in vitro* at concentrations of 0.25; 0.5; 0.75 and 1.0% to determine its effectiveness against monogenean parasites, for later testing *in vivo*. In these concentrations it was not possible to determine the oils efficacy, since the parasites died instantly. Thus, the experimental concentrations were changed for 10, 20, 40, 60, 80 and 100 mg L^{-1} based on similar studies in the literature (72, 50).

For *in vitro* tests, koi carps were randomly captured, anesthetized with clove oil (75 mg L^{-1}) and euthanized by medullar section for gill arches collection and confirmation of the presence of monogenean parasites with a dissecting microscope (Carl Zeiss Suzhou Co., model Square 3-B). After confirming the presence of parasites, solutions from the oils of the three species of *Lippia* were prepared. The essential oils were diluted in grain alcohol to a stock solution of 10% and soon thereafter the concentrations in the following proportions and treatments were prepared: control with water from fish tank, control with cereal alcohol, *L. alba* (10, 20, 40, 60, 80 and 100 mg L^{-1}), *L. origanoides* (10, 20, 40, 60, 80 and 100 mg L^{-1}), *L. sidoides* (10, 20, 40, 60, 80 and100 mg L^{-1}), *L. alba* + *L. origanoides* (1:1 to 10, 20, 40, 60, 80 and 100 mg L^{-1}), *L. alba* + *L. sidoides* (1:1 to 10, 20, 40, 60, 80 and 100 mg L^{-1}), *L. origanoides* + *L. sidoides* (1:1 to 10, 20, 40, 60, 80 and 100 mg L^{-1}), *L. alba* + *L. origanoides* + *L. sidoides* (1:1:1 to 10, 20, 40, 60, 80 and 100 mg L^{-1}).

The treatments were performed in triplicate in a six well microplate containing a parasitized gill filament per well. The plates were observed every 15 min for quantification of dead parasites and behavioral observations, with parasite death confirmed by absence of movement when stimulated with a needle. At concentrations of 10 and 20 mg L^{-1} of *Lippia* oils, parasite immobilization was observed in the first 20 min of exposure, followed by a recovery of the movements after 50 min, therefore, for effective confirmation of mortality, the parasites were stimulated with a needle for up to 5 h after the start of the experiment.

In vivo test with Lippia sidoides essential oil supplemented in the diet

The essential oil of *Lippia sidoides* was selected for the *in vivo* assay since it presented higher efficacy against parasites *in vitro*. The essential oil was added to a commercial diet already used in the farm, containing 50% crude protein. The essential oil at concentrations 0.25; 0.5; 0.75 and 1.0% was diluted in grain alcohol and added to the feed at the proportion 100 g of alcohol per kg. The oil was weighed with the aid of a digital scale. The essential oil with its respective concentrations was placed in a hand sprayer and sprinkled on the feed, which was left to dry for 24 hours at room temperature and then stored in a freezer until the day of its use (21).

In vivo assay in net cages

Three hundred carps from the same pond with mean weight of 3.56±0.68 g were distributed in 1 x 1 x 1.30 m net tanks with 0.5 cm mesh opening. The experimental design consisted of five treatments, in triplicate: control (commercial feed without addition of essential oil), 0.25, 0.50, 0.75 and 1.0% of *Lippia sidoides* essential oil. The fishes were fed twice a day (9 a.m. and 4 p.m.) for 60 days. During this period, water quality parameters remained at the following values: dissolved oxygen 6.08±1.55 mg L^{-1}, temperature 20.50±0.88ºC, pH 5.40±0.82, electrical conductivity 40.73±6.52 µS cm^{-3} and total suspended solids 19.25±4.20 mg L^{-1} measured with multiparameter instrument (Hanna HI 9828), total ammonia 0.58±0.27 mg L-1 and nitrite 0 mg L-1 measured with commercial colorimetric kit (Alcon pet®) and transparency 24.17±1.83 cm measured with Secchi disk. At the beginning of the experiment and at the times of 30 and 60 days, biometrics of all the fish were performed for the calculation of zootechnical indexes and samples of six fish per experimental unit were collected for parasitological analysis.

Growth performance parameters

The calculations of daily weight gain, protein efficiency rate, mean individual feed intake, apparent feed conversion, specific growth rate and survival rate were performed using the formulas (30):

Weight gain (g) = mean final weight (g) – mean initial weight (g).

Protein efficiency rate (PER) = weight gain (g) / ingested crude protein (g).

Mean Individual Feed Intake (MIFI) = provided feed (kg)/number of fish.

Apparent feed conversion (AFC) = MIFI / [(mean final weight (g) – mean initial weight (g)].
Specific Growth Rate (SGR) = 100 x (ln mean final weight – ln mean initial weight) / time.
Survival Rate (SR) = 100% x (final number of fish/initial number of fish).

Parasitological analysis

Six fish per experimental unit were anesthetized in clove oil solution (75 mg L^{-1}) and euthanized by brain section for collection of the branchial arches and mucus scraping. Gill arches were initially bathed in water at 55°C and then fixed in 70% ethanol. Mucus scraping was fixed in 70% ethanol and analyzed under microscope.

The quantification of parasites was calculated using the prevalence rate, mean intensity (41) and average parasite abundance (14). The monogeneans found in the experiment were mounted in Hoyer's medium between glass slide and cover slip for observation of sclerotized structures such as copulatory complex, horseradish bar, hooks and anchors (26), and identified according to (58) and (45). For trichodinids, the Klein method of silver impregnation (43) was applied for parasite identification (60, 77, 24).

Statistical analysis

To evaluate the differences among the means, ANOVA was applied with significance level of 0.05. Before the analysis, the data were tested for normality and homoscedasticity, and the data expressed as percentage were normalized by applying angular transformation. When necessary, Tukey test applied to confirm differences among means. Statistical analyzes were performed on GraphPad 4.03 software (La Jolla, CA, USA).

Results

Chemical composition of the essential oils

The analysis results of the chemical composition of *L. sidoides*, *L. origanoides* and *L. alba* are shown in Fig. 1. For *L. sidoides*, 100% of the chemical components were quantified and 99.4% identified. The compounds with the highest percentages were thymol (72.2%), p-cymene (8.15%) and (E)-caryophyllene (4.9%). In *L. origanoides*, 100% of the compounds were quantified, and the most abundant ones were identified as p-cymene (37%), carvacrol (14%), y-terpinene (11.6%) and linalool (6%). In *L. alba*, 100% of the compounds were quantified, and 97.9% of the compounds were identified. The major constituents were carvone (58.2%), limonene (19.2%) and D-germacrene (3.8%).

In vitro test of the essential oils of Lippia alba, Lippia origanoides and Lippia sidoides separately and in combination

The best result was assumed as the lowest concentration that killed monogenean *Dactylogyrus minutus* and *Dactylogyrus extensus* within the shortest time. Best results were obtained with *L. sidoides* and *L. origanoides* assessed separately, while the worst results were observed in the treatments containing only *L. alba* essential oil (Table 1). The treatments that presented 100% mortality with the lowest essential oil concentration within the shorted time were as follows: *L. sidoides* (≥ 40 mg L^{-1} in 8 min), *L. origanoides* (≥ 60 mg L^{-1} in 9 min) and *L. alba* + *L. origanoides* (≥ 60 mg L^{-1} in 12 min).

In addition, in solutions containing concentrations of essential oils above 20 mg L^{-1}, the monogenean parasites presented continuous contortions, followed by alterations in the body shape and the appearance of oedema, vacuolisation of internal structures and rupture of the internal membrane. There was a temporary neutralisation of parasite movements, even with needle stimulation, after the first 20 min of exposure to essential oils at concentrations of 10 and 20 mg L^{-1} and recovery of the movements after 50 min of the experiment in all treatments, with subsequent parasite mortality.

In vivo *test with essential oil of* Lippia sidoides

In the present study, the monogeneans *Dactylogyrus minutus* and *Dactylogyrus extensus* and the trichodinids *Trichodina reticulata* Hirschmann et Partsch, 1955, *Trichodina heterodentata* Duncan, 1977 and *Trichodina* sp. were identified. There was no fish mortality in any of the treatments; however, reductions in weight gain, protein efficiency rate and specific growth rate were observed in fish fed diets containing 0.75% of essential oil from *L. sidoides* in comparison to the control group and the animals fed a diet containing 0.25% of essential oil. The other parameters did not present significant differences ($p > 0.05$) among treatments (Table 2).

In the present study, the parasitological indices of koi carp fed 0.25; 0.5; 0.75 and 1% *L. sidoides* essential oil were similar to those of the control at 30 days of feeding. There was no reduction in the parasitic load of the fish in the experimental period, with a prevalence of 72.22% and a mean intensity of 1.91 at the concentration of 1%, while the control had a prevalence of 66.67% and a mean intensity of 2.06.

Throughout the experimental period, the highest and lowest parasite prevalence values were 83.33 to 55.56%, with mean intensities of 8.27 to 4.72 at concentrations of 0.25 and 0.50% of the oil, evidencing that *L. sidoides* essential oil was not effective at the concentrations tested for monogeneans (Table 3).

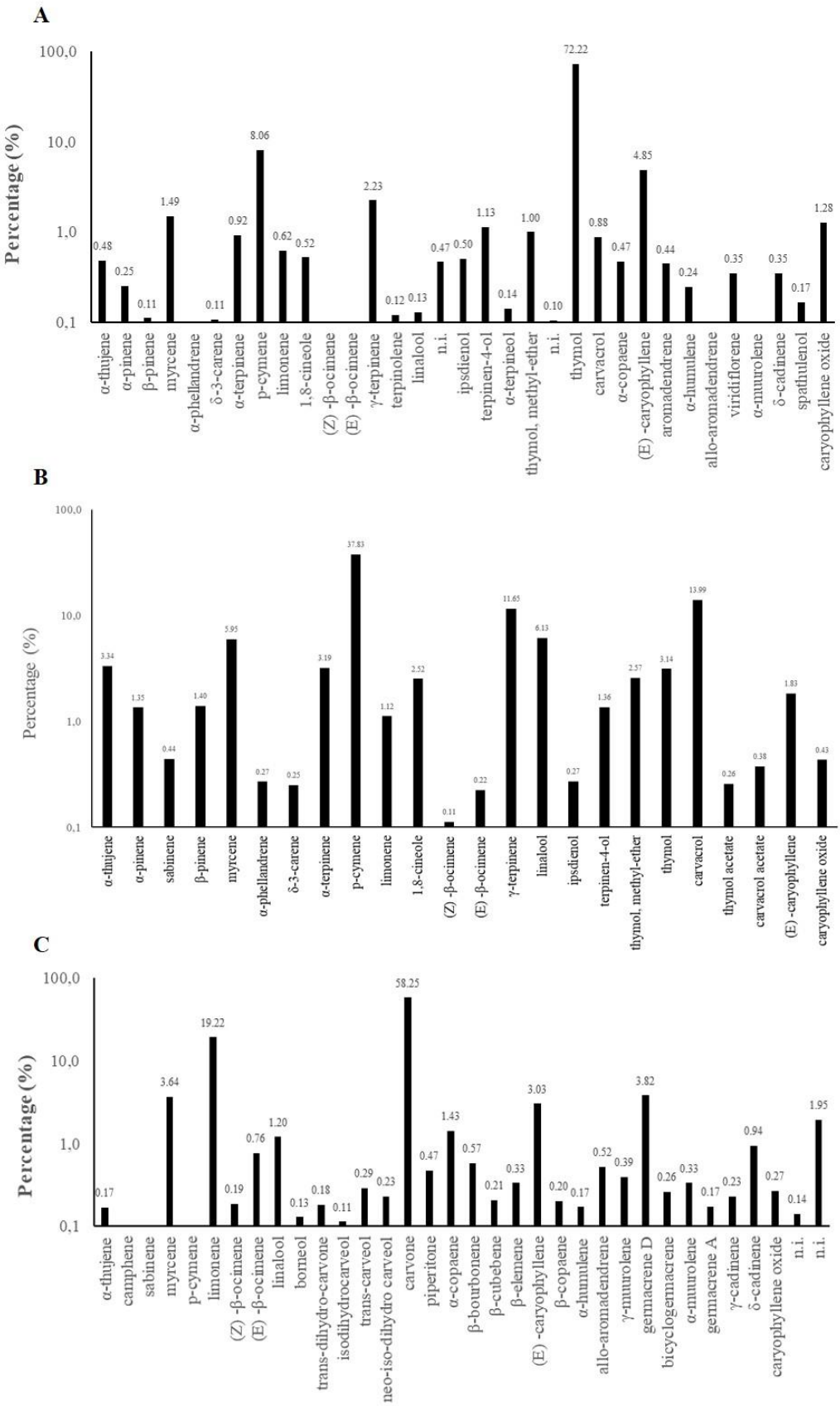

Figure 1. Chemical composition of the essential oils from **A.** *Lippia sidoides* **B.** *Lippia origanoides* and **C.** *Lippia alba*.

Table 1. *In vitro* test with crescent levels of essential oils from *Lippia alba*, *Lippia origanoides* and *Lippia sidoides*, separately and in combination, at 10, 20, 40, 60, 80, 100 mg L^{-1}, with water control and grain alcohol control.

Concentration of essential oils (mg L^{-1})	Mortality time	Initial number of parasites	Final number of dead parasites	Mortality rate (%)
Water control	3 h 57 min	23	23	100.00
Alcohol control	5 h 29 min	19	15	78.95
L. alba				
10	4 h 47 min$^{a*\#}$	19	16	84.21
20	4 h 48 min^{a*}	29	27	93.10
40	4 h 24 min^{a*}	38	29	76.32
60	4 h 12 min^{a*}	32	25	78.13
80	3 h 17 min^{b*}	25	23	92.00
100	3 h 12 min^{b*}	30	30	100.00
L. origanoides				
10	2 h 24 min^{a}	19	19	100.00
20	1 h 45 min^{a}	16	16	100.00
40	25 min^{b}	18	18	100.00
60	9 min^{c}	18	18	100.00
80	7 min^{c}	20	20	100.00
100	5 min^{c}	18	18	100.00
L. sidoides				
10	2 h 58 min^{a}	20	20	100.00
20	1 h 26 min^{b}	17	17	100.00
40	8 min^{c}	25	25	100.00
60	6 min^{c}	19	19	100.00
80	7 min^{c}	24	24	100.00
100	5 min^{c}	17	17	100.00
L. alba + *L. origanoides*				
10	4 h 27 min^{a*}	28	21	75.00
20	4 h 53 min^{a*}	25	16	64.00
40	4 h 5 min^{a*}	21	12	57.14
60	12 min^{b}	23	23	100.00
80	11 min^{b}	20	20	100.00
100	8 min^{b}	22	22	100.00
L. alba + *L. sidoides*				
10	4 h 6 min^{a*}	16	4	25.00
20	4 h 6 min^{a*}	24	14	58.33
40	2 h 23 min^{b}	17	17	100.00
60	20 min^{c}	21	21	100.00
80	19 min^{c}	30	30	100.00
100	6 min^{d}	16	16	100.00
L. origanoides + *L. sidoides*				
10	4 h 38 min^{a*}	18	12	66.67
20	4 h 37 min^{a*}	18	9	50.00
40	4 h 26 min^{a*}	47	22	46.61
60	1h 3 min^{b}	26	26	100.00
80	8 min^{c}	31	31	100.00
100	6 min^{c}	22	22	100.00
L. alba + *L. origanoides* + *L. sidoides*				
10	4 h 17 min^{a*}	14	12	85.71
20	4 h 12 min^{a*}	15	13	86.67
40	3 h 16 min^{b}	27	27	100.00
60	32 min^{c}	43	43	100.00
80	10 min^{d}	26	26	100.00
100	9 min^{d}	39	39	100.00

*Identical to water control. $^{\#}$Identical to alcohol control. Different letters indicate significant difference ($p<0.05$) among concentrations in the same treatment, by Tukey's test.

Table 2. Zootechnical parameters (mean±standard deviation) from Koi carp fed supplemented diets with different concentrations of *Lippia sidoides* essential oil. WG: weight gain, SL: standard length, PER: protein efficiency rate, MIFI: mean individual feed intake, AFC: apparent feed conversion, SGR: specific growth rate, SR: survival rate.

	Concentration of essential oil of *Lippia sidoides*					
Parameters	**Control**	**0.25%**	**0.50%**	**0.75%**	**1.0%**	***p***
WG	3.79±0.44^{a}	3.89±0.31^{a}	2.67±0.29^{ab}	2.38±0.70^{b}	3.23±0.56^{ab}	0.011
SL (cm)	3.54±0.18	3.48±0.22	3.29±0.57	3.26±0.32	3.61±0.65	0.812
PER	0.92±0.13^{a}	0.94±0.10^{a}	0.67±0.08^{ab}	0.58±0.17^{b}	0.80±0.13^{ab}	0.020
MIFI	0.07±0.00	0.07±0.00	0.07±0.00	0.07±0.00	0.07±0.00	0.294
AFC	0.02±0.00	0.02±0.00	0.03±0.00	0.03±0.00	0.02±0.00	0.069
SGR	6.32±0.73^{a}	6.48±0.52^{a}	4.45±0.48^{ab}	3.97±1.16^{b}	5.38±0.93^{ab}	0.011
SR (%)	100	100	100	100	100	-

WG: weight gain, SL: standard length, PER: protein efficiency rate, MIFI: mean individual feed intake, AFC: apparent feed conversion, SGR: specific growth rate, SR: survival rate. Different letters indicate significant difference among treatments by Tukey's test ($p<0.05$).

Table 3. Parasitological indexes (mean ± standard deviation) of monogeneans from Koi carp in net tanks, fed control and supplemented diets with 0.25%, 0.5%, 0.75% and 1% of essential oil from *Lippia sidoides*

		30 Days				**60 Days**			
TR	**IS**	**P (%)**	**MA**	**MD**	**MI**	**P (%)**	**MA**	**MD**	**MI**
0.00	M	0	0	0	0	0	0	0	0
	G	66.67	1.44±1.01	0.05±0.03	2.06±1.29	72.22	2.56±1.58	0.15±0.09	3.42±1.77
0.25	M	0	0	0	0	0	0	0	0
	G	66.68	3.22±1.82	0.82±1.16	3.38±1.84	83.33	6.89±2.83	0.21±0.08	8.27±3.40
0,50	M	0	0	0	0	0	0	0	0
	G	61.11	3.50±6.06	0.06±0.1	4.20±7.27	55.56	2.56±1.66	0.09±0.06	4.72±1.39
0.75	M	0	0	0	0	0	0	0	0
	G	61.48	1.67±1.74	0.06±0.06	2.22±1.95	66.67	3.83±2.20	0.09±0.05	5.40±1.31
1.00	M	0	0	0	0	0	0	0	0
	G	72.22	2.11±0.91	0.06±0.02	1.91±0.62	77.78	3.83±2.58	0.17±0.11	5.58±3.45

Treatment (TR), prevalence (P%), mean abundance (MA), mean dominance (MD), mean intensity (MI), infestation site (IS), gills (G), mucus from body surface (M).

Also, there was a prevalence of 83.33 to 55.56% of monogeneans in concentrations of 0.25 and 0.50% of the oil in the diet after 60 days of supplementation. Among the trichodinids, the prevalence was 100 and 66.67% at concentrations of 0.25 and 1.0% of the oil in the diet in 60 days. Parasitological indices such as prevalence, mean abundance, mean dominance and mean intensity of the identified groups did not show significant differences ($p > 0.05$) among treatments, both at 30 and 60 days (Tables 3 and 4).

Discussion

Chemical composition of the essential oils of Lippia spp.

In this study, different species of the genus *Lippia* presented different major compounds, corroborating other authors (76), who also found variations in the content and chemical composition of the essential oils of the same plant species. These variations in the chemical composition of *Lippia* may be related to soil type, environmental factors, collection period, seasonal variation and month (73, 37).

Analyses of the chemical composition of *L. sidoides* oil indicated that thymol and p-cymene are the major chemical components. These results corroborate those obtained in other studies (21, 28, 51, 77), which, although obtaining different percentage values, also listed thymol and p-cymene as the most abundant components in the essential oil of this species. Studies with isolated thymol have proven its antimicrobial activity. A comparison between the antimicrobial activity of *Lippia sidoides* oil and its major compound, thymol, against bacteria (*Streptococcus aureus*, *S. mutans*, *Klebsiella pneumoniae*, *Providencia rettgeri*, *Enterobacter cloacae*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli*) was performed, and there was no difference in antimicrobial effect between essential oil and isolated thymol, evidencing that it is the component responsible for the antimicrobial activity (86). Thymol alone, at a concentration of 10 mg mL^{-1}, has antibacterial activity against *S. mutans* (13).

Table 4. Parasitological indexes (mean±standard deviation) of trichodinids from Koi carp in net tanks, fed control and supplemented diets with 0.25%, 0.5%, 0.75% and 1% of essential oil from *Lippia sidoides*.

		30 Days				60 Days			
TR	**IS**	**P (%)**	**MA**	**MD**	**MI**	**P (%)**	**MA**	**MD**	**MI**
0.00	M	88.89	9.17±3.24	0.28±0.1	10.08±1.72	77.78	2.83±2.36	0.17±0.14	3.35±2.72
	G	0	0	0	0	0	0	0	0
0.25	M	83.33	4.61±0.75	0.18±0.03	5.56±0.21	88.89	3.61±1.41	0.11±0.04	4.00±1.24
	G	0	0	0	0	0	0	0	0
0.50	M	83.33	34.89±42.06	0.24±0.11	16.87±7.91	100	6.06±2.27	0.23±0.08	9.89±6.09
	G	0	0	0	0	0	0	0	0
0.75	M	77.78	7.78±6.01	0.27±0.21	9.37±6.64	100	9.28±6.09	0.23±0.15	9.28±6.09
	G	0	0	0	0	0	0	0	0
1.00	M	94.44	8.56±4.58	0.25±0.13	8.98±4.33	66.67	3.67±2.72	0.16±0.12	6.33±6.04
	G	0	0	0	0	0	0	0	0

Treatment (TR), prevalence (P%), mean abundance (MA), mean dominance (MD), mean intensity (MI), infestation site (IS), gills (G), mucus from body surface (M).

In *L. origanoides* oil, p-cymene was the most abundant compound, followed by carvacrol and γ-terpinene. These results differ from those obtained in other studies (51, 4), which found a higher proportion of carvacrol (41.1-49.7%), with p-cymene being the second most abundant compound. Isolated carvacrol from *L. origanoides* showed antifungal activity against *A. fumigatus* and *A. flavus* (10), and p-cymene from the same plant was effective against *Trypanosoma cruzi* and *Leishmania chagasi* (28). In *L. alba* essential oil, the major compounds were carvone and limonene. Carvone ranged from 54.5 to 61.7% and limonene from 17.5 to 23.1%, similar to previous results (39, 76). Isolated carvone has antifungal, antimicrobial, anti-quorum sensing, insecticidal and insect-repellent properties (66, 11, 67).

In vitro analysis of the essential oils of Lippia spp. against monogeneans of koi carp

Essential oil of *L. sidoides* showed 100% efficacy *in vitro* against koi carp monogeneans at 40 mg L^{-1} for 8 min. This dose was lower than those used in other effective treatments against monogenean parasites from other fish species (50, 72). A 100% efficacy in vitro for *L. sidoides* essential oil was observed against monogeneans from tambaqui (*Colossoma macropomum*) after 10 min exposure to 320 mg L^{-1}, while those exposed to 160 mg L^{-1} showed total mortality only after 1 h (76). Other studies report 100% mortality of the monogeneans *Cichlidogyrus tilapiae* Paperna, 1960, *Cichlidogyrus thurstonae* Ergens, 1981, *Cichlidogyrus halli*, Price and Kirk, 1967 and *Scutogyrus longicornis* Paperna and Thurston, 1969 from Nile tilapia with *L. sidoides* oil at 160 mg L^{-1} for 60 s. (38). One hundred efficacy was found for *Melaleuca alternifolia* and *Mentha piperita* essential oils against monogeneans from pacu *Piaractus mesopotamicus* at 400 mg L^{-1} and for *Copaifera duckei* oleoresin at 100 mg L^{-1} after 60 min (20).

In the present study, oedema, vacuolisation and lysis of monogenean parasites were produced by essential oils from all *Lippia* species at concentrations of 40, 60, 80 and 100 mg L^{-1}. Similar observations have been reported in other studies (20) with copaiba (*C. duckei*) oleoresin, which produced swelling and lysis of the monogenean parasites from *P. mesopotamicus*, suggesting that its mode of action affecting cell membrane permeability. The same effect occurred with oils from *Lavandula angustifolia*, *Melaleuca alternifolia* and *Mentha piperita*, resulting in signs of swelling, vacuolisation, lysis and death of trophonts of *Ichthyophthirius multifiliis* Fouquet, 1876 from pacu *P. mesopotamicus* (85). Swelling and lysis have also been reported in studies with trophonts of *I. multifiliis* from grass carp (*Ctenopharyngodon idella*), which showed transparent bubbles in the plasma membrane, cytoplasmic damages, loss of membrane integrity and death after exposure to cynatratoside-C extracted from *Cynanchum atratum* root (35). These effects, classified as permeabilization of the cellular membrane, were observed in the protozoan parasite *I. multifiliis* from the gills of the channel catfish *Ictalurus punctatus* when exposed to pentagalloylglucose, extracted from *Galla chinensis* (90). The temporary neutralisation of parasites at concentrations of 10 and 20 mg L^{-1} in all treatments may be related to the anaesthetic action exerted by *L. alba*, *L. origanoides* and *L. sidoides* (70, 76). Thus, this result serves as an alert for a careful use of *Lippia* essential oils for in vitro tests against monogeneans, since this neutralising effect may mask the result over the mortality time of the parasites. Therefore, cautious monitoring of the exposure time of organisms to solutions that present compounds with anaesthetic potential is recommended.

In vivo test with essential oil of Lippia sidoides

Monitoring zootechnical performance during *in vivo tests* with phytotherapics is fundamental to verify if the tested product has some anti-nutritional factors, which could result in impaired growth and survival. In the present study, fish fed 0.75% essential oil showed reductions in zootechnical performance compared to control fish; however, those fed 1% essential oil did not demonstrate such results. Regarding growth, some studies have shown positive effects of other kind of essential oil in the diet (14, 2, 8, 16).

In the present study, *L. sidoides* oil did not influence parasite load after 60 days. For trichodinids, there was a prevalence of 100 and 66.67%, a mean intensity of 6.33 and 9.89 at concentrations of 0.25 and 1.0% of *L. sidoides* essential oil, respectively. In the form of therapeutic baths, 3 ppt of garlic oil showed 74% efficacy after 1 h exposition against *Trichodina* in Nile tilapia and 100% efficacy using crude garlic and Indian almond extracts at 800 ppm 2 days after treatment; however, the parasites reappeared after two weeks (1). The aqueous extract from *Artemisia vulgaris* at 800 mg L^{-1} of garlic for 5 days was effective against *Trichodina* sp. and *Aeromonas hydrophila* in tilapia (62).

Similarly, in studies with pufferfish *Takifugu rubripes* fed peppermint and cinnamon oils at 2.5 g kg^{-1} for 20 days, there was no effect on parasitism by *Heterobothrium okamotoi* Ogawa, 1991, (41). Ineffectiveness was also identified regarding dietary supplementation with ginger extract against *Gyrodactylus turnbulli* Harris, 1986, in *Poecilia reticulata* (49). In this study, both the control and treatments had similar results regarding parasitological indices.

Different from the present study, in other studies, phytotherapeutics in the diet have shown positive results against monogeneans (59, 24, 32, 93). It is possible that in the present study, the tested doses of *L. sidoides* essential oil were not high enough to eliminate parasites. Therefore, higher concentrations would be required but always assessing the possible toxic aspects about these doses. In another study, grass carp (*Ctenopharyngodon idella*) co-infected with *D. ctenopharyngodonid* and *I. multifiliis* were fed 4% *Astragalus membranaceus*, *Allium sativum*, *Morus alba* and *Glycyrrhiza uralensis* and treated with baths containing ginger extract at 4 mg L^{-1}, reaching 100% effectiveness at 28 days of treatment (34).

Dietary treatment is the preferred way for the administration of therapeutic products to fish, allowing treatment of fish of all sizes and in all life stages and preventing mortality. As there is no need to capture individuals, stress and injuries are largely avoided, and in addition, other diseases can be treated simultaneously, resulting in lower costs and increased immune responses and resistance to diseases (48, 14).

Dactylogyrus minutus and *D. extensus*, observed in this study parasitising koi carp gills, are generally found in these organs and can also occur in the nasal cavities of fish (65). This corroborates the findings of other studies with koi carp, where *D. minutus* was the most abundant parasite in the gill filaments of this fish (26, 71). In the present study, *T. reticulata*, *T. heterodentata* and *Trichodina* sp. were identified on the body surface of koi carp (54), while in another study, *T. reticulata* was identified for the first time in Brazil, parasitising the skin of *C. auratus*, which belongs to the same family as the koi carp (71). The species of *Trichodina* are not specific for koi carp, with *T. reticulata* occurring in *C. carpio* (25) and *T. heterodentata* in the common carp *C. carpio* (9) and in the piauçu *Leporinus macrocephalus* (84). *Trichodina heterodentata* has also been reported in *Rhinella pombali* tadpoles (29). Trichodinids are the parasitic agents that most affect fish worldwide (43); they are generally found in the skin and gills, where they cause itching, irritation and other clinical signs. In outbreaks of these parasites, fish present necrosis of the epidermis and erosion of the fins. Carps with immunosuppression or disease may be more affected by trichodinids (68), which serves as a justification for future studies to check the effects of the essential oils on the immune response, and consequently, on disease resistance.

Conclusion

The essential oil from *L. sidoides* was effective *in vitro* against monogenean parasites at concentrations of 40, 60, 80 and 100 mg L^{-1}. However, it was not effective in the *in vivo* test against koi carp parasites at 0.25, 0.5, 0.75 and 1.0% in the diet. No improvement of the zootechnical performance of the fish was found. Further studies are recommended with other doses to verify its potential to increase disease resistance by enhancing the immune response, as well as the use of the oil in therapeutic baths to reduce parasitism.

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