Aquaculture 450 (2016) 182–186

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

# Essential oils of *Lippia sidoides* and *Mentha piperita* against monogenean parasites and their influence on the hematology of Nile tilapia

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ARTICLE INFO

*Article history:*
Received 10 July 2015
Received in revised form 27 July 2015
Accepted 28 July 2015
Available online 1 August 2015

*Keywords:*
Fish
Phytotherapy
Rosemary pepper
Peppermint
Anti-parasitic
Blood

ABSTRACT

This study evaluated the use of therapeutic baths containing essential oils of *Lippia sidoides* (pepper rosemary) and *Mentha piperita* (peppermint) on the hematological parameters of Nile tilapia parasitized by the monogeneans *Cichlidogyrus tilapiae*, *Cichlidogyrus thurstonae*, *Cichlidogyrus halli*, and *Scutogyrus longicornis*. A total of 320 juvenile fish were distributed into 16 tanks of capacity 100 L (20 fish per tank), divided into 4 treatments in quadruplicates: fish exposed to a bath of *L. sidoides* at 20 mg $L^{-1}$; fish exposed to *M. piperita* at 40 mg $L^{-1}$; fish exposed only to a water bath; and fish exposed to water + DMSO (dimethyl sulfoxide) bath. The fish were subjected to 3 baths for 10 min, at intervals of 24 h between treatments. After the third bath, parasitological and hematological analyses were performed. The parasite prevalence in fish treated with essential oils was seen to have decreased by 70%. The efficacy attained among fish treated with *L. sidoides*, in comparison with control water and water + DMSO, was 1.96% and 14.16%, respectively; and among fish treated with *M. piperita*, it was 33.33% and 41.63%, respectively. The total numbers of red blood cells (RBC) and thrombocytes were lower in fish treated with *L. sidoides*. Glucose concentration and neutrophil count were significantly higher in fish treated with *L. sidoides*. Because of the efficacy and positive hematological results, we suggest that baths of *M. piperita* at 40 mg $L^{-1}$ should be used as anthelmintic action.

*Statement of relevance:* Authors believe on the use of essential oils to treat ectoparasites of cultured fish and consequently no damages for hematological profile of Nile tilapia were found.



## 1. Introduction

In aquaculture, phytotherapeutics for use among farmed aquatic animals may present several advantages, such as reduced environmental impact, biodegradability, lower residue levels in animals, low toxicity, and several modes of action resulting in low likelihood of causing resistance and low cost for farmers (Soares and Tavares-Dias, 2013; Chagas et al., 2014). A recent review has shown that the use of plant extracts in aquaculture has been responsible for increased immune response and hematological and biochemical improvements (Bulfon et al., 2015).

*Lippia sidoides*, commonly known as rosemary pepper, is a type of bush found in northeastern Brazil that presents antiseptic and antimicrobial properties (Costa et al., 2002). Species of *Lippia* have been exploited in several fields such as veterinary medicine, microbiology, parasitology, zootechny, and aquaculture because of their bioactive potential and ease of use on a large scale (Soares and Tavares-Dias, 2013).

The genus *Mentha*, known as peppermint, has been exploited for its flavor and is used in medicine as an antimicrobial and antioxidant agent (Tsai et al., 2013). It produces essential oil containing menthol and several components used in the pharmaceutical and natural product industry (Kumar and Patra, 2012). Menthol is the main component after oil extraction (Freire et al., 2011; Tsai et al., 2013).

A study by Moghaddam et al. (2013) showed that *Mentha piperita* had antifungal activity against three species of fungi: *Dreschlera spicifera*, *Fusarium oxysporum* f.sp. *ciceris* and *Macrophomina phaseolina*. Similar data were obtained by Freire et al. (2011), who demonstrated the inhibitory potential of this essential oil against the fungi *Aspergillus flavus*, *Aspergillus glaucus*, *Aspergillus niger*, *Aspergillus ochraceous*, *Colletotrichum gloesporioides*, *Colletotrichum musae*, *Fusarium oxysporum*, and *Fusarium semitectum*.

Compared with the use of plant extracts for immunostimulant effects, they have been little used against parasites, especially against monogeneans (Bulfon et al., 2015). The anthelmintic activity of oils or plant extracts has been evaluated mainly against monogenean parasites

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http://dx.doi.org/10.1016/j.aquaculture.2015.07.029
0044-8486/

of goldfish (*Carassius auratus*) (Steverding et al., 2005; Wang et al., 2009; Wang et al., 2010a, 2010b, 2011).

The efficacy of seed extracts from *Piper guineense* against the monogenean parasites *Gyrodactylus* and *Dactylogyrus* of goldfish has been studied *in vitro* and *in vivo* (Ekanem et al., 2004). Monogenean helminths are one of the most important parasites affecting fish farming and are found mainly in the gills and skin (Jerônimo et al., 2011).

Several natural substances can be used in aquaculture, but studies involving the use of extracts or essential oils in therapeutic baths against fish parasites are scarce (Reverter et al., 2014). The present study evaluated therapeutic baths using *L. sidoides* and *M. piperita* against monogenean gill parasites in Nile tilapia (*Oreochromis niloticus*), *in vitro* and *in vivo*.

## 2. Material and methods

### 2.1. Essential oil extraction

Essential oils used in this study were obtained from the leaves of *L. sidoides* and *M. piperita* cultured in the Section of Medicinal Plants of EMBRAPA Western Amazon situated in Manaus, AM (03°06′23.04″S and 60°01′35.14″W). Mean altitude is 50 m and mean air temperature is 25.6 °C with annual rainfall of 2200 mm. Plants were collected in the morning and the material processed in the Medicinal Plants and Phytochemistry Laboratory of Embrapa Western Amazon, Manaus, Brazil. Oil extraction was performed by the hydrodistillation method using a Clevenger-like equipment. After that, the oils were maintained refrigerated at −18 °C in dark glasses. Briefly, for chemical composition analysis a gas chromatograph Agilent (Palo Alto, USA) 7890A equipped with capillary column HP-5 (5%-diphenyl-95%-dimethyl silicon 30 m × 0.32 mm × 0.25 μm) was used. Temperature was programmed in 60 to 240 °C, a 3 °C $min^{-1}$, and using hydrogen as carrier gas (1.5 mL $min^{-1}$). 1.0 μL of 1% essential oil solution in dichloromethane (Merck Millipore, Darmstadt, Germany) with flux division (1:100, injector at 250 °C) was injected. The mass spectrum was obtained in a system Agilent 5973 N operated in the mode electron impact (EIMS) at 70 eV, coupled in a chromatograph Agilent 6890 using the same procedure of injection and temperature cited above. Retention indices were calculated from the retention times of the compounds and those of a serial of n-alkanes ($C_7$–$C_{26}$). Constituent identification was made by comparison of the mass spectrum obtained with the data of spectral library (Wiley 6th Ed.) and by the indices of the retention calculated and compared to published values (Adams, 2007).

### 2.2. In vitro immobilization assay

The monogenean parasites *Cichlidogyrus tilapiae* Paperna, 1960, *Cichlidogyrus thurstonae* Ergens, 1981, *Cichlidogyrus halli* Price and Kirk, 1976 and *Scutogyrus longicornis* Paperna and Thurston, 1969 were used in this assay and identified according to Douëllou (1993), Pariselle and Euzet (1995), Pariselle et al. (2003), and Thatcher (2006). The parasites were collected directly from the gills of parasitized tilapia, immediately prior to beginning assay.

Six concentrations of *L. sidoides* and *M. piperita* essential oils were used to obtain the most efficient in causing the parasite mortality. A stock solution was composed by 1 g of essential oils diluted in 9 mL of dimethyl sulfoxide (DMSO — $C_2H_6OS$) in a proportion of 1:10.

Parasitized gill filaments of Nile tilapia were separated in sterile Petri dishes 5.5 cm comprising in each concentration with three replicates. From the stock solution, essential oils were diluted in 25 mL water to obtain 320, 160, 80, and 40 mg $L^{-1}$. For the lowest concentrations of 20 mg $L^{-1}$ and 10 mg $L^{-1}$, 10 μL in 50 mL and 100 mL water divided in 8 mL in each Petri dish was diluted. Two controls were used, one of them diluting 80 μL DMSO in 25 mL water and the other only water.

For the lowest concentrations the parasites were observed each 15 min and for the highest concentrations (160 and 320 mg $L^{-1}$) the observation was continuous. Parasites were considered dead when detected the absence of movement when stimulated with a needle or body wrinkling.

### 2.3. Toxicity test

The toxicity test aimed to evaluate the tolerance of the fish to exposure to the oil. For each concentration, three fish were used in 3 L of water and the oil solution was added in order to observe their behavior. Water quality was measured before and after the treatment. In situations of abnormal behavior such as agitation, anoxia, intense swimming or tipping over, the fish was immediately transferred to another bucket and the time was registered. Fish handling and samples collection were approved by Ethic Committee of Federal University of Santa Catarina (CEUA/UFSC PP00869).

### 2.4. In vivo assay

A total of 320 healthy juvenile Nile tilapia (weight of 9.76 ± 0.48 g and length of 8.47 ± 0.18 cm) from the same spawning were acquired from tilapia fish farmer and acclimatized for 7 days prior to distribution into 16 circular tanks of capacity 100 L.

There were 4 treatments and 4 replicates: fish exposed to *L. sidoides* bath at 20 mg $L^{-1}$; fish exposed to *M. piperita* bath at 40 mg $L^{-1}$; fish exposed to water; and fish exposed to water + dimethyl sulfoxide (DMSO).

During the assay, 50% of the water was renewed and the fish were fed three times a day with commercial dry ration for omnivorous fish, containing 28% crude protein. The water quality parameters did not alter among the treatments: temperature 27.67 ± 0.99 °C, dissolved oxygen 6.83 ± 0.92 mg $L^{-1}$, pH 5.86 ± 0.65 measured using a multiparameter portable Hanna HI9829® (Hanna Instruments Brazil, SP), and ammonia 3.00 ± 1.01 mg $L^{-1}$, nitrite 0.04 ± 0.01 mg $L^{-1}$ and nitrate 0.70 ± 0.22 mg $L^{-1}$ measured by colorimetric kit Alfakit® (Alfakit, SC, Brazil).

Each treatment consisted of three baths of 10 min at intervals of 24 h. The therapeutic solution was distributed at the edge of the tank: 14 mL of stock solution of *L. sidoides*; 28 mL of *M. piperita* solution; water alone; and water + 224 mL of DMSO. After the third bath, 10 fish from each replicate were collected for parasitological and hematological analysis.

### 2.5. Parasitological and hematological analyses

Fish were anesthetized in clove oil solution (75 mg.$L^{-1}$), euthanized and the gills of 5 fish per replicate were collected for immediate parasitological analysis and the other 5 bathed in water 60 °C and fixed in alcohol 70% for posterior parasite counting. Monogenean quantification followed the method of Jerônimo et al. (2011).

The efficacy was calculated according to the formula: EF = MNPC − MNPT × 100 / MNPC (EF: efficacy, MNPC: mean number of parasites in control fish, MNPT: mean number of parasites in treated fish) (Dotta et al., 2015). Prevalence, mean intensity, and mean abundance of parasites were calculated according to Bush et al. (1997).

After fish were anesthetize the blood was withdrawn from the caudal vein with syringes containing a drop of EDTA 10% and used for blood smears stained with May Grunwald/Giemsa/Wright, hematocrit percentage, hemoglobin rate and calculated the hematimetric parameters: mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (CHCM) (Ranzani-Paiva et al., 2013). For total erythrocyte count (RBC) 5 μL of the blood was diluted in 1 mL of fixative Dacie solution for posterior counting (Blaxhall and Daisley, 1973). Total number of thrombocytes and leukocytes (WBC) were calculated by the indirect method (Ranzani-Paiva et al., 2013). After hematocrit determination the capillary was broken at the level of white blood cells and the plasma

transferred to refractometer for total protein determination according to Feldman et al. (2000) method. Hemoglobin concentration was analyzed in spectrophotometer Spectrum Meter® at 545 nm and glucose with the kit Accu-Chek®.

### 2.6. Statistical analysis

The data were submitted to factorial variance analysis (ANOVA) using a Statsoft STATISTICA 7.0 software. The means among the treatments were compared at $p < 0.05$ significance.

## 3. Results

In this study, the essential oil of *L. sidoides* showed thymol (76.6%) as the main compound followed by p-cimeno (6.3%) (Fig. 1A) being identified 21 constituents representing 98.6% of the essential oil composition. *Mentha piperita*, showed 29 constituents and 99.5% of the essential oil composition having menthol (27.5%), menthofurano (22.5%), pulegone (12.8%), menthyl acetate (12.5%) and menthone (11.0%) as the main compounds (Fig. 1B).

In the present assays, both *L. sidoides* and *M. piperita* had an anesthetic effect on the fish after a few minutes (Fig. 2).

In the *in vitro* immobilization test, the concentrations of 160 mg $L^{-1}$ and 320 mg $L^{-1}$ for *L. sidoides* and *M. piperita*, respectively, were the most efficient for causing parasite mortality, taking 1 min and 58 s, and 8 min and 11 s, respectively. Parasites exposed to water died after 4 h and 21 min and those exposed to water + DMSO after 2 h and 51 min. When comparing the efficacy in relation to a water bath and water + DMSO bath there was 1.96% and 14.16% for *L. sidoides* and 33.33% and 41.63% for *M. piperita*, respectively (Fig. 3).

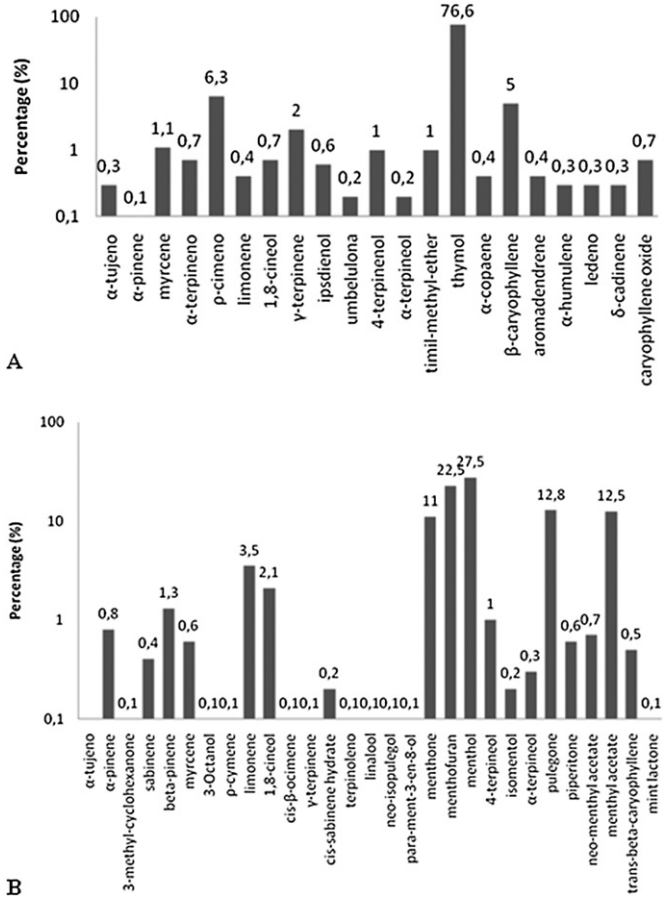


**Fig. 1.** Composition of essential oils used in this study. A — *Lippia sidoides* and B — *Mentha piperita*.

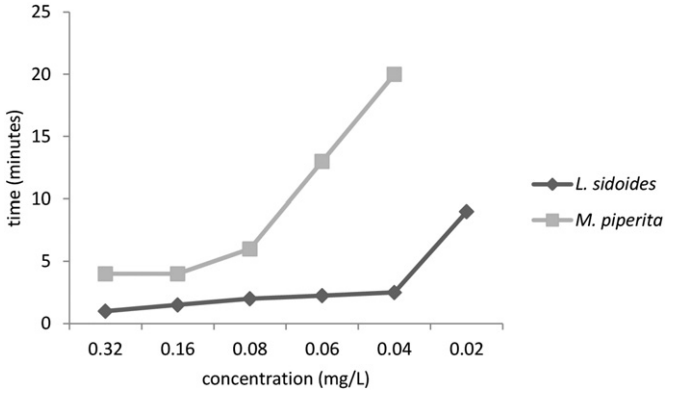


**Fig. 2.** Mean time for anesthetizing Nile tilapia exposed to toxicity test with essential oils of *Lippia sidoides* and *Mentha piperita*.

From the toxicity test results, the therapeutic doses were defined as 20 mg $L^{-1}$ for *L. sidoides* and 40 mg $L^{-1}$ for *M. piperita* (Table 1).

Regarding hematological parameters, an increase in hematocrit percentage accompanied by a decrease in RBC and thrombocyte count ($p < 0.05$) was observed in fish treated with *L. sidoides*, in comparison with the water bath (Table 2). Glucose concentration and neutrophil count were significantly higher in fish treated with *L. sidoides* than in the other treatments.

## 4. Discussion

In this study, the composition of *L. sidoides* was similar to that found by Cavalcanti et al. (2010), who observed thymol as the main substance (80.8%). Differently, Botelho et al. (2007) and Silva et al. (2013) found that the proportions of thymol were 56.7% and 68.40% respectively. Menthol was the main substance found in the composition of *M. piperita* in the present study (27.5%), *i.e.* similar to what was reported by Tsai et al. (2013) (28.19%) and Freire et al. (2011) (54.2%).

According to Bakkali et al. (2008) the major compounds determine the biological essential oil activity. *L. sidoides* showed elevated percentage of thymol and p-cimeno. The main compound found in *L. sidoides* was the monoterpene thymol in which has been confirmed its antibacterial and antiparasitic potential (Bakkali et al., 2008; Oliveira et al., 2009). Menthol, the main compound found in *M. piperita*, is a monocyclic monoterpene that stands out for its great industrial importance with antibacterial, antifungal, and anthelminthic properties (McKay and Blumberg, 2006).

Similarly, Cunha et al. (2010) observed that *Lippia alba* was an efficient anesthetic for *Rhamdia quelen*, inhibiting an increase in the plasma cortisol level at 100 to 500 mg $L^{-1}$. Similar results were found by Boijink et al. (2011), who used therapeutic baths of basil *Ocimum*

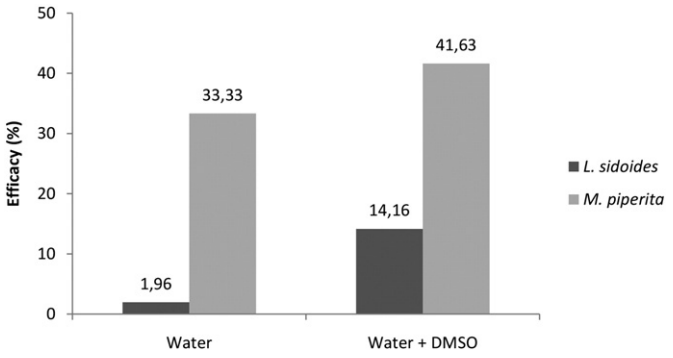


**Fig. 3.** Efficacy of essential oil of *Lippia sidoides* (20 mg $L^{-1}$) and *Mentha piperita* (40 mg $L^{-1}$) compared to control water and water + DMSO in therapeutic baths against monogenean parasites in Nile tilapia.

**Table 1**
Prevalence values (%), mean intensity and mean abundance (± standard deviation) of monogenean parasites in Nile tilapia after therapeutic bathes of *Lippia sidoides* (20 mg $L^{-1}$), *Mentha piperita* (40 mg $L^{-1}$), water, and water + DMSO.

| | *Lippia sidoides* | *Mentha piperita* | Water | Water + DMSO |
|---|---|---|---|---|
| Prevalence (%) | 25.0 | 27.5 | 57.5 | 60.0 |
| Mean intensity | 2.00 ± 1.24 | 1.36 ± 0.67 | 2.04 ± 1.22 | 2.33 ± 1.65 |
| Mean abundance | 0.50 ± 1.06$^{ab}$ | 0.38 ± 0.70$^{c}$ | 1.18 ± 4.70$^{bc}$ | 1.40 ± 1.72$^{a}$ |

Different letters indicate significant difference among the treatments ($p < 0.05$).

*gratissimum* on tambaqui (*Colossoma macropomum*) at 10 mg $L^{-1}$ and 15 mg $L^{-1}$ for 15 min. These authors did not observe any damage among the treated fish. Even though *L. sidoides* demonstrated anesthetic activity towards Nile tilapia at lower concentrations, it could be another economic alternative for use in aquaculture.

The immobilization test was efficient, as also observed by Militz et al. (2014), in evaluating garlic extract against the monogenean *Neobenedenia* sp. in *L. calcarifer*. These authors found that the garlic suppressed embryo development, thereby reducing the eclosion and longevity of the oncomiracidium.

Similar to what was found in the present study, tea tree oil has shown potential for use against the parasite *Gyrodactylus* spp. in sticklebacks (*Gasterosteus aculeatus*) (Steverding et al., 2005). Saponins and polyphyllin D extracted from *Paris polyphylla* were efficient against *Dactylogyrus intermedius*, a monogenean parasite of goldfish (Wang et al., 2010b). The efficacy of these agents against *D. intermedius* has been shown to be variable. Wang et al. (2010a) found that the efficacy of steroidal saponins from *Dioscorea zingiberensis* was up to 90% and Wang et al. (2011) found that osthol extract from *Radix angelicae pubescentis* had efficacy of 100%. On the other hand, Huang et al. (2013) reported that ethyl acetate extract from *Lysima chiachristinae* presented 50% efficacy against *D. intermedius*.

According to Tu et al. (2013), a bath consisting of chloroform extracts from the plant *Santalum album* applied to goldfish at 40 mg $L^{-1}$ showed 100% efficacy against the monogeneans *Dactylogyrus* sp. and *Gyrodactylus* sp.

Therapeutic bath comprising 2.9 mg/L of *Azadirachta indica* for 5 days reduced the numbers of the monogenean parasite *Anacanthorus penilabiatus* in pacu (*Piaractus mesopotamicus*) by 82% (Cruz et al., 2008). Similar results were found after a bath of *Piper aduncum* at 80 ml $L^{-1}$ for 24 h applied to pirarucu (*Arapaima gigas*) parasitized by the monogenean *Dawestrema cycloancistrium*, which showed 80% efficacy (Queiroz, 2012). The study of Claudiano et al. (2009) also proved that *Terminalia catappa* extract was effective against the monogenean and dinoflagellate protozoan *Piscinoodinium pillulare* in juvenile tambaqui at 120 ml $L^{-1}$.

A study using a diet supplemented with 0.5% and 1% propolis and *Aloe barbadensis* extracts showed efficacy of 83% and 85% respectively against monogenean parasites in Nile tilapia (Dotta et al., 2015). In the present study, it must be emphasized that the treatment with *M. piperita* showed the best results as an anti-parasitic agent.

Regarding the toxicity test, similar results were found by Boijink et al. (2011) after a bath with *O. gratissimum* applied to tambaqui at 10 mg $L^{-1}$ and 15 mg $L^{-1}$ for 15 min, without damage to the fish. Fish treated with *L. sidoides* and *M. piperita* showed significantly lower parasite loads than those exposed to water or water + DMSO (75% and 72.5%, respectively).

As reported by Ekanem et al. (2004), the toxicity increases as the concentration administered to the fish increases. The results from steroidal saponins used as an acute toxicity assay for goldfish vary according to the compounds extracted (Wang et al., 2010b). In fact, this was confirmed in observing the difference in toxicity over time in the tests of the present study. *L. sidoides* showed higher toxicity than *M. piperita*.

Fish exposed to different environments, stress or treatments may present hematological alterations. Hematocrit percentage reflects the proportion of RBCs in the blood in relation to WBCs and plasma. On the other hand, RBCs are responsible for oxygen and carbon dioxide transport (Ranzani-Paiva et al., 2013). Greater hematocrit and lower RBC count observed in fish treated with *L. sidoides* suggest that some stressor and/or respiratory dysfunction was present, thereby suppressing the RBC count. Apart from the low RBC count in the fish treated with *L. sidoides*, the values for the other parameters were within the range of amplitude for the species (Tavares-Dias et al., 2009).

Reduced thrombocyte counts indicate damage to coagulation and organism defense (Martins et al., 2008). It can be inferred that the low thrombocyte counts found in fish treated with *L. sidoides* may have been associated with migration of these cells, thereby demonstrating the irritative action of the oil, as confirmed by increased neutrophil count and glucose concentration. Neutrophils are granulocytes responsible for defense of the organism (Ranzani-Paiva et al., 2013), and situations of neutrophilia in fish have been reported to be due to stress and management (Jerônimo et al., 2011) or infection (Martins et al., 2008). Glucose concentration may be affected by several factors such as stress and fish health (Ranzani-Paiva et al., 2013). It is possible that, as a result of stress caused by the treatment with *L. sidoides*, the neutrophil count and glucose concentration became altered.

## 5. Conclusions

The present study reported on the efficacy of using the essential oils of *L. sidoides* and *M. piperita* against monogenean parasites, for the first time. However, the mode of action and chemical component responsible for parasite mortality remain unknown. Although the essential oil of *L. sidoides* (20 mg $L^{-1}$) presented efficacy, it cannot be recommended because of the hematological alterations that it caused in the fish. Use of *M. piperita* at 40 mg $L^{-1}$, as a therapeutic bath, may be safe and recommendable, given that it did not cause hematological alterations. After the treatments, the fish did not show neither alterations on the body surface nor any mortality.

**Table 2**
Hematological parameters (± standard deviation) of Nile tilapia after therapeutic bathes of essential oil of *Lippia sidoides* (20 mg $L^{-1}$), *Mentha piperita* (40 mg $L^{-1}$), water, and water + DMSO.

| Parameters | *Lippia sidoides* | *Mentha piperita* | Water | Water + DMSO |
|---|---|---|---|---|
| Erythrocytes (×$10^6$ μ$L^{-1}$) | 1.85 ± 0.33$^{c}$ | 2.79 ± 0.50$^{a}$ | 2.22 ± 0.70$^{b}$ | 2.76 ± 1.08$^{a}$ |
| Hematocrit (%) | 28.25 ± 3.27$^{a}$ | 26.50 ± 3.66$^{ab}$ | 25.50 ± 3.93$^{b}$ | 27.50 ± 3.26$^{ab}$ |
| Hemoglobin (g $dL^{-1}$) | 7.89 ± 0.99$^{b}$ | 8.62 ± 0.99$^{a}$ | 7.25 ± 1.11$^{b}$ | 7.69 ± 1.09$^{b}$ |
| Total protein (g $dL^{-1}$) | 5.45 ± 0.60$^{a}$ | 5.40 ± 0.40$^{a}$ | 5.70 ± 0.54$^{a}$ | 5.40 ± 0.46$^{a}$ |
| Glucose (mg $dL^{-1}$) | 83.00 ± 34.19$^{a}$ | 65.00 ± 15.82$^{b}$ | 52.50 ± 26.12$^{b}$ | 51.50 ± 34.37$^{b}$ |
| Leukocytes (×$10^3$ μ$L^{-1}$) | 91.70 ± 37.9$^{a}$ | 91.45 ± 30.46$^{a}$ | 107.14 ± 46.62$^{a}$ | 94.77 ± 53.85$^{a}$ |
| Thrombocytes (×$10^3$ μ$L^{-1}$) | 42.12 ± 24.01$^{b}$ | 89.47 ± 34.66$^{a}$ | 83.30 ± 42.12$^{a}$ | 90.19 ± 68.92$^{a}$ |
| Lymphocytes (×$10^3$ μ$L^{-1}$) | 85.58 ± 35.79$^{a}$ | 83.81 ± 29.66$^{a}$ | 103.35 ± 45.10$^{a}$ | 87.95 ± 50.85$^{a}$ |
| Neutrophils (×$10^3$ μ$L^{-1}$) | 3.35 ± 4.12$^{a}$ | 0.75 ± 1.31$^{b}$ | 0.83 ± 3.07$^{b}$ | 1.64 ± 3.25$^{b}$ |
| Monocytes (×$10^3$ μ$L^{-1}$) | 3.60 ± 2.84$^{ab}$ | 4.78 ± 2.59$^{a}$ | 2.97 ± 2.14$^{b}$ | 5.29 ± 5.43$^{a}$ |

Different letters indicate significant difference among the treatments ($p < 0.05$).

## Acknowledgments

The authors thank EMBRAPA (MP2-01/2012) (Brazilian Corporation of Agricultural Research) for financial support, CAPES (Coordination for the Improvement of Higher Education Personnel) for master scholarship to G.S.O. Hashimoto and CNPq (National Council of Scientific and Technological Development) for financial support and grant to M.L. Martins (CNPq 305869/2014-0) and Drs. Debora M. Fracalossi, Marcos C. Albuquerque and Natalia Marchiori (Aquaculture Department, Federal University of Santa Catarina, SC, Brazil) for critical review prior to submission.

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