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In vitro antimicrobial activity of ethanol extracts of *Lippia sidoides*, *Ocimum gratissimum* and *Zingiber officinale* against isolates of *Aeromonas* spp.

Patrícia Castro Monteiro[1], Cláudia Majolo[2], Francisco Célio Maia Chaves[3], Edsandra Campos Chagas[4]

Abstract

The objective of this study was to evaluate the *in vitro* antimicrobial activity of ethanolic extracts of *Lippia sidoides*, *Ocimum gratissimum* and *Zingiber officinale* against 10 isolates of *Aeromonas* spp. The ethanolic extracts were obtained by cold static maceration of the plant´s leaves and rhizomes. It was used ethanol 95% as a solvent, and the extraction underwent to a rotary evaporation process. Moreover, to determine their antimicrobial activity, the technique of microdilution in broth was used to obtain the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The extracts were tested at concentrations of 10,000, 5,000, 2,500, 1,250, 625 and 312.5 μg mL^{-1}, with three replicates for each plant species. The three ethanolic extracts evaluated showed bactericidal and bacteriostatic action against isolates of *Aeromonas* spp., with MIC values ranging from 625 to 10,000 μg mL^{-1}. Of these, the ethanolic extract of *L. sidoides* presented the lowest MIC (625 μg mL^{-1}) against five isolates of *Aeromonas* spp., thus showing the best antimicrobial activity. The results from this study provide information that may support the use of these medicinal plants for treating fish infections caused by *Aeromonas* spp.

Keywords: medicinal plants, minimal inhibitory concentration, minimum bactericidal concentration, bacteriosis, fish culture.

Atividade antimicrobiana *in vitro* de extratos etanólicos de *Lippia sidoides*, *Ocimum gratissimum* e *Zingiber officinale* contra isolados de *Aeromonas* spp.

O objetivo deste estudo foi avaliar a atividade antimicrobiana *in vitro* de extratos etanólicos de *Lippia sidoides*, *Ocimum gratissimum* e *Zingiber officinale* contra 10 isolados de *Aeromonas* spp. Os extratos etanólicos foram obtidos por meio de maceração estática a frio das folhas e rizomas das plantas, utilizando álcool etílico a 95% como solvente, o qual foi evaporado pelo processo de rotoevaporação. Após o preparo dos extratos, para a determinação da sua atividade antimicrobiana foi utilizada a técnica de microdiluição em caldo visando obter a concentração inibitória mínima (CIM) e a concentração bactericida mínima (CBM). Os extratos foram testados nas concentrações de 10.000, 5.000, 2.500, 1.250, 625 e 312,5 μg mL^{-1}, com três repetições para cada espécie de planta. Os

[1]Pós-graduação Ciências Pesqueiras nos Trópicos, UFAM, Manaus, AM, Brazil. pcmonteiro17@gmail.com
[2] Embrapa Amazônia Ocidental, Manaus, AM, Brazil. claudia.majolo@embrapa.br
[3] Embrapa Amazônia Ocidental, Manaus, AM, Brazil, celio.chaves@embrapa.br
[4] [2]Embrapa Amazônia Ocidental, Manaus, AM, Brazil. Corresponding author: edsandra.chagas@embrapa.br

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três extratos etanólicos avaliados apresentaram atividade bactericida e bacteriostática frente isolados de *Aeromonas* spp., com valores de CIM variando de 625 a 10.000 µg mL^{-1}. Destes, o extrato etanólico de *L. sidoides* apresentou a menor CIM (625 µg mL^{-1}) frente cinco isolados de *Aeromonas* spp., demonstrando a melhor atividade antimicrobiana. Os resultados deste estudo fornecem informações que podem subsidiar o uso dessas plantas medicinais no tratamento de infecções em peixes causadas por *Aeromonas* spp.

Palavras-chave: plantas medicinais, concentração inibitória mínima, concentração bactericida mínima, bacteriose, piscicultura.

1. Introduction

Motile *Aeromonas* species are opportunistic Gram-negative bacterium that is present in aquatic environments (PEIXOTO et al., 2012; GRIFFIN et al., 2013; PESSOA et al., 2019). *Aeromonas hydrophila* stands out for being the most frequently isolated species in fish farming worldwide (NOGA, 2010; PESSOA et al., 2019). In fish, it causes hemorrhagic septicemia, with cutaneous lesions, hemorrhages and ulcers (NOGA, 2010; SEBASTIÃO et al., 2015; BAUMGARTNER et al., 2017). The high degree of infection that this bacterium causes is responsible for increasing the morbidity and mortality rates in the aquaculture sector (FIGUEIREDO & LEAL, 2008; TAVARES-DIAS & MARTINS, 2017; PESSOA et al., 2019), thus affecting native species such as tambaqui (*Colossoma macropomum*), pacu (*Piaractus mesopotamicus*), pintado (*Pseudoplatystomafasciatum*) and jundiá (*Rhamdia quelen*) (COSTA & CYRINO, 2006; BARCELLOS et al., 2008; SEBASTIÃO et al., 2015, ARIEDE et al., 2018; PESSOA et al., 2019; 2020).

Only a few antibiotics have been approved by the United States Food and Drug Administration for treating bacterial infections in fish. These include sulfadimethoxine/ ormetoprim, florfenicol and oxytetracycline (FDA, 2018). In Brazil, only the latter two have been released for use by the Ministry of Agriculture, Livestock-rearing and Supply (MAPA, 2011). However, use of unregistered antibacterials has become a common practice within aquaculture and has generated environmental pollution, accumulation of residues in fish and development of bacterial strains that are resistant to antibiotics (RINGO et al., 2010). This has already been observed in Brazil, in relation to *A. hydrophila* isolated from pacus (*Piaractus mesopotamicus*) and tilapias (*Oreochromis niloticus*) (COSTA & CYRINO, 2006).

In this context, natural products have been evaluated regarding their possible antimicrobial activity, since large numbers of bioactive components are present in the extracts and essential oils of plants. The aim has been to select new molecules for treating bacterial diseases in fish (REVERTER et al., 2014; TAVARES-DIAS, 2018).

Among the medicinal plants with potential, *Lippia sidoides* Cham. (Verbenaceae), known as pepper-rosmarin, can be highlighted. This is an erect branched deciduous shrubby herb (LORENZI & MATOS, 2008) that has aroused great botanical and pharmaceutical interest because of its extensive use in folk medicine. In addition to antimicrobial potential, the extract of *L. sidoides* has

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been used as an antifungal and anthelmintic agent (SILVA et al., 2010, SOUZA et al., 2010; BATISTA et al., 2013).

Ocimum gratissimum L. (Lamiaceae), known as clove basil, is native to eastern Brazil and subspontaneous throughout this country, and it presents characteristics of rapid growth. It is widely used in a variety of therapeutic applications because of the antimicrobial, anesthetic and antioxidant properties of its extracts and/or essential oils (AGUIYI et al., 2000; ALO et al., 2012; BOIJINK et al., 2016).

Zingiber officinale (Zingiberaceae), known as ginger, is a perennial herbaceous plant whose rhizome is widely marketed for food and industrial use (LORENZI & MATOS, 2008). It has been proven to have antioxidant, anti-inflammatory, antibacterial, larvicidal and repellent properties (KHANDAGLE et al., 2011; GULL et al., 2012; MAJOLO et al., 2014; EL-SHERBINY, 2015).

Given the potential of medicinal plants to form natural alternatives for controlling pathogens in fish farming, instead of the conventional chemicals that are used, with their deleterious effects, the objective of this study was to evaluate the *in vitro* antimicrobial activity of ethanolic extracts of *Lippia sidoides*, *Ocimum gratissimum* and *Zingiber officinale* against *Aeromonas* spp. isolated from tambaquis (*Colossoma macropomum*).

2. Materials and methods

2.1. Plant material

Specimens of *L. sidoides*, *O. gratissimum* and *Z. officinale* (genetic heritage register accession number AB13781) were cultivated in the medicinal plants sector of Embrapa Amazônia Occidental, Manaus, Amazonas (AM), Brazil, from where leaves and/or rhizomes were collected for preparation of ethanolic extracts. The plant material thus collected was identified and deposited in the herbarium of the Federal Institute of Education, Science and Technology of Amazonas (Instituto Federal de Educação, Ciência e Tecnologia do Amazonas, IFAM), Manaus, AM, under registration numbers 13882, 13889 and 13887, respectively.

2.2. Preparation of ethanolic extract

To obtain the ethanolic extract, the *in natura* plant material (leaves and rhizomes) was subjected to a drying process in a heated chamber with air circulation and forced air renewal at 45 °C, until a constant weight was reached, in accordance with the recommendations of ANVISA (2018). After drying, the material was crushed in a mill and stored in plastic bags until ethanol extraction was performed. Ethanolic extracts were obtained by means of static cold maceration. The solvent that was used was 95% ethyl alcohol. Initially, 100 g of each plant material (*L. sidoides*, *O. gratissimum* and *Z. officinale*) were weighed and placed in a one-liter amber flask. Then, 900 ml of ethanol (extractant liquid) was added to the flask. This extractive system was kept under full light at room temperature for seven days. After this period, the extracts were filtered and subjected to the rotoevaporation process, under reduced pressure, in a thermostat-controlled bath, at a temperature of 45 °C, for solvent evaporation, and drying at 50 ^{0}C for

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24 h and subsequent resuspension with dimethyl sulfoxide (DMSO, 1:1).

2.3. Antimicrobial assays

2.3.1. Clinical isolates

Ten isolates of *Aeromonas* spp. (248, 249, 284, 351, 432, 561, 562, 565, 568 and 570) were evaluated in this study. The isolates were obtained from tambaqui (*C. macropomum*) and the biochemical identification was done using the kit API 20E® (Biomérieux, EUA) in the Fish Farming Laboratory at Embrapa Amazônia Ocidental, Manaus, AM. The isolates were stored in brain heart infusion broth (BHI; Himedia, Mumbai, India), supplemented with 30% sterile glycerol, at -80 °C. For reactivation, the isolates were inoculated in BHI and incubated for 24 h at 35 °C. For purity assessment, the isolates were plated on tryptone soy agar (TSA; Himedia, Mumbai, India) and were Gram-stained. After this, samples of the bacterial growth were diluted in sterile saline solution and the absorbance was adjusted at 600 nm in a spectrophotometer to obtain a viable cell count of 10^8 CFU mL^{-1}. The diluted and adjusted samples were then diluted in Mueller-Hinton broth (MHB; Kasvi, Italy) to obtain 10^5 CFU in 100 µL, and it was added to each well containing the extracts evaluated in this study.

2.3.2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays

Assays to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were conducted using the broth microdilution technique, in accordance with the procedures for using 96-well plates described by the Clinical and Laboratory Standards Institute (CLSI-M7-A6), National Committee for Clinical Laboratory Standards (2003). Positive and negative controls were used in these assays: the medium without the bacterial inoculum and with the ethanolic extracts was used as the negative control; and the medium with the bacterial inoculum was used as the positive control. The antibiotic gentamicin sulfate (Inlab, Diadema, SP, Brazil) was used as a positive control against bacterial isolates. Microdilutions were performed for each extract, to reach final concentrations of 10,000, 5,000, 2,500, 1,250, 625 and 312.5 µg mL^{-1}, and these were then incubated at 35 °C for 24 hours.

After incubation, the results were confirmed by means of aseptic addition of 0.5% sterile aqueous solution of triphenyl tetrazolium chloride (TTC) to the wells of the plates, with incubation at 35 °C for 1 hour. The results were obtained in triplicate and expressed in µg mL^{-1}. The minimum inhibitory concentration (MIC), defined as the lowest concentration of an antimicrobial that inhibited visible growth of a microorganism after 24 hours of incubation, was determined. The bactericidal action of the extracts was evaluated through addition of 20 µL of the microbial culture, withdrawn from the wells at or above the MIC. This was then inoculated into Mueller-Hinton agar plates (MHA; Kasvi, Italy) and incubated at 35° C for 24 hours, with three replicates. After incubation, the plates were read and the minimum bacterial concentration (MBC) was defined as the lowest concentration without bacterial growth. Strong activity was defined when MIC < 100 µg mL^{-1} (KUETE, 2010).

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3. Results and discussion

In this study, the MIC and MBC values of ethanolic extracts of *L. sidoides*, *O. gratissimum* and *Z. officinale*, in relation to 10 *Aeromonas* spp. isolates, ranged from 625 to 10,000 µg mL^{-1} (Table 1). The ethanolic extracts of the species studied presented bacteriostatic and bactericidal activity against *Aeromonas* spp. isolates. However, the ethanolic extract of *L. sidoides* showed a better response regarding antimicrobial activity, compared with the extracts of *O. gratissimum* and *Z. officinale*.

Table 1 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ethanolic extracts from the *O. gratissimum, Z. officinale* and *L. sidoides* on *Aeromonas hydrophila* strains.

Strains	*L. sidoides*		*O. gratissimum*		*Z. officinale*	
	MIC	MBC	MIC	MBC	MIC	MBC
248	625	625	5,000	5,000	10,000	10,000
249	625	625	5,000	5,000	5,000	5,000
284	1,250	1,250	5,000	5,000	5,000	5,000
351	625	625	5,000	5,000	5,000	5,000
432	625	625	5,000	5,000	5,000	5,000
561	625	625	5,000	5,000	1,250	1,250
562	625	625	5,000	5,000	5,000	5,000
565	625	625	5,000	5,000	2,500	10,000
568	625	625	5,000	5,000	10,000	10,000
570	625	625	5,000	5,000	10,000	10,000

MIC and MBC values were expressed in µg mL^{-1}.

For the ethanolic extract of *L. sidoides*, the MIC and MBC values in relation to *Aeromonas* spp. ranged from 625 to 1,250 µg mL^{-1} (Table 1). Similar results were obtained by FABRI et al. (2011) in relation to the Gram-negative bacteria *Shigella sonnei* and *Pseudomonas aeruginosa*, using the methanolic extract of *L. sidoides*, for which the MIC values were 625 µg mL^{-1}. In relation to the Gram-positive bacterium *Staphylococcus aureus*, the MIC value was higher (5,000 µg mL^{-1}) (FABRI et al., 2011). Studies have shown that Gram-negative bacteria have lower sensitivity than Gram-positive bacteria, because Gram-negative bacteria have an outer membrane that is capable of inhibiting or even postponing, the penetration of extracts/essential oils (MUNYENDO et al., 2011). In the present study, it was observed that the extract of *L. sidoides* was able to inhibit different isolates of *Aeromonas* spp., at lower concentrations compared to the other extracts, despite the fact that it is a Gram-negative bacteria.

For the ethanolic extract of *O. gratissimum*, the MIC and MBC values were 5,000 µg mL^{-1} in relation to all isolates of *Aeromonas* spp. evaluated (Table 1). With the same ethanolic extract, NWINYI et al. (2009) obtained MIC values of 2,500 and 10,000 µg mL^{-1} in relation to *Staphylococcus aureus* and *Escherichia coli*, respectively. Compared with *E. coli* (Gram-negative), the results obtained in the present study showed that the *Aeromonas* spp. isolates evaluated were more sensitive. STANLEY et al. (2014) obtained a MIC of 12,500 µg mL^{-1} in relation to *S. aureus*, also through evaluating the eth-

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anolic extract of *O. gratissimum*. Using the essential oil of *O. gratissimum*, BANDEIRA-JR et al. (2017) obtained MICs ranging from 400 to 800 µg mL^{-1} in relation to *A. hydrophila* strains, thus showing better essential oil responses than those from the ethanolic extract evaluated in the present study.

For the ethanolic extract of *Z. officinale*, the MIC and MBC variation was from 1,250 to 10,000 µg mL^{-1} (Table 1). In this study, the ethanolic extract of *Z. officinale* showed the lowest sensitivity in relation to most of the strains tested (except for strains 561 and 565). EL-SHERBINY et al. (2015), evaluating aqueous and ethanolic extracts of *Z. officinale* against *Enterococcus faecalis*, *Streptococcus mutans*, *Streptococcus acidominimus* and *Porphyromonas gingivalis*, found MIC ranges of 500 to 1,300 µg mL^{-1} and 300 to 1,000 µg mL^{-1}, respectively. This indicated that there were variations in the sensitivity of different microorganisms, compared with different extracts.

According to KUETE (2010), the antimicrobial activity of extracts can be considered strong when MIC < 100 µg mL^{-1}, moderate when 100 < MIC ≤ 625 µg mL^{-1} and weak when MIC > 625 µg mL^{-1}. Therefore, according to this classification, the antimicrobial activity of the *L. sidoides* ethanolic extract was considered moderate in relation to all isolates of *Aeromonas* spp. evaluated, except for isolate 284. For the ethanolic extracts of *O. gratissimum* and *Z. officinale*, the antimicrobial activity was considered to be weak for all the isolates of *Aeromonas* spp. These results are of great importance as support for future actions regarding the use of these medicinal plants as an alternative method for treating infections in fish caused by *Aeromonas* spp.

Other extracts and essential oils from medicinal plants such as *Hesperozygis ringens*, *Aloysia triphylla*, *Lippia alba*, *Ocimum americanum* and *O. gratissimum* have been described as potent antibacterial agents against *A. hydrophila* isolated from fish (SUTILI et al., 2016; BANDEIRA-JR et al., 2017; ROSA et al., 2019). Synergistic effects from the essential oils of *A. triphylla* or *L. alba*, used with the antibiotic florfenicol, have also been described, with achievement of better antibacterial activity (SOUZA et al., 2017). These combinations thus contribute towards the aim of new therapeutic alternatives that will also enable reduction of resistance to antibiotics.

Presence of motile *Aeromonas* species is considered to be an important factor limiting aquaculture production in many countries because this generates large losses, which may make it impossible to produce several fish species around the world. In this study, it was possible to describe the *in vitro* antimicrobial activity of ethanolic extracts of *L. sidoides*, *Z. officinale* and *O. gratissimum* against *Aeromonas* spp. isolated from tambaqui, from which the best results were obtained using *L. sidoides* extract. These extracts have different phytochemical compounds that can inhibit the bacterium through different mechanisms.

In general, the common phenolic compounds in *L. sidoides* ethanolic extracts reported by Braga et al., (2019) were tannins, catechins, flavonoids, steroids and triterpenes, coumarin derivatives, saponins and alkaloids. For the *O. gratissimum* the compounds alkaloids and saponins

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were present in higher quantities, but phenols, tannins, phlobatannins, and anthraquinones were also present (TALABI & MAKANJUOLA, 2017). For *Z. officinale* the phytochemical constituints were alkaloids, phlobotannins, flavanoids, glycosides, saponins, tannins and terpenoids (BHARGAVA et al., 2012). It is important to highlight that some authors attributed the antimicrobial activity to the components of these plant extracts due to the disturbance in the cell structure and permeability (NAZZARO et al., 2013; MOSTAFA et al., 2018)

It is intended to continue the present studies, with the aims of isolating active compounds from the extracts and evaluating combination of these compounds with antibiotics. Through this, it is sought to reduce the minimum inhibitory concentration and minimum bactericidal concentration values of all plants evaluated, including those that have shown efficacy in *in vivo* evaluations on farmed fish, and to validate the results and make new options for treating bacteriosis in farmed fish.

4. Conclusion

The ethanolic extracts of *Lippia sidoides*, *Ocimum gratissimum* and *Zingiber officinale* presented antimicrobial activity against 10 isolates of *Aeromonas* spp. The extract of *L. sidoides* presented the best response, and its activity was considered moderate.

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