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CHEMICAL COMPOSITION AND EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF TWO ESSENTIAL OILS*

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ABSTRACT

This study aimed to identify and quantify the chemical constituents and to evaluate the in vitro antimicrobial activity of essential oils from lemongrass (*Cymbopogum flexuosus* - EOCF) and basil (*Ocimum basilicum* - EOOB) against 24 isolates of *Aeromonas* spp. The main components of EOCF were α-citral (50.13%) and ß-citral (40.31%), while those of EOOB were linalool (53.35%) and eucalyptol (11.49%). The EOCF showed high inhibitory activity (≥ 195.31 µg mL^{-1}), whereas EOOB showed moderate inhibitory activity (≥ 781.25 µg mL^{-1}) for *Aeromonas* spp. Both essential oils have potential for application as antimicrobial agents, in particular EOCF.

Key words: *Aeromonas* spp.; citral; linalool; minimum bactericidal concentration.

COMPOSIÇÃO QUÍMICA E AVALIAÇÃO DA ATIVIDADE ANTIMICROBIANA DE DOIS ÓLEOS ESSENCIAIS

RESUMO

Este estudo objetivou identificar e quantificar os constituintes químicos e avaliar a atividade antimicrobiana *in vitro* dos óleos essenciais de capim limão (*Cymbopogum flexuosus* - OECF) e manjericão (*Ocimum basilicum* - OEOB) frente a 24 isolados de *Aeromonas* spp. Os componentes majoritários de OECF foram o α-citral (50,13%) e ß-citral (40,31%), enquanto que de OEOB foram o linalol (53,35%) e eucaliptol (11,49%). OECF demonstrou atividade inibitória elevada (≥ 195,31 µg mL^{-1}), enquanto que OEOB apresentou atividade inibitória moderada (≥ 781,25 µg mL^{-1}) para *Aeromonas* spp. Ambos os óleos essenciais têm potencial para aplicação como agentes antimicrobianos, principalmente OECF.

Palavras-chave: *Aeromonas* spp.; citral; linalol; concentração bactericida mínima.

INTRODUCTION

Sanitary control is one of the key measures that need to be implemented to improve intensive fish production systems. Bacteria of the genus *Aeromonas* spp. are among the microorganisms that most affect fish health, causing problems such as haemorrhagic septicemia, skin ulcers, ascites, exophthalmos, corneal opacity, anaemia and liver and kidney damage (PEEPIM *et al*., 2016); they can be transmitted sporadically to humans through contaminated water or food (CASTRO-ESCARPULLI *et al*., 2003). In fish farms, *Aeromonas* spp. frequently cause outbreaks with high mortality and considerable economic losses (PEEPIM *et al*., 2016).

The intensification of fish farming systems has promoted the use of synthetic antibiotics by fish farmers and, consequently, contributed to the development of bacterial resistance to multiple drugs (SOUZA *et al*., 2017). Essential oils are mixtures of substances derived from the secondary metabolism of plants; their therapeutic and organoleptic properties are due to the presence of volatile compounds (e.g. monoterpenes) (SIKKEMA *et al*., 1995) and represent a promising alternative to synthetic antibiotics in aquaculture. The mode of action of essential oils is mainly due to its ability to promote the rupture of the plasma membrane of the pathogen, increase non-specific permeability, which promotes the extravasation of intracellular ions and proteins, inactivate enzymatic systems, and interfere with nucleic acid synthesis (DEVI *et al*., 2010; GYAWALI and IBRAHIM, 2014). These findings highlight an exciting scientific interest in which essential oils warrant special attention due to their chemical and structural variance, making them functionally versatile (YAP *et al*., 2014) and a potential source of novel drug compounds.

Previous studies have shown that essential oils from lemongrass (*Cymbopogon flexuosus* - EOCF) and basil (*Ocimum basilicum* - EOOB) present intense antimicrobial activity (KAKARLA and GANJEWALA, 2009; AHMAD and VILJOEN, 2015; RADAELLI *et al*., 2016). Due to the importance of these plants, as well as the high incidence of infections caused by *Aeromonas* spp. in fish, the present study aimed to identify and quantify the chemical constituents as well as to report the antimicrobial activity of EOCF and EOOB against *Aeromonas* spp.

METHODS

Cymbopogon flexuosus and *Ocimum basilicum* were cultivated in Três Passos, RS, Brazil. Essential oils were obtained from the Pólo de Tecnologia da Universidade do Noroeste do Rio Grande do Sul (Unijui), Três Passos-RS. The leaves were separated in the laboratory and subjected to oil extraction by drag steam distillation for two hours, using a Clevenger-type apparatus.

The essential oils were stored at -4 °C in amber glass bottles until chemical analysis by GC-MS (gas chromatography-mass spectrometry) in the Laboratório de Extratos Vegetais, Universidade Federal de Santa Maria, Brazil (PINHEIRO *et al*., 2016). The components of the essential oils were identified by comparing the Kovats retention index and the mass spectra in a mass spectral library (NIST, 2010). The experiments were conducted in accordance with the Ethical Committee of the Biology Institute Universidade Federal da Bahia (UFBA) (number 14-2014).

In vitro analysis was performed in the Laboratório de Microbiologia e Imunologia Animal da Universidade Federal do Vale do São Francisco (LAMIA-UNIVASF), Petrolina, PE, Brazil. Twenty-four isolates of *Aeromonas* spp. were obtained from LAMIA-UNIVASF to test the antimicrobial activity of EOOB and EOCF. The bacterial isolates were obtained from the kidney, integument, intestine and lesions of specimens of pacamã (*Lophiosilurus alexandri*) and Nile tilapia (*Oreochromis niloticus*). Bacteria were identified according to morphology, biochemical tests and dyeing.

The equivalent of 0.25 g of each essential oil was weighed after prior density calculation and they were diluted in 5 mL of dimethylsulfoxide (DMSO) and 5 mL of methanol and stock solutions were obtained at the concentration of 25,000 μg mL^{-1}. The antimicrobial activity was evaluated by the broth microdilution method, and the determination of MBC (minimum bactericidal concentration) was performed according to the document M07-A9 (CLSI, 2014) and applied separately for each essential oil. Initially, 200 μL of Müller-Hinton Agar (MHA) were added to microtiter plates. Subsequently, 200 μL of the EOOB or EOCF stock solutions were added to the first well, homogenised, transferred to the second and so on successively, obtaining the following final concentrations: 12,500.00, 6,250.00, 3,125.00, 1,562.50, 781.25, 390.62, 195.31 and 97.60 μg mL^{-1}.

While preparing the inoculum, colonies in MHA were used to prepare a bacterial suspension with turbidity equivalent to the McFarland standard 0.5. For this, 10 μL of this suspension were inoculated in the microplate wells containing EOOB or EOCF and the diluent (5 mL methanol + 5 mL DMSO). The plate was incubated at 35°C for 24 h under aerobiotic.

Subsequently, to determine minimum bactericidal concentration (MBC), an aliquot of 10 μL was seeded on the surface of the MHA. After 48 h of incubation at 35°C, the MBC was defined as the lowest concentration of EOOB or EOCF required to kill the bacteria. In addition to the positive control (MHA broth and bacteria) and negative control (MHA broth), the antimicrobial activity of the diluent was determined (methanol, DMSO and bacteria). Each bacterial isolate was considered a unit and the tests were performed in triplicate.

RESULTS AND DISCUSSION

The major constituents of EOCF were α-citral (50.13%) and β-citral (40.31%) which represented 90.44% of the total constituents (Table 1). For EOOB, linalool (53.35%) was the major constituent, followed by eucalyptol (11.49%) (Table 2).

The chemical composition and antibacterial activity of an essential oil are influenced by several aspects, such as mode of extraction, intrinsic factors of the plant (e.g. age, species or cultivar) and environmental conditions of culture and collection (SILVA *et al*., 2011). Despite this, major compounds generally do not change, serving as excellent chemical markers (PINTO *et al*., 2013), although their activity can be modulated by chemical components in smaller amounts (BAKKALI *et al*., 2008). Thus, the antimicrobial activity verified for EOCF and EOOB in this study can be attributed to its major components citral and linalool, respectively (Tables 1 and 2).

Citral is a monoterpene formed by the mixture of α-citral and ß-citral isomers, while linalool is an alcoholic monoterpene. Previous studies have reported similar results for the major components for EOOB (RADAELLI *et al*., 2016; SNOUSSI *et al*., 2016; LIMMA-NETTO *et al*., 2016) and EOCF (AHMAD and VILJOEN, 2015; AZEVEDO *et al*., 2016; LIMMA-NETTO *et al*., 2016). The antimicrobial action of monoterpenes has been explained by the toxic effect on the structure and function of the cellular membrane of microorganisms (SIKKEMA *et al*., 1995).

In vitro assays showed that 50% of *Aeromonas* spp. isolates were sensitive to EOCF at the concentration of 195.31 μg mL^{-1}, while the other isolates were sensitive at concentrations of 390.62 to 3,125.00 μg mL^{-1}. For EOOB, bactericidal activity was verified in 54% of the *Aeromonas* spp. isolates at the concentration of 781.25 μg mL^{-1} and in the remaining isolates at concentrations of 1,562.50 to 3,125.00 μg mL^{-1} (Figure 1). The *Aeromonas* spp. isolates were sensitive to the diluent (control) in the concentrations of 3,125.00 to 6,250.00 μg mL^{-1}.

Table 1. Chemical composition of the essential oil of *Cymbopogon flexuosus* determined via the GC-MS technique.

Chemical Compound	RIC	RIL	%
Cyclofenchene	878	882	3.02
Camphene	978	969	1.46
Z-Verbenol	1,185	1,188	2.02
β-citral (Neral)	1,243	1,242	40.31
α-citral (Geranial)	1,273	1,270	50.13
Identified Compounds			**96.93**

RIC: Calculated Retention Index; RIL: Literature Retention Index (NIST, 2010).

Table 2. Chemical composition of the essential oil of *Ocimum basilicum* determined via the GC-MS technique.

Chemical Compound	CRI	LRI	%
Sabinene	937	944	1.85
β-Myrcene	982	985	0.96
Eucalyptol	1,029	1,031	11.49
β-*E*-ocimene	1,050	1,048	1.06
Linalool	1,102	1,101	53.35
Camphor	1,143	1,146	0.91
Bornyl acetate	1,287	1,284	1.96
Eugenol	1,359	1,356	4.25
β-Elemene	1,392	1,394	1.29
α-bergamotene	1,437	1,436	6.58
Germacrene D	1453	1453	0.65
α-Amorphene	1,481	1,490	2.58
δ-Guaiene	1,506	1,505	2.65
γ-Cadinene	1,515	1,514	3.19
τ-Cadinol	1,642	1,640	3.12
Identified Compounds			**95.89**

RIC: Calculated Retention Index; RIL: Literature Retention Index (NIST, 2010).

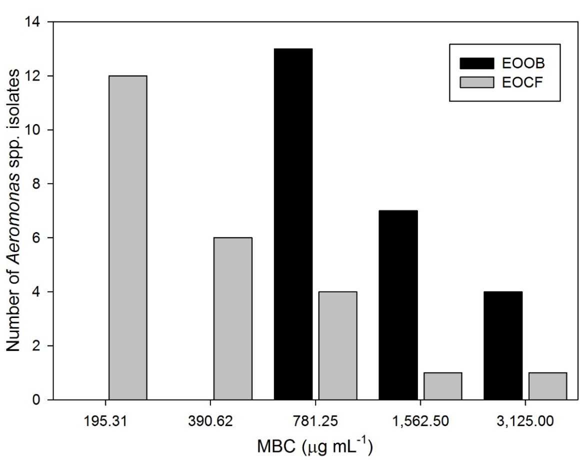

Figure 1. Minimum bactericidal concentration (MBC) of the essential oils of *Ocimum basilicum* (EOOB) and *Cymbopogum flexuosus* (EOCF) against *Aeromonas* spp. (n = 24).

When an essential oil, in lower concentrations, is able to sensitise a large amount of bacteria, it is considered to be of moderate to high antibacterial activity. The MBC values between 50 and 500, 600 and 1500 or above 1600.0 µg mL^{-1} are considered strong, moderate and weak activity, respectively (SARTORATTO *et al*., 2004; SOUZA *et al*., 2017). The EOCF showed high antibacterial activity, causing the death of half of the inoculums of *Aeromonas* spp. at an MBC of 195.31 µg mL^{-1}. In contrast, EOOB showed moderate bactericidal activity, since an MBC of 781.25 µg mL^{-1}was used to obtain a bacterial mortality similar to that for EOCF. In similar study, essential oils from *Aloysia triphylla* and *Lippia alba* demonstrated strong to moderate antimicrobial activity against most *Aeromonas* spp. isolates (SOUZA *et al*., 2017).

CONCLUSIONS

Based on the results of this study, EOCF and EOOB have great therapeutic potential and may contribute to the reduction of the use of synthetic antibiotics in intensive fish production. The components α-citral and ß-citral and linalool, respectively, influenced the antimicrobial activity of EOCF (high) and EOOB (moderate) against *Aeromonas* spp. In this sense, the antimicrobial activity of these essential oils may stimulate perspectives in the development of effective phytotherapeutics used in the treatment of microbial infectious diseases in fish. In addition, it is suggested that new studies evaluate the effects of EOCF and EOOB against an indigenous fish microbiota.

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