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Artículo de investigación

In vitro assay of *Lippia graveolens and Lippia alba* extracts against *Aeromonas* spp isolated from tilapia (*Oreochromis niloticus*)

Ensayo in vitro de extractos de Lippia graveolens y Lippia alba contra Aeromonas spp aisladas de tilapia (Oreochromis niloticus)

Ensaio in vitro de extratos da Lippia graveolens e Lippia alba sobre Aeromonas spp isolados de tilápia (Oreochromis niloticus)

Josué García-Pérez [1*] BS, MSc, PhD. ✉ ORCID, Juan Francisco Pérez-Sabino [2] BS, MSc, PhD. ORCID, Susana Mendoza-Elvira [3] BS, MSc, PhD. ✉ ORCID, Antonio Ribeiro da Silva [4] Blgo, Ph.D. ✉ ORCID, Juan B. Ulloa-Rojas [5] BS, MSc, PhD. ✉ ORCID

[*] Autor de correspondencia.
[1] Doctorado en Ciencias Naturales para el Desarrollo (DOCINADE), Instituto Tecnológico de Costa Rica, Universidad Nacional, Universidad Estatal a Distancia, Costa Rica. Centro de Estudios del Mar y Acuicultura, Universidad de San Carlos de Guatemala, Guatemala, Guatemala.
[2] Escuela de Química, Facultad de Ciencias Químicas y Farmacia, Universidad de San Carlos de Guatemala, Guatemala, Guatemala.
[3] Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autónoma de México, Estado de México, México.
[4] Instituto de Pesquisas de Productos Naturales, Universidad de Río de Janeiro, Brasil.
[5] Escuela de Ciencias Biológicas. Universidad Nacional, Heredia, Costa Rica.

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Abstract

This study evaluated the antimicrobial activity of *Lippia graveolens* and *Lippia alba* extracts and essential oils against nine strains of *Aeromonas* spp., isolated from *Oreochromis niloticus*. The crude extracts were obtained by percolation technique and CO_2 supercritical fluid extraction, while the essential oils by applying hydro-distillation technique using a Clevenger apparatus. The antimicrobial activity for each extract and essential oils was verified through the disc diffusion method at 24 hours. The essential oil of *L. graveolens* showed higher effectiveness to inhibit fish bacterial pathogens (100%) than the extracts, revealing an inhibition zone diameter that ranged from 25.20 - 36.94 mm. The extracts from supercritical fluid and from the percolation technique with ethyl acetate and cyclohexane presented the same

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effectiveness (77.78%). The crude extract obtained with ethanol 95% showed limited antimicrobial effect (22.22%), presenting the smallest inhibition zone (ranged from 8.34 to 9.57 mm). On the other hand, *L. alba* displayed a lower antibacterial activity, being the essential oil 66.67% effective, presenting an inhibition zone ranging between 10.68 to 16.29 mm. The result of this study indicates that essential oils from both *L. graveolens* and *L. alba* offer a promising alternative for the control of *Aeromonas* spp. growth.

Keywords: *Aeromonas hydrophila; Aeromonas sobria; antimicrobial activity; aquaculture*.

Resumen

El presente estudio evaluó la actividad antimicrobiana de extractos y aceites esenciales de *Lippia graveolens* y *Lippia alba,* frente a nueve cepas de *Aeromonas* spp., aisladas de *Oreochromis niloticus*. Los extractos crudos se obtuvieron por la técnica de percolación y a través de la extracción de CO_2 supercrítico, mientras que los aceites esenciales se realizaron mediante la técnica de hidrodestilación empleando un equipo Clevenger. La actividad antimicrobiana para cada extracto y aceite esencial se verificó mediante el método de difusión en disco a las 24 horas. El aceite esencial de *L. graveolens* mostró mayor efectividad para inhibir patógenos bacterianos de peces (100%), en comparación con los extractos. Se obtuvo un diámetro de inhibición que osciló entre 25,20 a 36,94 mm. Los extractos de fluido supercrítico y de la técnica de percolación con acetato de etilo y ciclohexano presentaron la misma efectividad (77,78%). El extracto crudo obtenido con etanol al 95% mostró efecto antimicrobiano limitado (22,22%), presentando el menor halo de inhibición (8,34 y 9,57 mm). Por otro parte, *L. alba* mostró menor actividad antibacteriana. El aceite esencial inhibió únicamente el 66,67% de las bacterias patógenas, presentando un halo de inhibición que oscila entre 10,68 a 16,29 mm. El resultado de este estudio indica que los aceites esenciales de *L. graveolens* y *L. alba* son una alternativa prometedora para el control del crecimiento de *Aeromonas* spp.

Palabras clave: *Aeromonas hydrophila*; *Aeromonas sobria; actividad antimicrobiana; acuicultura.*

Resumo

Neste estudo avaliou-se a atividade antimicrobiana de extratos e óleos essenciais de *Lippia graveolens* e *Lippia alba*, contra nove cepas de *Aeromonas* spp., isoladas de *Oreochromis niloticus*. Os extratos brutos foram obtidos pela técnica de percolação e por extração supercrítica com CO_2, enquanto os óleos essenciais foram obtidos por hidrodestilação em aparelho tipo Clevenger. A atividade antimicrobiana para cada extrato e óleo essencial foi

verificada pelo método de disco-difusão em 24 horas. O óleo essencial de *L. graveolens* apresentou maior eficácia na inibição de patógenos bacterianos em peixes (100%), comparado aos extratos. Obteve-se um diâmetro de halo de inibição que variou entre 25,20 e 36,94 mm. Os extratos do fluido supercrítico e da percolação com acetato de etila e ciclohexano apresentaram a mesma eficácia (77,78%). O extrato bruto obtido com etanol 95% apresentou efeito antimicrobiano limitado (22,22%), apresentando o menor diámetro de halo de inibição (8,34 e 9,57 mm). Por outro lado, *L. alba* apresentou menor atividade antibacteriana. O óleo essencial inibiu apenas 66,67% das bactérias patogênicas, apresentando um halo de inibição que varia de 10,68 a 16,29 mm de diámetro. Os resultados deste estudo indica que os óleos essenciais de *L. graveolens* e *L. alba* são uma alternativa promissora para o controle do crescimento de *Aeromonas* spp.

Palavras-chave: *Aeromonas hydrophila; Aeromonas sobria; atividade antimicrobiana*; *aquicultura*.

Introduction

Tilapia (*Oreochromis* spp.) is considered worldwide as the second-largest aquaculture finfish species group by volume [7, 20]. In Guatemala, the tilapia aquaculture industry, according to the Food and Agriculture Organization of the United Nations (FAO), produced approximately 10,914 MT of tilapia in 2018. This industry has shown an annual growth of 10.53% since 2011, supplying an alternative source of animal protein and providing an income to Guatemalans with different economic development levels. In recent years, the tilapia culture industry has been significantly affected worldwide by the breakout of infectious diseases, especially from viral and bacterial origin, being the most frequently cited: *Streptococcus agalactiae, S. iniae, S. dysgalactiae, Lactococcus garvieae, Flavobacterium columnare, Francisella noatunensis* subsp*. orientalis, Edwardsiella ictaluri, E. tarda, E. anguillarum, E. piscicida, Aeromonas hydrophila, A. veronii, A. jandaei, A. shuberti, A. dhakensis* [16, 17, 18]. Moreover, the conventional approaches to control fish diseases have limited success in fish disease prevention, control and treatment. In aquaculture traditional health management, to avoid mortalities, to control and prevent the infection from several bacterial pathogens, a broad-spectrum of veterinary drugs are used [19, 33, 41]. Fish diseases related to parasites and fungal pathogens have mostly been treated and controlled by using formalin, quaternary ammonia and hydrogen peroxide [38]. While, for treating bacterial diseases, the antibiotics most widely used are florfenicol, oxytetracycline, enrofloxacin, chloramphenicol, erythromycin and trimethoprim + sulfametoxazol [33]. It is well known that chemical and antibiotic drugs can have negative effects not only in the environment, through all trophic levels, but also on human health by consumption of contaminated food and water [29] and most importantly, their use can result in drug-resistant pathogenic microbes. Thus, their routine use is now restricted or banned in some countries.

Therefore, medicinal plants and their extracts have been widely used as antibacterial, antiparasitic, antiviral and immunostimulants [10, 51, 54]. Due to their phytochemical components,

such as flavonoids, terpenes, tannins, alkaloids and coumarins, among others, they have been assessed for the treatment and prophylaxis of different pathogens in the aquaculture industry [3, 46, 47]. Moreover, most of these natural products are environmentally friendly, because they are biodegradable compared to synthetic products such as antibiotics, and they are less likely to generate drug resistance on bacteria due to their molecule's diversity [36].

Several plant extracts have been shown to display antibacterial effects in aquaculture, including those from *Lippia* spp. *Lippia* belongs to the Verbenaceae family, comprising 200 species widely distributed in America [56]. In Guatemala, approximately 13 species have been identified, being the most common *L. graveolens* (Khunth) and *L. alba* (Mill). They are also frequently used for medical purposes in public health [11, 50]. Among the phytochemical components in *Lippia* spp., the most frequently found are *p*-cymene, *cis*-sabinene hydrate, terpinen-4-ol, carvacrol, thymol, caryophyllene oxide, citral, geranial, neral and linalool [21, 37]. Most of these major components from different *Lippia* species have an antibacterial capacity in different fish bacterial pathogens, such as *Aeromonas* spp., *Vibrio* spp., *Streptococcus* spp., *Edwardsiella* spp. and *Flavobacterium columnare*, among others, but there is no evidence for *L. graveolens* against fish bacteria pathogens [3, 48].

The efficiency of these metabolites against different bacterial pathogens depends on the method of extraction and solvent. Although several studies have evaluated the efficacy of herbal medicine [24,40,57], limited information exists on the efficacy of extracts obtained using different extraction methods and solvents. Therefore, this study aimed to determine the (*in vitro*) antimicrobial capacity of different types of extracts and essential oils of *L. alba* and *L. graveolens* against nine pathogenic strain bacteria isolated from tilapia cultures in Guatemala.

Material and methods

Plant collection and extraction methodology

Two *Lippia* species (Verbenaceae) were sampled in Guatemala during April 2019. Fresh and healthy leaves of mature *L. graveolens* (LG) and *L. alba* (LA) plants were collected from wild suburbs in Zacapa department (14°59' 50.2'' / 89° 40' 26.2'') and from the medical plant collection from Centro Experimental Docente de Agronomía, Facultad de Agronomía, Universidad de San Carlos de Guatemala (CEDA-FAUSAC) in Guatemala City (14° 35' 6.45'' / 90° 33' 24.34''); respectively. The plant species were identified at Herbario de la Escuela de Biología, Facultad de Ciencias Químicas y Farmacia, Universidad de San Carlos de Guatemala. The leaves were oven-dried at 45 ^{o}C for 48 h, crushed into powder using an electric grinder, sieved through a 300-500 µm mesh size and finally stored at room temperature until use.

Once the plant-powder was prepared, three methods of extraction with different solvents were used to obtain the crude extracts and essential oils from *L*. *graveolens* and *L*. *alba*. The extraction methods were the following:

Percolation technique. Crude extracts were obtained using the percolation technique (PE) through consecutive extractions with three different solvents: cyclohexane (CLH), ethyl acetate (ACE), and ethanol 95% (ETH) [44]. Sequential extractions were carried in amber glass bottle (5 L), filled with 300 g of plant-powder and 3,000 mL of the selected solvent (ratio 1:10 w/v). Each solution was maintained for 48 h at room temperature (28 ± 2 ^{o}C). Afterwards, the solution was separately filtered through a Whatman No. 1 filter paper (Whatman, USA). Finally, the filtered solution was evaporated using a rotary vacuum evaporator at 45 ^{o}C and 115 mB until the solvent faded completely. The resulting crude extracts were separately stored at 4 ^{o}C in 150 mL amber glass bottles.

Supercritical fluid technique. The supercritical fluid extracts (FSC) were obtained using a homemade apparatus based on the model according to Loaiza-Salguero, Ortíz & Robles (1994). For each plant, the extraction vessel (2,000 mL) was filled with 1,000 g of plant-powder and 1,000 mL of ethanol 95% (ratio 1:1). The extractions were done at 45 ^{o}C and 1,300 psi of CO_2 for 45 min. The extracts obtained were collected in a glass flask. To improve the efficiency of this extraction method, the procedure was repeated three times with the same plant material and the same solvent ratio. Finally, the extracts were evaporated using a rotary vacuum evaporator at 45 ^{o}C and 115 mB.

The crude extracts were separately stored at 4 ^{o}C in 150 mL amber glass bottles *Essential oil.* Essential oils (EOS) were extracted by hydro-distillation using a Clevenger type apparatus [30]. Each extraction used 150 g of plant-powder, placed in a 2,000 mL volumetric flask and covered with tap water until submerged. The extraction lasted 2 h after water boiling. The essential oils were collected and stored at 4 ^{o}C in 5 mL amber glass bottles.

For each of the extraction methods, the extract yield was estimated as $Y_{(\%)}$ = M/MV x 100, where M is the mass of extract obtained with each method and MV is the amount of ground dried plant material used.

Microorganisms and culture conditions

Nine bacterial pathogens (six strains of *Aeromonas sobria* and three of *A. hydrophila*) were obtained from the bacterial collection of Centro de Estudios del Mar y Acuicultura, Universidad de San Carlos de Guatemala, and used in the study. These bacteria strains were isolated from diseased tilapia (*Oreochromis* spp.) and identified using the API 20NE (BIOMERUX, France). The bacteria were cultured in trypticase soy agar at 28 ^{o}C for 24 h before used in the antimicrobial susceptibility tests.

Antimicrobial screening

The effect of the different extracts obtained from *L. graveolens* and *L. alba* were assayed on the nine bacteria strains using the disc diffusion method as described by Tkachenko et al. (2016). Each bacterial suspension was adjusted to a concentration of 1-2 x 10^8 CFU/mL in sterile

phosphate buffer solution (PBS), then inoculated on the surface of Muller Hinton agar and spread all around. Subsequently, three blank discs were permeated with 10 mg of each extract, and in the case of EOS 10 µL were used. For positive control, standard discs of oxytetracycline (OXI: 40 µg) and florfenicol (FLC: 40 µg) were obtained from AVIMEX®. For negative controls, blank discs individually impregnated with 20 µL of cyclohexane, ethyl acetate, and ethanol 95% were used. All discs were placed in an incubator for 10 min at 30 ^{o}C to volatilize the solvents. Then, the discs for each treatment and controls were separately set upon the surface of the plates containing the bacteria strains. Each of the extracts were tested twice in triplicate for the nine bacteria strains. The plates were incubated at 28 ^{o}C for 24 hours. The inhibition zone diameters (IZD) were measured to an accuracy of 0.01 mm after 24 h of incubation. The interpretation of the IZD was done according to García-Pérez & Marroquin-Mora, (2020) for the following criteria: extracts or essential oils were considered effective when their IZD was equal or greater than half of the IZD measured for each bacteria strain against the antibiotics. In this case, the most widely used in Guatemala aquaculture, which is oxytetracycline.

Analysis of the plant's mayor components by Gas chromatography–mass spectrometry (GC/MS)

Due to their high effectiveness in the antimicrobial screening, the EOS chemical components of *L. graveolens* and *L. alba* were determined. The GC/MS analyses were performed using a Shimadzu 2010 Plus system coupled with a Shimadzu QP-2010 Plus selective detector (MSD), equipped with a DB5-MS capillary fused silica column (60 m, 0.25 mm I.D., 0.25 µm film thickness). The oven temperature was programmed from 60 ºC to 246 ºC at 3 ºC/min and then held isothermally at 246 ºC for 20 min. He (99.999%) was used as carrier gas with a flow rate of 1.03 mL/min; split ratio of 1:50. Mass spectra were taken at 70 eV. The m/z values were recorded in the range of m/z 40–700 Da. The components were identified by their mass spectra and retention indices compared to values reported in the literature [(1)]. Relative amounts of components were calculated based on GC peak areas without using correction factors.

Data analysis

All experiments were conducted twice in triplicate. Mean values and standard deviations of the inhibition zone diameters in disk diffusion assay were calculated from the experimental data obtained. Mean significance of inhibition zone diameter for different bacterial strains, plant material and type of extraction were analyzed using Generalize Liner Mixed Model (GLM: Negative binomial), to predict the effect of different plant and extraction methods. The differences between the mean values were considered significant when $p < 0.05$. The statistical analysis was carried out using R statistic software [(39)].

Results

Among the different extraction methods used, the highest yield in both plants was obtained with the FSC method (5.29% for *L. alba* and 5.06% for *L. graveolens*), followed by PE extraction

(solvent: CLH + ACE + ETH). Of the PE methods, 95 % ethanol produced the highest yield (4.16 % for *L. graveolens* and 3.28% for *L. alba*); while cyclohexane (2.48% for *L. graveolens* and 0.73% for *L. alba*) was the least efficient. The lowest yield was obtained with the essential oil extraction using water as solvent for *L. alba* with 0.53% (Table 1).

Table 1. Extraction yield (%) for *L. graveolens* and *L. alba* obtained with different extraction methods.

Method	Solvent	Yield (%)	
		L. alba (LA)	L. graveolens (LG)
Percolation extraction (PE)	Cyclohexane (CLH)	0.78 ± 0.04	2.48 ± 0.03
	Ethyl acetate (ACE)	2.37 ± 0.06	3.16 ± 0.06
	Ethanol 95% (ETH)	3.28 ± 0.02	4.16 ± 0.09
	PE Total	6.43	9.80
Supercritical fluid extraction (FSC)	CO_2 + Ethanol 95%	5.29 ± 0.08	5.06 ± 0.05
Essential oil extraction (EOS)	Water	0.71 ± 0.18	4.00 ± 0.12

Note: derived from research.

Concerning the aroma displayed by the plants and extracts, the difference was evident. *L. graveolens* had a spicy and intense pungent odor, while *L. alba* had a distinctly penetrating herb-like and an intense lemon citrus odor. For both plants, the supercritical fluid and essential oil extraction had similar aroma, as well as the extract obtained by PE with 95% ethanol. In contrast, the extracts obtained with cyclohexane and ethyl acetate showed a milder aroma.

The results of antimicrobial activities are presented in table 2 and figure 1. No inhibition zones were recorded in the negative controls (cyclohexane, ethyl acetate, and ethanol 95%), while the positive controls (oxytetracycline: OXI and florfenicol: FLC) showed strong antibacterial activity against *Aeromonas* spp. Their inhibition zones diameter ranged from 9.58 to 31.79 mm (Figure 1c). The inhibition zone diameter for OXI ranged from 9.74 to 27.17 mm. To be considered effective, the inhibition zone of plant extracts must be equal or greater than 5.10 – 13.59 mm, depending on the bacteria strain. A lower value was considered ineffective in this study.

Table 2. Antimicrobial potential of *L. graveolens* and *L. alba* against *Aeromonas* spp.

Bacteria strain	ID	Commercial antibiotics		*Lippia alba*					*Lippia graveolens*				
		OXI	**FLC**	**ACE**	**CLH**	**EOS**	**ETH**	**FSC**	**ACE**	**CLH**	**EOS**	**ETH**	**FSC**
A. sobria	MX01	12.41 ± 0.47	32.37 ± 0.21	00.00 ± 0.00	00.00 ± 0.00	13.53 ± 0.58	00.00 ± 0.00	00.00 ± 0.00	09.40 ± 0.81	07.66 ± 0.04	32.00 ± 1.47	00.00 ± 0.00	11.27 ± 0.55
	MX06	27.11 ± 0.64	30.77 ± 0.57	00.00 ± 0.00	00.00 ± 0.00	13.24 ± 0.39	00.00 ± 0.00	00.00 ± 0.00	17.86 ± 0.68	15.21 ± 0.58	27.96 ± 0.69	08.67 ± 0.35	17.95 ± 0.83
	MX07	12.62 ± 0.52	28.88 ± 0.90	00.00 ± 0.00	00.00 ± 0.00	15.81 ± 0.76	00.00 ± 0.00	00.00 ± 0.00	10.10 ± 0.38	08.66 ± 0.54	28.37 ± 0.74	00.00 ± 0.00	11.89 ± 0.64
	MX09	25.77 ± 0.52	31.14 ± 0.65	00.00 ± 0.00	00.00 ± 0.00	13.96 ± 0.58	00.00 ± 0.00	00.00 ± 0.00	08.01 ± 0.89	00.00 ± 0.00	25.2 ± 0.49	00.00 ± 0.00	08.65 ± 0.43
	MX10	27.17 ± 0.86	26.79 ± 0.28	00.00 ± 0.00	00.00 ± 0.00	11.78 ± 0.20	00.00 ± 0.00	00.00 ± 0.00	08.81 ± 0.49	00.00 ± 0.00	30.48 ± 0.74	00.00 ± 0.00	08.96 ± 0.50
	MX11	12.63 ± 0.62	30.16 ± 0.41	00.00 ± 0.00	00.00 ± 0.00	13.27 ± 0.53	00.00 ± 0.00	00.00 ± 0.00	18.07 ± 0.80	13.98 ± 0.96	29.6 ± 0.41	09.1 ± 0.49	20.2 ± 0.75
A. hydrophila	MX02	24.09 ± 0.89	28.45 ± 0.61	00.00 ± 0.00	00.00 ± 0.00	15.71 ± 0.56	00.00 ± 0.00	00.00 ± 0.00	14.33 ± 0.68	13.53 ± 0.49	36.94 ± 1	09.57 ± 0.42	17.33 ± 0.72
	MX14	22.23 ± 1.01	26.51 ± 0.59	00.00 ± 0.00	00.00 ± 0.00	10.68 ± 0.45	00.00 ± 0.00	00.00 ± 0.00	13.13 ± 1.07	12.38 ± 0.77	30.65 ± 3.56	08.72 ± 0.41	15.34 ± 0.46
	MX15	09.59 ± 0.36	25.52 ± 0.45	00.00 ± 0.00	00.00 ± 0.00	16.29 ± 0.58	00.00 ± 0.00	00.00 ± 0.00	13.51 ± 0.99	11.56 ± 0.55	33.34 ± 4.42	08.34 ± 0.38	15.22 ± 0.73

Note: Values (mean ± standard error) of inhibition zones diameter of oxytetracycline (OXI), florfenicol (FLC), ethyl acetate (ACE), cyclohexane (CLH), essential oil extraction (EOS), ethanol 95% (ETH), Supercritical fluid extraction (FSC).

Among all tested extracts and EOS, *L. graveolens* (LG) exhibited a higher inhibitory effect. The EOS-LG was the most effective, which inhibited 100% of bacteria strains and presented inhibition zone diameters that ranged from 25.2 to 36.94 mm (Table 2, Figure 1a). The other extracts showed lower inhibitory effects, FSC-LG, CLH-LG, ACE-LG (77.78%) respectively and ETH-LG (22.22%), respectively. In the case of ETH-LG, its inhibition zone diameters ranged from 8.34 to 9.57 mm, for this reason, it was classified as an extract with limited antibacterial effect according to the criteria established in this study. The crude extracts from *L. alba* did not show any antibacterial activity, while its EOS inhibited 66.67% of the *Aeromonas* spp. strains, presenting inhibition zone diameters ranging from 10.68 to 16.29 mm (Figure 1b).

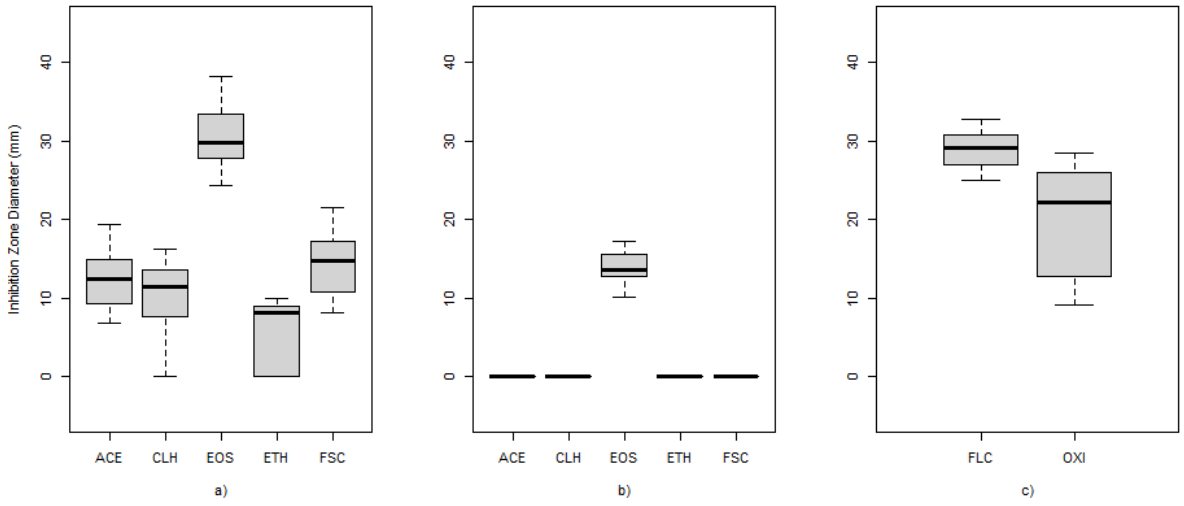

Figure 1. Antimicrobial activity of different organic extracts and essential oils from *L. graveolens* and *L. alba.*

Note: data expressed as means of IZD and the standard deviation: **a):** *L. graveolens (LG)*, **b):** *L. alba (LA)*, **c):** Antibiotic; **OXI:** oxytetracycline; **FLC:** florfenicol, **EOS:** essential oil; **ACE:** ethyl Acetate; **CLH:** cyclohexane; **ETH:** Ethanol 95%, **FSC:** Supercritical fluid.

According with the GLM analysis, the glmer.nb (Y~ (PLANT/EXTRACT), with two random effects (1| SAMPLES) + (1| PLANT), was the best model to predict the extracts strong dependent activity against all bacteria species evaluated. *L. graveolens*, as essential oils, is the best predictor to inhibit *Aeromonas* spp. ($p < 0.001$), compared with the antibiotic and *L. alba*. Although the inhibition zone diameter of *L. alba* essential oils was smaller than *L. graveolens,* these inhibited 67% of bacteria strains, also proving to be effective.

Among the samples, oxytetracycline showed resistance in 44.44% (*A. hydrophila* = 1 strain / *A. sobria* = 3 strain) of the bacteria strains. This study showed that *L. graveolens* and *L. alba* antimicrobial activity does not depend on the antibiotic susceptibility pattern (Figure 2).

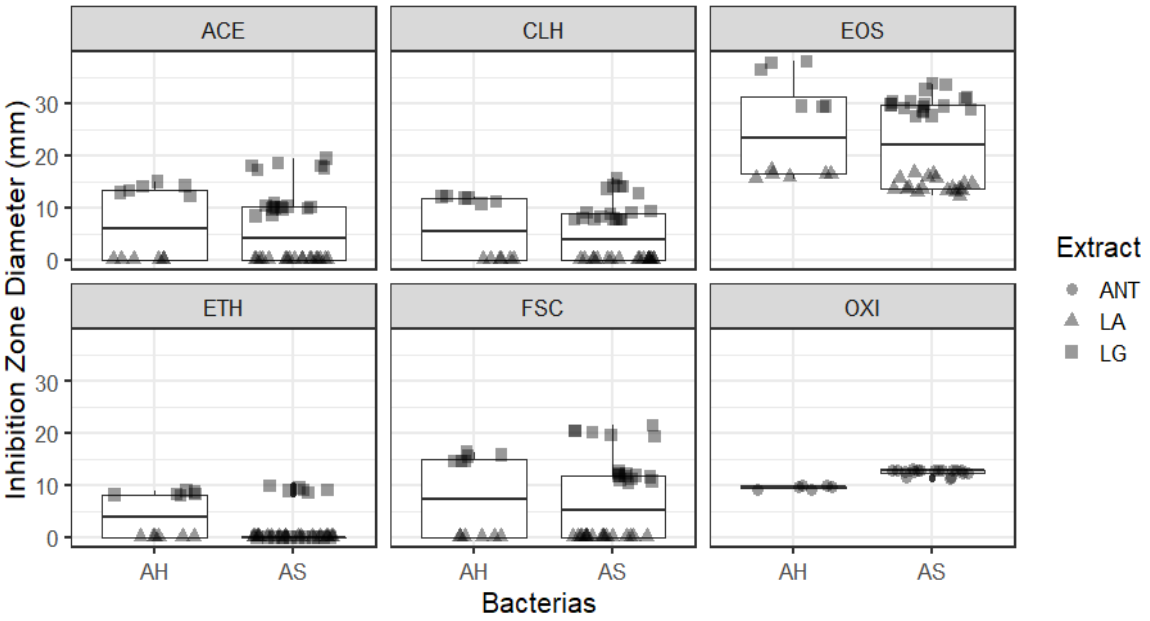

Figure 2. Antimicrobial activity of *L. graveolens* and *L. alba* against oxytetracycline resistant *Aeromonas* spp.

Note: AH: *Aeromonas hydrophila*, **AS:** *A. sobria*. **LG:** *L. graveolens*, **LA:** *L. alba*, **ANT:** Antibiotic. **ACE:** ethyl Acetate; **CLH:** cyclohexane; **EOS:** essential oil; **ETH:** Ethanol 95%, **FSC:** Supercritical fluid, **OXI:** oxytetracycline.

The volatile components identified in the essential oil of the *L. graveolens* and *L. alba* essential oils are reported in table 3. For *L. graveolens* 10 components were identified, representing nearly 98.22% of the total essential oil's composition. The major compound was thymol (81.31%), follow by *o*-cymene (8.44%), while the remainder components were below 2%. Moreover, in *L. alba* 20 components were identified, representing nearly 97.74% of the total essential oil's composition. The major compounds were geranial (36.8%), followed by neral (25.7%) and 1,8-cineole (13.45%), while the rest of the compounds were below 4%.

Table 3. Composition of essential oils (%) in *L. graveolens* and *L. alba*.

Components	Area (%)		RI
	Lippia graveolens	***Lippia alba***	
g-amorphene	-	0.55	1495
δ-cadinene	-	0.54	1522
Carvacrol	0.51	-	1298
(E)-caryophyllene	1.62	1.89	1417
Caryophyllene oxide	1.95	-	1582

Components	Area (%)		RI
	Lippia graveolens	***Lippia alba***	
Caryophyllene oxide	-	3.87	1582
1,8-Cineole	-	13.45	1026
cubebol	-	1.01	1514
o-cymene	8.44	-	1022
Geranial	-	36.80	1264
Geraniol	-	0.58	1249
Geranyl acetate	-	1.33	1379
Geranyl isobutanoate	-	0.55	1514
6-methyl-5-hepten-2-one	-	2.29	981
α-humulene	1.09	-	1452
Humulene epoxide II	0.97	-	1608
Linalool	-	1.52	1095
Myrcene	0.91	-	988
Neral	-	25.70	1235
1-Octen-3-ol	-	1.46	974
trans-sabinene hydrate	-	0.86	1104
Terpinen-4-ol	0.81	0.56	1174
α-terpineol	-	1.21	1186
δ-Terpineol	-	0.42	1162
Thymol	81.31	0.60	1289
Thymol, methyl ether	0.61	-	1232
α-Ylangene	-	2.55	1373
Total area (%)	98.22	96.65	

Note: RI: Retention index.

Discussion

The essential oils from *L. graveolens* and *L. alba* offer a promising alternative for the control of *Aeromonas* spp. growth. The oil extracted from *L. graveolens* exhibited *better antibacterial properties than L. alba*, which included a significantly larger inhibition zone diameter against *Aeromonas* spp. and inhibition against all bacteria strains. It is well documented that *L. graveolens* have an antibacterial effect against a broad group of pathogens, especially against Gram-negative bacteria [2, 6, 42], however, until now there was no evidence for the effectiveness of *L. graveolens* against fish bacteria pathogens. There are many studies with plants with similar metabolites profile as *L. graveolens* such as *Origanum vulgare.* This plant known as European

oregano has well documented [4, 26, 49] uses against fish bacterial pathogens especially Gram-negative bacteria. These properties may be attributed to several active metabolites, such as carvacrol, thymol, *c*-terpinene and *o*-cymene [25] that have antibacterial activity and have been detected in various species of *Origanum*.

The chemical composition of *L. graveolens* in this study was slightly different compared to other studies from Guatemala and Mexico [9, 32, 43]. For example, Pérez-Sabino et al., (2012) also reported thymol (67.4 – 73.5%) and *o*-cymene (1.9 – 5.9%) as the major constituents in *L. graveolens* from Zacapa department, but did not report the presence of Myrcene, Humulene epoxide II and *α*-Humulene. Also, the same authors reported that *L. graveolens* presented three different chemotypes (Thymol, Carvacrol, and Mixed), indicating that these volatile compounds were affected by the prevailing geographical region in Guatemala, genetic factors, nutrients in the soil, harvest time and method of extraction among other factors.

In addition, there are other species of *Lippia* with similar chemical profile as *L. graveolens* which have a strong antibacterial effect, such as *L. sidoides* and *L. origanoides* [23, 27, 30], which have thymol (9-76.6%) and carvacrol (49.7%) as major components. The essential oils of *L. origanoides* and *L. sidoides* had inhibitory effects against *A. hydrophila* with a minimal inhibitory and bactericidal concentration ranging from 1250 to 5000 μg mL^{-1}. This indicates that probably those components, thymol and carvacrol, are responsible for the antimicrobial activity, showing an inhibitory effect against Gram-negative such as *A. hydrophila*, but also Gram-positive bacteria. It should be noted that both compounds (thymol and carvacrol) have a similar chemical structure. Although, the chemical composition does not appear to have any major effect on the antibacterial activity, both metabolites provoke the disintegration of the external membrane of bacteria [27].

In the present study, thymol, which is responsible for the inhibition of *Aeromonas* spp, was found to be the major compound in the *L. graveolens* essential oils. This phenolic terpene exhibits strong antimicrobial activity capable of rupturing the external membrane of bacteria and increasing its permeability to ATP [27]. On the other hand, the efficiency of *L. graveolens* extracts could be related to the extraction methodology and solvent used, which can affect the amount of thymol extracted. In the present study, *L. graveolens* showed antibacterial activity with FSC-LG extract, which indicated that the polarity of CO_2 combined with ethanol 95% as co-solvent might increase the extraction of polar compounds such as thymol [12]. However, this method probably didn't extract the total amount of the polar compounds, since its efficiency was lower than the EOS, which could contain higher concentration of thymol. Moreover, the efficiency of the PE method was related to the type of solvent used, varying the amount of the major component with the higher antibacterial activity. In this case, CLH-LG and ACE-LG were the solvents with the higher affinity to extract the thymol, however, they apparently did not extract enough of that compound. Consequently, those extracts showed lower antibacterial activity compared to FSC and EOS. This indicates that it is necessary to properly select the type

of solvent and technique for extracting a target compound efficiently. Another important factor to be considered is the amount of extract used to permeate the discs in the assays. In this case, 10 mg for the crude extract and 10 µL for the EOS appears to be enough to incorporate a specific proportion of the compounds to show antibacterial activity.

Lippia alba EOS inhibited the growth of *A. hydrophila* and *A. sobria* agreeing with results found by other authors, who reported the effectiveness of *L. alba* EOS in tests against several *Aeromonas* spp. strains and other Gram-negative and positive bacteria [15, 30, 35], indicating that the volatile compounds with antibacterial activity were geranial, neral and 1,8-cineole. Other authors reported that the antibacterial activity of *L. alba* is mainly against Gram-positive bacteria [5, 31, 45]. This is due to the morphological structure of the bacterial cell wall of Gram-positive bacteria, which is less complex than Gram-negative bacteria [55]. The present study suggests that the lipophilic character of essential oils of *L. alba* may interact with lipopolysaccharides, therefore, reducing its efficiency to cause changes in Gram-negative bacteria structure, which could result, in some cases, in no bacterial lysis. Moreover, the chemical composition of *L. alba* EOS could vary depending on location, soil type, weather conditions, among other factors, showing different chemotypes with varying proportions of major compounds (e.g., geranial, neral, carvone, linalool, myrcene) [13, 21, 52]. The antimicrobial activity of these compounds may be correlated with a specific chemotype.

Finally, bacteria can develop resistance to antimicrobials due to the continuous and prolonged exposure to them [14, 34, 40]. Contrarily, due to the complex composition of *L. graveolens* and *L. alba* essential oils, it is expected that bacteria rarely develop a resistance because plants have different mechanisms of action that implies several cell targets [8]. This work confirms that *L. graveolens* (extracts and EOS) and *L. alba* (EOS) have a high antimicrobial activity, indicating that this is independent on the antibiotic susceptibility pattern, since at least one byproduct of *Lippia* spp. influences the most resistant bacteria. The efficiency of the plant is related to the plant´s chemical composition, but also to the extraction procedure. The effectiveness of *L. alba* was limited compared to *L. graveolens*, due to 1) a lower content of the major metabolites, 2) different types of metabolites. This research also addresses the importance of choosing the proper extraction method and solvent to reach a high effectiveness in the extracts.

Conclusion

This study shows that essential oils from *Lippia* spp. have high antibacterial activity against *Aeromonas* spp., isolated from tilapia. The greatest inhibitory effect was obtained with *L. graveolens* EOS. The results suggest that the antibacterial activity of EOS is attributed to the abundance of monoterpenes and terpenoids compounds. However, further investigations are required to evaluate the mechanisms of the antibacterial activity of the essential oils from *Lippia* spp. Additionally, future studies should include the isolation of major bioactive components of each EOS and assess their individual effects. In this sense, special attention

should focus on thymol for *L. graveolens* and geranial for *L. alba*, to evaluate their efficiency in controlling pathogens in aquaculture.

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