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Original article

Antibacterial activity of *Lemna minor* extracts against *Pseudomonas fluorescens* and safety evaluation in a zebrafish model

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ABSTRACT

The treatment of bacterial diseases in aquaculture is done using antibiotics, their applications has resulted in contamination and bacterial resistance. Natural extracts are a potential alternative as an antimicrobial, they have demonstrated effectiveness in their use aimed at treating conditions. The purpose of this study was to evaluate the antimicrobial activity of *Lemna minor* extracts against *Pseudomonas fluorescens* with different solvent for extraction. Methanol, chloroform and hexane were used. Subsequently, the safety assessment of the extracts in *Danio rerio* embryos and larvae was performed to validate as ecologically harmless. Antibacterial activity was detected in three extracts with significant differences ($p = 0.001$). Hexane extract had the highest antibacterial activity, followed by chloroform and methanol extracts. The three extracts have differences with respect to the control, between times and concentrations tested ($p = 0.001$). Minimum inhibitory concentration values (MIC) at 24 h methanolic extract ME $0.05 \mu\text{g mL}^{-1}$. In embryos and larvae increased safety of the LC₅₀ methanolic extract was evidenced followed by the hexane and chloroform extract. No morphological or tissue changes were observed in embryos and larvae. The hexane extracts of *L. minor* had a greater bactericidal effect against *P. fluorescens* and are functional because of their antibacterial activity, but methanolic extract is more safety in embryos and larvae of *D. rerio*, making it a potential alternative for use in the treatment and control of septicemia in fish.

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1. Introduction

During the last decades, aquaculture has become the fastest growing animal food production activity in the world with an annual rate of 2.3% (FAO, 2020). Diseases constitute an important limitation in the growth of animal production systems and the aquaculture sector is no exception. The diseases are caused by a series of events associated with interactions among the host,

environment and presence of pathogens (Henriksson et al., 2018). According to the World Organization for Animal Health (OIE), infectious diseases of fish and crustaceans could reduce the world's productive potential by 20%, due to the associated economic losses (Perumal et al., 2015), and many of them could constitute a threat to human health and animal welfare (Haenen et al., 2013). Although the pathogens that affect farmed fish are diverse, bacteria are the most frequent and the ones that cause the most damage (Figueroa et al., 2019). Among the main bacterial species that affect species of consumption and ornament are: *Aeromonas salmonicida*, *A. hydrophila*, *Pseudomonas fluorescens*, *Edwardsiella ictaluri*, *Edwardsiella tarda*, *Streptococcus agalactiae*, *Streptococcus iniae*, *Vibrio alginolyticus*, *Flavobacterium columnaris* among others (Hawke et al., 2013; Sahoo et al., 2016). For the treatment and control of bacterial diseases, various pharmaceutical chemicals and antibiotics, such as oxytetracycline, erythromycin and tetracycline have been used (Jani et al., 2011; Economou and Gousia, 2015);

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however, indiscriminate use has generated resistant strains and gene transfer through various mechanisms among bacterial populations (Xiong et al., 2015). In addition, antibiotics have severe environmental repercussions due to the discharge of aquaculture effluents as well as resistance and bioaccumulation in animals and humans, a situation that makes them a potential risk for consumers (Smaldone et al., 2014; Reverter et al., 2014; Subasinghe, 2009; Xiong et al., 2015).

Faced with this problem in 2010, the World Health Organization (WHO), the World Organization for Animal Health (OIE), and the Food and Agriculture Organization of the United Nations (FAO) agreed to combat antimicrobial resistance with the AMR (antimicrobial resistance) global action plan, and in 2016 reaffirmed the commitment and strategy to improve health services and promote research aimed at the use of natural extracts that help to reduce and control infections (FAO, 2016). In addition to the above, there are studies on the use and efficacy of plant extracts, based on the activity of secondary metabolites that have naturally been associated with important physiological defense functions in plants and that have proven useful for the treatment of viral, parasitic, and fungal diseases in fish. (Harikrishnan et al., 2009; Stratev et al., 2018; Van Hai, 2015).

Regarding Lemnaceas, the information is scarce, some evaluations carried out by various authors have shown that they have compounds with bactericidal effect, which are mainly attributed to phenolic compounds, flavonoids among others, that have been shown to have antimicrobial properties (Maddox et al., 2010; Akbary, 2014; Al-Abd et al., 2015). In the same way, it has been reported that they have a favorable effect on the health of aquatic organisms since they can also act as antioxidants (Çalışkan and Aytelkin Polat, 2011). Thus, performed research has shown antibacterial activity with species of the Lemnoideae family, such as *Lemna gibba*, *L. minor*, *Spirodela polyrrhiza* and *S. punctata*, and other less widespread such as *Wolffia globosa* (Stomp, 2005; Gülçin et al., 2010; Das et al., 2012; Dafalla, 2015a; Al-Snafi, 2019). Genus *Lemna* has demonstrated antagonism against *Bacillus subtilis*, *B. cereus*, *Staphylococcus aureus*, *S. saprophyticus*, *S. warneri*, *Proteus vulgaris*, *Citrobacter freundii*, *C. koseri*, *Neisseria lactamica*, *Micrococcus luteus* and *Streptococcus pneumoniae*, among other. Antibacterial activity has occurred in different degrees of sensitivity, depending on the susceptibility and biology of the bacterial species being tested (Brain et al., 2004; Dafalla, 2015; Gülçin et al., 2010; Jani et al., 2011; Ramirez and Marin Castaño, 2009). However, there is no research showing direct antimicrobial effect against *Pseudomonas fluorescens*, an opportunistic Gram-negative bacterium that infects fish, birds, and humans (Miyazaki et al., 1984; Scales et al., 2014). It is a pathogen of farmed fish in marine, brackish, and freshwater worldwide and it has reported in grass carp (*Ctenopharyngodon idella*), Indian major carp (*Catla catla*), Japanese flounder (*Paralichthys olivaceus*), tilapia (*Oreochromis*), black carp (*Mylopharyngodon piceus*) and turbot (*Scophthalmus maximus*) (Wang et al., 2009; Darak and Barde, 2015). The development of bacterial diseases constitute one of the major challenges facing sustainable aquaculture production, provoking several mortalities caused by hemorrhage and ulceration, and represent economic losses to fin-fish aquaculture in around the world (Foyosal et al., 2011).

Considering that the use of phyto-pharmaceutical products in aquaculture is growing (Bulfony et al., 2015) and derived from the interest in determining their efficacy and safety, research is required to validate its efficacy and ensure the safety of extracts. In this sense, despite higher animals have been for many years models of excellence used to evaluate drug toxicity, the zebrafish presents itself as a reliable vertebrate model to determine developmental toxicity, general toxicity and to perform an initial drug screening (Caballero and Candiracci, 2018), their use for toxicity assessment of pharmaceutical compounds has been greatly

increased (Jayasinghe and Jayawardena, 2019). The main benefits of using zebrafish as a toxicological model over other vertebrate species are with regards to their size, husbandry, early morphology and the requirement of small quantities of test compounds (Hill et al., 2005). From the egg stage, the transparent embryos can survive for several days through the absorption of yolk and can be visually assessed for malformation (MacRae and Peterson, 2003). Therefore, the present study aimed to evaluate the *in vitro* antibacterial activity of *L. minor* extracts against *P. fluorescens* and the safety evaluation of these extracts using *D. rerio* as a model.

2. Materials and methods

2.1. Plant material and extracts

Lemna minor was collected in channels of the lake zone of Xochimilco, México during June to July 2016, and identified by the herbarium of the Metropolitan Autonomous University. The macrophyte culture was performed under controlled conditions, 8.34 ± 2.1 pH and $19.8 \text{ }^\circ\text{C} \pm 0.2$ temperature. For this culture, organic liquid earthworm humus fertilizer (Humiviv[®]) 10 mL L^{-1} humic acids and $0.25 \text{ mg day L}^{-1}$ sea salt was used as a contribution of essential minerals. The drying of the plant was carried out at room temperature and dark for 20 days, it was ground by an industrial blender. For the extraction, three organic solvents were used individually with different polarity: technical grade methanol, chloroform, and hexane (J.T Baker[®]).

To obtain the methanolic extract (ME), 200 g of *L. minor* powder was used, placed inside a 1 L round-bottom flask with 600 mL of methanol, the mixture was placed in a reflux position for 4 h, the extract was filtered under vacuum through Whatman No. 1 filter paper in a 2 L Kitasato flask and Buchner funnel; the solvent was removed under reduced pressure and the extract was concentrated by a rotary evaporator (IKA[®] RV10). The same procedure was performed for another 200 g of the *L. minor* powder but using chloroform (EC) as a solvent. In the case of hexane (EH), the reflux was during 4 h (Oreopoulou et al., 2019).

The extracts were stored at $4 \text{ }^\circ\text{C}$ in Schott Duran[®] glass bottle hermetically closed until further use. For the evaluation of these extracts, six aqueous concentrations were prepared from $5000 \text{ } \mu\text{g mL}^{-1}$ to $0.05 \text{ } \mu\text{g mL}^{-1}$ per extraction method. The extraction yield was calculated by the formula (crude extract weight/plant material weight) $\times 100$ (Victório et al., 2010). To prepare the aqueous extracts, with the methanolic extract, water was added and polyvinylpyrrolidone (PVP) (Sigma-Aldrich[®]) at proportion of 1:4 (crude extract: PVP, (w/w), was used to allow to make a miscible solution with hexane and chloroform extracts.

2.2. Bacterial strain and growth conditions

The *Pseudomonas fluorescens* collection strain (ATCC 13525) was resuspended in 100 mL of brain heart solution (BHI) broth. Cultures were homogenized at 100 rpm and incubated at $28 \text{ }^\circ\text{C}$ for 48 h in a digital incubator Luzeren[®] without shaker. To obtain the number of cells necessary for standardization, aliquots of the standardized inoculum were transferred to 100 mL in Muller-Hinton broth (Difco[®]) at a final concentration of $1.5 \times 10^7 \text{ CFU mL}^{-1}$. The growth of *P. fluorescens* was monitored at 1–10, 12, 16, 24, 30, 36, 48, 54, 60, 72, 80, 84, 90, 96, 108 and 120 h of incubation. The values were determined by measuring the optical density (OD) with respect to time from a concentration $1.5 \times 10^7 \text{ CFU mL}^{-1}$ with an incubation temperature of $28 \text{ }^\circ\text{C}$. Furthermore, at each interval, an aliquot was transferred and diluted 10-fold in tube containing 9 mL of 0.1% (w/v) saline solution, and 100 μL were spread on to brain heart agar plate in triplicate. The plates were incubated at $28 \text{ }^\circ\text{C}$ for 24 h,

and the colonies were counted CFU mL⁻¹. The microbial count data were correlated with optical density and the data were fitted using non linear regression by the modification Gompertz equation $y = a - \exp[-\exp(b - ct)]$ (Zwietering et al., 1990).

2.3. In vitro evaluation of antimicrobial effect

The antibacterial capacity of the extracts in six concentrations of 5000, 500, 50, 5, 0.5 and 0.05 µg mL⁻¹ were evaluated by the microdilution method and counted in the box of colony forming units (CFU), according to the methodology proposed by (Ramirez and Marin Castaño, 2009; Balouiri et al., 2016) due to its usefulness in the use of polar and non-polar substances as antimicrobials. For the tests, the *P. fluorescens* inoculum was used at 1.5×10^7 CFU mL⁻¹ in Muller-Hinton broth (Difco®).

The minimum inhibitory concentration (MIC) was determined at 24 h post-incubation, at 28 °C according to the methodology proposed by (Andrews, 2001). Once the process was finished, Petri dishes were seeded in triplicate, and the CFU mL⁻¹ colony forming units were counted for the quantitative estimation of viable cells with the help of a magnifying viewer (Ramirez and Marin Castaño, 2009).

2.4. In vivo toxicology evaluation

2.4.1. Zebrafish maintenance

Adults of zebrafish (*Danio rerio*) were used, which were raised and cultivated, until generation F3. The initial strain was subjected to quarantine period to ensure the health of the organisms. The reproduction began with the selection of breeders, 2:1 male:female ratio in 10 L aquariums with a previous 24 h fast to prevent contamination of the eggs with the excreta of their parents and prevent proliferation of fungi like *Saprolegnia parasitica*. The maintenance conditions were, dissolved oxygen 6 ± 0.5 mg L⁻¹, temperature $26 \text{ °C} \pm 1$, NO₂ 0.3 mg mL^{-1} , NO₃ $23 \text{ mg mL}^{-1} \pm 2$, pH 7.5 ± 0.5 , photoperiod 12 h:12 h light:dark cycle, with 25% water replacement every 7 days. Juveniles and adults were fed commercial flake (Wardley®) *Ad livitum* and the larvae were fed with a mixture of *Chlorella vulgaris* and *Scenedesmus* sp. Microalgae, in 50:50 ratio at a concentration of 1×10^4 cells mL⁻¹ and once the yolk sac was completely absorbed after two days of hatching, commercial fry food from Azoo® was provided.

2.4.2. Determination of the median lethal concentration LC₅₀ of *L. minor* extracts in zebrafish embryos and larvae.

The median lethal concentration LC₅₀ of the extracts in *D. rerio* embryos and larvae was determined by means of a static-type bioassay without renewal of the test solution. In this test, the fish were exposed to the extracts and mortality and immobility of test organisms were monitored and recorded at 4, 8, 12, 24, 48, 72 and 96 h.

For carrying out the embryo tests, only those fertilized eggs that did not present any external anomaly (asymmetries, vesicles) or whose membrane was not damaged were used. The essay began immediately after fertilization and continued for 96 h post fertilization (hpf) (OECD, 2013), using extracts at concentrations 5000, 500, 50, 5, 0.5, and 0.05 µg mL⁻¹ and a control group with reconstituted semi-hard type water (United States Environmental Protection Agency, 2002). A 24-well microplate (Corning®) was used per extraction method (hexane, methanol, chloroform), n = 4 embryos per well, and 3 replicates, a total of n = 12 embryos by concentration evaluated. The same was done for testing with larvae in post-absorption stage of yolk sac by concentration and 3 replicates. The volume of extract in solution was 2 mL per well. The count of live or dead embryos throughout the test was performed using the criterion of four apical points (oculated eggs, seg-

ment formation, detailed tail, and presence of heartbeat) according to Organization for Economic Co-operation and Development (OECD, 2013), with the help of a stereoscopic microscope (Olympus® SZX12, 4X). Eggs that did not have the described characteristics were considered dead at the corresponding time. The same procedure was performed with the larvae, except that only organisms were monitored alive or dead, regarding treatments.

2.4.3. Tissue analysis

D. rerio embryos and larvae were fixed in 10% neutral buffered formalin (NBF). The samples were processed in a tissue histotechnique (Leica Microsystems, TP 1020), serial sections of 5 µm were prepared on a microtome (Leica® HM 315) coupled to a tissue transfer system with a hot bath at 50 °C. The sections were mounted on glass slides. Slides were deparaffinized, rehydrated, and stained with Hematoxylin-Eosin (Merk®) (Copper et al., 2018). All the histological sections were examined in an optical microscope (Carl Zeiss® Cx31) and processed using the associated Axio Vision Rel. 4.8 software. The tissue evaluation was performed qualitatively, alterations such as pericardial edema, yolk sac edema, skeletal deformations, defined eye, and axial malformations (abnormal notochord) were verified. These elements are part of the most sensitive acute embryo and larvae stage sub-lethal endpoints (Anuradha and Katti, 2009; Dubinska-Magiera et al., 2016).

2.5. Statistical analysis

Arithmetic mean number bacterial CFU calculated from triplicate were log₁₀ transformed and used to calculate overall means of log CFU. Assumptions of normality were reviewed using Levene's test at 0.05 significance level. Data was analyzed by general linear model (GLM), with effects and interaction analysis. The GLM was followed by mean separation using Tukey's honest significant difference test. All analyses were conducted using IBM SPSS Software®; significance was assigned at p < 0.05. Lethal concentrations of the extracts (LC₅₀) were estimated using larval and embryo mortality values by log Probit analysis of the MATLAB® statistical package, at 95% confidence level p = 0.05.

3. Results and discussion

3.1. Extracts yield

The extraction of the *L. minor* extracts gave different yields. The highest yield was obtained with methanolic (17.6%) followed by chloroform (1.43%) and hexanic (1.35%) extracts. The yield of the methanol extract of this study is similar to reported by Gülçin et al., (2010), who obtained yields of 13.2% aqueous extract and 18.4% ethanol extract of *L. minor*. There are other studies that evaluated extracts of *L. minor* such as Dafalla, (2015a), Peng et al., (2018), *L. pauciscostata* (Effiong and Sanni, 2009); however, they did not report their extraction yields. The quantitative and qualitative extraction yield depends on the polarity of the solvents used, as in this study, also the chemical composition of the compounds extracted, the amount and position of their hydroxyl groups, the molecular size, factors such as solvent concentration, temperature, contact time, particle size and mass-solvent ratio (Azmir et al., 2013; Ngo et al., 2017).

3.2. *Pseudomonas fluorescens* ATCC 13525 growth curve

The growth curve of *P. fluorescens* ATCC 13525 is observed by Gompertz model for 72 h in Fig. 1. The r = 0.98, and coefficient of determination R² = 0.96 indicates a positive average relationship

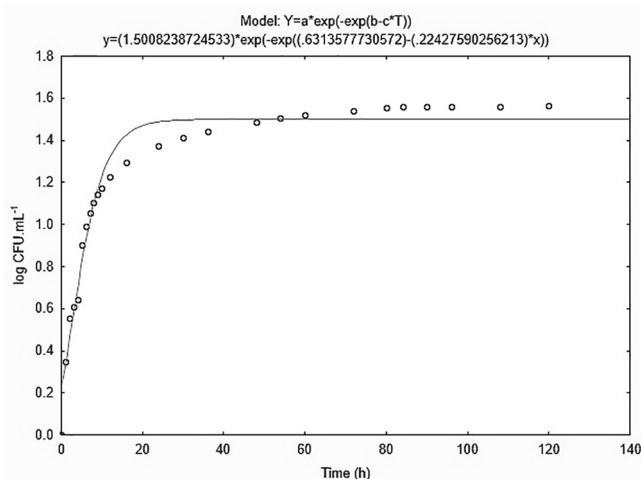


Fig. 1. Growth curve of *Pseudomonas fluorescens* ATCC 13525 at 28 °C fitted by Gompertz model.

of 96%. between the bacterial growth and the incubation time, with values of $\mu m = 0.336 \text{ h}^{-1}$, $\lambda = -1.643 \text{ h}$, $G = 2.059 \text{ h}$.

According to this trend, the first three phases of its growth were identified from the initial concentration of $1.5 \times 10^7 \text{ CFU mL}^{-1}$. In phase I, the bacterium metabolized, but did not multiply; in phase II, it showed exponential growth; in phase III, the cell concentration remained constant (stationary phase). Phase IV (death) was not appreciated in this method, since dead bacteria and metabolic wastes were suspended in the medium, maintaining turbidity.

3.3. In vitro antimicrobial effect

L. minor extracts studied in this work showed II *In vitro* antibacterial activity against *P. fluorescens*; the effect analysis developed showed significant differences between treatments and control ($p = 0.001$). The differences were between the methanolic and chloroform extract ($p = 0.002$) and between the methanolic and hexane extract ($p = 0.000$).

There are significant differences ($p = 0.001$) between bacterial inhibition with respect to exposure times of 24, 48, and 72 h with the three tested extracts and significant differences ($p = 0.001$) between concentrations evaluated by extract Table 1. In this way, the greater effect of the chloroform and hexane extracts is reflected between 24 and 48 h and in time the bactericidal effect reduce at 72 h Fig. 2B, 2C., however, with the effect of the methanolic extract becomes more noticeable at 72 h with significant differences between concentrations ($p = 0.001$).

Table 1

Analysis of the antimicrobial activity of extracts of *L. minor* on *P. fluorescens* at different times and concentrations evaluated.

Treatment	Hours (h)	Mean \pm SD Log CFU mL ⁻¹	Time (h)		Concentrations
			p values	HSD Tukey	
ME	24	6.95 \pm 0.29	0.009**	24–48 h***	p = 0.001***
	48	6.57 \pm 0.24		24–72 h	
	72	6.81 \pm 0.16		48–72 h**	
CE	24	7.00 \pm 0.10	0.000***	24–72 h***	p = 0.001***
	48	6.49 \pm 0.27		48–72 h	
	72	6.66 \pm 0.36		24–48 h***	
HE	24	6.95 \pm 0.10	0.000***	24–48 h*	p = 0.001***
	48	6.70 \pm 0.35		24–72 h***	
	72	6.55 \pm 0.16		48–72 h	

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ by Tukey's Honest Significant Difference test.

On the other hand, the minimum inhibitory concentration (MIC) values of this study at 24 h were $0.05 \mu\text{g mL}^{-1}$ with the three extracts. As it has been shown, in this study antimicrobial activity was presented against *P. fluorescens*, particularly in Lemnaceae. The information is scarce; however, some evaluations have demonstrated bactericidal effect, and studies such as that of Jani et al. (2011) where they evaluated aqueous extracts with ethyl acetate, methanol and hexane from *Lemna gibba*., and only the latter showed inhibitory activity at the concentration of 0.5 mg mL^{-1} . In this case, the extracts of *L. gibba* presented inhibition against *Bacillus* sp., *Staphylococcus aureus*, and *Proteus vulgaris*; the MIC reported by Jani et al., (2011) is higher than the one obtained in our study, being of $0.05 \mu\text{g mL}^{-1}$ with the hexane *L. minor* extract at 24 h.

In addition, Dafalla, (2015) evaluated the bactericidal effect of methanol extracts of *L. minor*, the MIC values for *Shigella flexneri*, *Bacillus subtilis*, *Micrococcus luteus* and *Staphylococcus aureus* were 12, 40, 60 and $170 \mu\text{g mL}^{-1}$ respectively. In contrast to our study, MICs were lower at 24 h with $0.05 \mu\text{g mL}^{-1}$ of the three evaluated extracts. What could be observed is that the MIC values of the extracts have a wide range of action depending on the bacterial species and whether they were gram positive or negative.

In another study conducted by Gülçin et al. (2010), antimicrobial activity of aqueous and ethanolic extracts of *L. minor* was found in a concentration of $1 \mu\text{g mL}^{-1}$ against gram positive and negative bacterial strains, producing inhibition halos of 7–11 mm, with the exception of *P. fluorescens* in which none of the two extracts showed inhibition. This contrasts with what was found in this study for there was inhibition and the comparison is difficult due to the difference between evaluation methods.

More recent studies, such as Peng et al., (2018), methanol extract of *L. minor* was evaluated, finding MIC values of $1.8\text{--}2.00 \text{ mg mL}^{-1}$ against gram positive and negative bacteria. These concentrations are higher than those reported in this study of $0.05 \mu\text{g mL}^{-1}$. However, despite the MIC values, the authors found no inhibitory effect under the disk diffusion test, so they disagree with authors such as Gülçin et al., (2010) seeming contradictory.

However, the results of our research validate the efficiency of *L. minor* extracts against *Pseudomonas fluorescens*, and according to Balouiri et al., (2016); Ramirez and Marin Castaño, (2009) refers that the microdilution methodology is important due to its usefulness in the use of polar and non-polar substances as antimicrobials and avoid diffusion problems. These problems are supported by the fact that agar well diffusion methods are recommended for polar substances because the Whatman filter paper is composed of cellulose and a high amount of free hydroxyl groups that gives the disc hydrophilic properties, which directly affects some cationic compounds of natural products, as these are absorbed by the surface of the disc, preventing their diffusion in the media. Therefore, this can distort the results of the true antibacterial potential of the

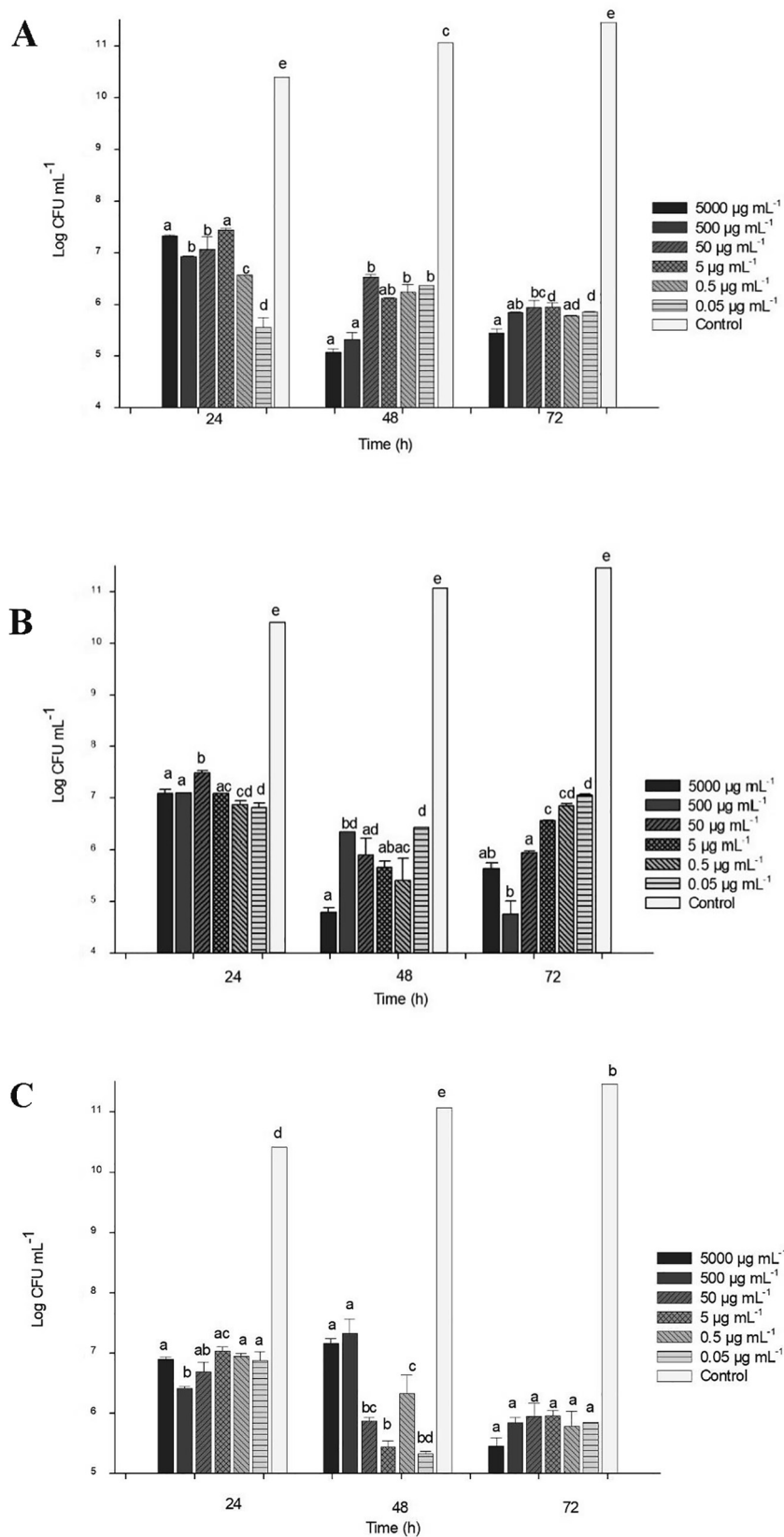


Fig. 2. Antimicrobial activity of *L. minor* extracts A) methanolic extract, B) chloroform extract, and C) hexane extract. 5000 µg mL⁻¹, 500 µg mL⁻¹, 50 µg mL⁻¹, 5 µg mL⁻¹, 0.5 µg mL⁻¹ and 0.05 µg mL⁻¹. Different superscript letters indicate significant difference ($p < 0.05$) among concentrations by extraction method.

evaluated products (Corrales et al., 2013). In this sense, the method is selected according to the characteristics of the substance used. In this study, extracts with different polarity, polar, moderately polar, and non-polar were evaluated, for this reason the microdilution method was used, also because it presents advantages of sensitivity and reproducibility (Klančnik et al., 2010; Rivas-Morales et al., 2016). As it was mentioned earlier Peng et al., (2018) evaluated methanolic extracts of local duckweed and did not show any of the inhibitory effects on the bacteria tested under the disc diffusion assay, but in the MIC evaluation by microdilution method, results show inhibitory of bacterial growth for all bacterial tested, at the concentration of 1.8–2.0 mg mL⁻¹.

In this study, the evaluated extracts were of different polarity and demonstrated antimicrobial activity with significant differences of antimicrobial action among them, deriving from the different components extracted according to the nature of the available compounds and their affinity with the organic solvent used in the extraction. The inhibitory effects in this study are attributed to the availability of metabolites and the methanolic extract showed the highest antimicrobial activity, which indicates that the available compounds with activity are mostly polar. In this regard, have been reported in *Lemna* species the presence of phenolic compounds, such as gallic acid, tannins, flavonoids, anthocyanins, quercetin, and other compounds like thiol and terpene as steroids. Which are known to have antimicrobial properties (Effiong and Sanni, 2009; Gülçin et al., 2010; Leão et al., 2014; Vladimirova and Georgiyants, 2013; Al-Snafi, 2019; Mahizan et al., 2019). In hexane extract, terpene compounds as steroids should have the principal antimicrobial activity.

3.4. Toxicity evaluation in zebrafish embryos and larvae

Given the exposure of embryos to the different extracts of *L. minor*, embryonic development was carried out from the concentration 0.05, 0.5, 5, 50 µg mL⁻¹ with the three extracts, at 500 and 5000 µg mL⁻¹ the embryos died at 4 h of exposure. The calculated lethal concentrations that produce 50% of embryonic mortality were for 54.59 µg mL⁻¹ for HE, 495.32 µg mL⁻¹ for ME and 26.70 µg mL⁻¹ for CE (Table 2), without deformations or developmental delays according to the times described by Kimmel et al. (1995), Fig. 3. and in comparison, with the control group. At a macroscopic level, the correct formation of the spine, eyes, yolk sac with abundant reserve lipid, and heartbeat were appreciated.

Table 2
Toxicological evaluation of *Lemna minor* extracts in *Danio rerio* embryos and larvae.

ME	Apical points	Embryos								Larvae			
		Exposure time								CL ₅₀ 96 h (µg mL ⁻¹)	95% confidence limit (µg mL ⁻¹)	CL ₅₀ 96 h (µg mL ⁻¹)	95% confidence limit (µg mL ⁻¹)
		1h	4h	8h	12h	16h	24h	36h	48h				
ME	1	+	+	+	+	+	+	+	+	495.32	317.48–1141.94	149.44	61.52–379.38
	2		+	+	+	+	+	+	+				
	3						+	+	+				
	4							+	+				
CE	1	+	+	+	+	+	+	+	+	26.70	0.00–113.33	6.70	0.00–11.44
	2		+	+	+	+	+	+	+				
	3						+	+	+				
	4							+	+				
HE	1	+	+	+	+	+	+	+	+	54.59	24.62–112.58	92.69	39.72–249.31
	2		+	+	+	+	+	+	+				
	3						+	+	+				
	4							+	+				

Note: ME: extraction method, ME: methanol extract, CE: chloroform extract and HE: hexane extract. Apical points: (1) oculated eggs, (2) segment formation, (3) tail detachment, (4) presence of heart beat. + symbol indicates presence of apical points.

In a toxicity analysis of methanol extracts from *L. minor* by Peng et al. (2018), they obtained LC₅₀ of 140.64 µg mL⁻¹ in *Artemia salina*, being similar to the calculated concentration of ME in *D. rerio* larvae of 149.44 µg mL⁻¹ and higher in embryos with ME 495.32 µg mL⁻¹ of this study.

According to the calculated LC₅₀, the methanolic extract showed the highest tolerance in *D. rerio* larvae and embryos at high concentrations, and the chloroform extract with the highest mortality in embryos and larvae at lower concentrations during the 96 h that the experiment lasted. Table 2.

According to Braunbeck et al. (2005) the chorion in *D. rerio* embryos provides protection against substances; in the case of exposure to hydrophilic substances, the absence of the chorion does not affect the acute toxicity of the embryo, but clearly shows sublethal effects, such as a disturbance of the swimming balance in larvae, indicating that the chorion acts at least as a barrier form, even for hydrophilic substances. This explains the greater tolerance of the embryos to the extracts than the newly hatched larvae.

The authors also report that in the case of moderately lipophilic substances there is a significant increase in toxicity. The lethal concentrations of the substances reported by (Braunbeck et al. (2005) indicate greater toxicity at lower concentrations compared to those found with the present study. Regarding the increase in toxicity, it is similar to the values obtained in this study with CE and HE, since it presented a lower tolerance due to its affinity with lipophilic type compounds.

3.5. Tissue analysis of embryos and larvae

The tissue analysis of the *D. rerio* larvae and embryos exposed to the three extracts revealed that the embryos exposed to the hexane and chloroform extracts in concentrations of 50, 500, and 5000 µg mL⁻¹ stopped their development; they did not develop somites and died in the first 24 h. With the methanolic extract, they died at a concentration of 5000 µg mL⁻¹. Embryos exposed to concentrations lower than the LC₅₀ calculated values in Table 2 continued with their development, being no evidence of abnormalities in the morphogenesis of vital structures and in the differentiation of the central nervous system, cardiovascular system; there were no obvious tail deformations or alterations in the anteroposterior axis and death.

Danio rerio larvae, post-absorption of the yolk sac, that survived from the calculated LC₅₀, showed no damage at the epithelium level and nor alterations at the bone level, such as lordosis or

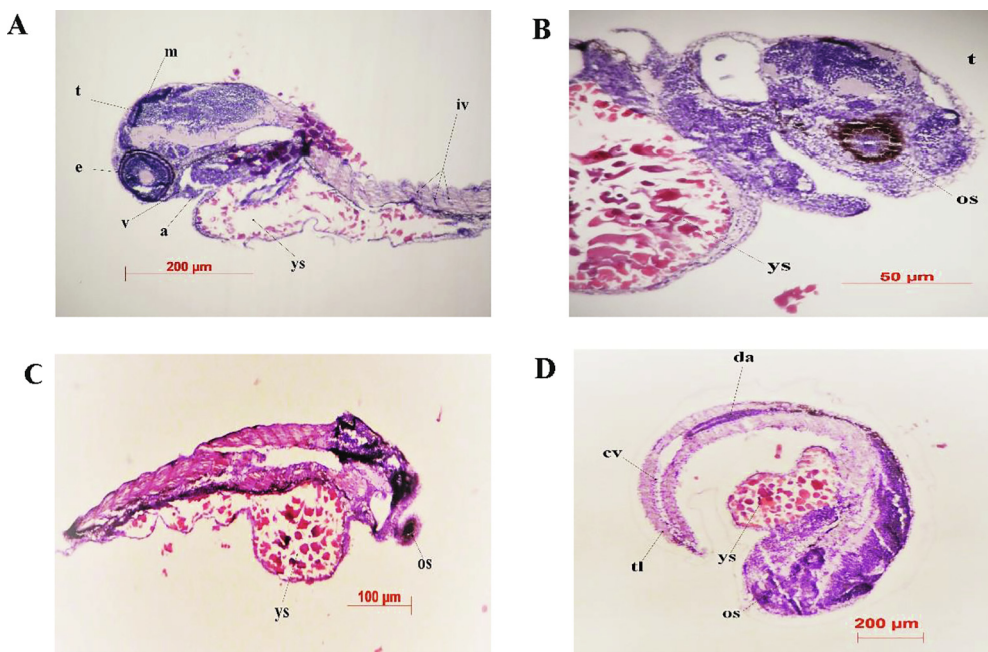


Fig. 3. Histological microphotographs of zebrafish larvae and embryos, H&E staining. (A) Larval negative control 10X at 72 hpf., (B) 72 hpf embryo exposed to methanolic extract 40X., (C) 72 hpf embryo 10X exposed to hexane extract, (D) 72 hpf embryo without hatching exposed to chloroform extract 40X. Key: (ys) yolk sac, (de) defined eye and retinal ganglion cell presence, (t) telencephalous, (m) mesencephalous, (os) ocular spot, developing, (tl) tail, (ca) caudal artery, (a) atrium, (v) ventricle, (e) eye, (iv) intersegmental vessels, (ccv) common cardinal vein, (cv) caudal vein, (da) dorsal aorta, (ca) caudal artery.

kyphosis. Evidence is presented in Fig. 3. Blood vessels with distinct lumens and evident movement of blood within them were appreciated at 48 hpf. The ontogenic development of *D. rerio* is indirect, so they are underdeveloped at the time of hatching (Zavala-Leal et al., 2011).

In the 96 hpf stage post-test, some organs such as kidney and liver and gill system were not yet fully developed as was the mouth, which the embryo naturally opens once the yolk sac has been fully absorbed 3 days post hatching. However, it is reported that the development time of the organs and their functionality can be affected by various factors, such as exposure to substances. One of the main effects in response to a toxic substance are the disturbances to: the development of the cardiovascular system, deformations can be seen in the skeleton, osmoregulatory dysfunction, neural defects, growth reduction, and decrease survival (Dubansky and Bautista, 2017), which, according to those observed in this study, did not show these affectations at concentrations below the calculated LC₅₀. In the cases of death, it could be derived from alterations in the heart tissue.

4. Conclusions

This study demonstrates that *L. minor* extracts have antibacterial activity against *P. fluorescens* with respect to control. The method used in this study for the evaluation of post-incubation antimicrobial capacity allowed us to discern the quantification of live bacterial cells from the dead that could interfere with the quantification and appreciation of the results. The MIC variations in research with this plant, suggest that it is necessary to characterize the antimicrobial effects with other bacterial species. According with our study we suggest using hexanic extract due to its greater antimicrobial effect than others, but we recommend methanolic extract because it is more safety according with our study. In this study, it was shown that embryos developed adequately at concentrations below the calculated LC₅₀ and in the tissue evaluation of the larvae no abnormalities that affected their

vital function were identified. In this sense, the zebrafish model allowed to evaluate the safety of the extracts tested for use in the control and treatment of bacterial septicemia *In vivo*.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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