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Original Research

Biological effects caused by the pharmaceuticals losartan and diclofenac, and their mixture on marine organisms

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Abstract

Studies show that pharmaceuticals are being taken to the oceans causing contamination and toxicity to aquatic organisms. The present study evaluated the survival rate of microcrustaceans *Artemia salina* and the abnormal embryo larval development rate of sea urchin *Echinometra lucunter*, after exposure to the drug Losartan, an antihypertensive drug, and the drug Diclofenac, a non-steroidal anti-inflammatory drug (NSAID), in addition to its mixture among its compounds. Acute toxicity tests were carried out using *A. salina* and chronic toxicity tests were carried out using embryos of *E. lucunter*. Organisms were exposed to isolated pharmaceuticals at different concentrations: 1.56 mg.L^{-1}; 3.12 mg.L^{-1}; 6.25 mg.L^{-1}; 12.5 mg.L^{-1}; 25 mg.L^{-1}; 50 mg.L^{-1} and 100 mg.L^{-1}, and their mixture at concentrations: 0.78 mg.L^{-1}; 1.56 mg.L^{-1}; 3.12 mg.L^{-1}; 6.25mg.L^{-1}; 12.5 mg.L^{-1}; 25 mg.L^{-1} and 50 mg.L^{-1}. The result obtained in the acute toxicity test did not show toxicity to *A. salina*. Chronic toxicity test with losartan did not show toxicity to sea urchin embryos, in contrast, the isolated diclofenac showed chronic toxicity at NOEC = 6.25 mg.L^{-1}, LOEC = 12.5 mg.L^{-1} and IC50 = 62.15 mg.L^{-1}. The result obtained with embryos exposed to the mixture of losartan and diclofenac, showed chronic toxicity at NOEC= 6.25 mg.L^{-1} and LOEC= 12.5 mg.L^{-1}, not being possible to show the IC50. Our results suggest that the mixture of the two studied pharmaceuticals might decrease the toxicity, since diclofenac showed higher chronic toxicity to *E. lucunter* embryo larval development when it was isolated than when it was mixed with losartan. However, there is a need for further ecotoxicological studies that clarify the pathways of these pharmaceuticals in non-target organisms.

Keywords: Diclofenac; Losartan; Mixtures; Toxicity; Coastal organisms.

1. INTRODUCTION

The concentrations of pharmaceuticals found in ocean waters change the quality of aquatic ecosystems due to the complexity of human activities, whether domestic, commercial, or industrial (Fent *et al.*, 2006; Van der Aa, 2011; Al Aukidy *et al*., 2012; Gutperlet *et al.*, 2015; Lolic *et al.*, 2015; Pereira *et al.*, 2016; Sangion and Gramatica, 2016; Comber *et al*., 2018; Tak and Kakde, 2019). Presenting high dispersion in the ocean, pharmaceuticals contaminate the aquatic environment (Borova *et al*., 2014; Pereira *et al*., 2016) and when absorbed by organisms, become a harmful threat (Salgot, 2006; Ternes, 2007; Fürhacker, 2008).

To minimize the aggravating factors of contamination in the environment, Brazilian Federal Decree No. 10,388 (June 5th, 2020) institutes the reverse logistics system for expired or unused household medicines, for human

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use, industrialized and manipulated, and for their packaging after disposal by consumers. Internationally, the European integrated system classified pharmaceutical products according to the specific toxicity results, through the directive 93/67/EEC-Council of the European Community (CEC, 1996). The normative classify chemical substances into different classes (extremely toxic, very toxic, toxic, harmful, and non-toxic).

Two pharmaceutical products were selected for this study. Losartan and diclofenac are pharmaceuticals widely used with high prevalence in the elderly, which their concomitant use of these two pharmaceuticals is frequent, causing the emergence of significant drug interactions in humans (Nascimento and Pigoso, 2013). About 4% of an oral dose of losartan is excreted unchanged in urine and about 6% is excreted in urine as the active metabolite (Rahman *et al.*, 2015). About diclofenac, approximately 65% is excreted in the urine and approximately 35% in the bile as conjugates of unchanged diclofenac plus metabolites (Williams *et al*., 2011). The removal efficiency of losartan and diclofenac was relatively low (<50%) in a wastewater treatment plant (WWTP) in Kumamoto (Japan) with activated sludge treatment (Matsuo et al., 2011).

The drug losartan is indicated for the treatment of systemic arterial hypertension (SAH) and acts in humans by blocking the calcium channel and the AT1 receptor of angiotensin II (Malachias et al., 2016; Silva et al., 2018). In surface waters, it has a low potential to bind iodine from sewage treatment plants, it is a weak acid, which does not completely dissociate its ions in water (Godoy *et al.,* 2015). The occurrence of losartan concentrations in the surface waters has been reported in several countries (Table 1), as at Santos Bay (São Paulo, Brazil) where it was found the concentration of 0.032 **µg.L^{-1}**. Previous studies addressed the toxicity effects of losartan in marine species as sea urchins *Lytechinus variegatus* and mussels *Perna perna* exposed to mg.L^{-1} (Table 2).

Table 1. Concentrations found in surface waters of the drug Losartan.

Country	Concentration (µg.L^{-1})	Environmental Matrices	Reference
Brazil	0.032	Coastal Waters	Pereira *et al.*, 2016.
Spain	0.62	Mediterranean Sea	Gros *et al.*, 2017.
India	2500	Close to factories	Larsson *et al.,* 2007.
Portugal	0.91	Close to hospital	Santos *et al*., 2013.
Spanish Coast	0.62	Surface Water	Huerta-Fontela *et al.*, 2011.
Germany	0.000333	Effluents	Gurke *et al*., 2015.
Sweden	0.98	Effluents	Gros *et al.*, 2017.

Table 2. Toxicity effects on aquatic species from losartan exposure.

Species	Concentration (mg.L^{-1})	Parameters	Reference
Lenma minor	63.9	EC50	Godoy *et al*., 2015.
Pimephales promelas	1000	EC50	FDA, 2001.
Oncorhynchus mykiss	9029	CL50	FDA, 2001.
Lytechinus variegatus	50	NOEC	Yamamoto *et al.,* 2014.
Lytechinus variegatus	70	LOEC	Yamamoto *et al*., 2014.
Perna perna	50	NOEC	Cortez *et al.,* 2018.
Perna perna	75	LOEC	Cortez *et al.,* 2018.
Perna perna	84.6	IC50	Cortez *et al.*, 2018.

Diclofenac, a non-steroidal anti-inflammatory drug (NSAID), is an inhibitor of cyclooxygenase 1 and 2, thus inhibiting the production of prostaglandin and thromboxane, responsible for physiological factors (Brunton et al., 2012). When diclofenac is exposed to sunlight, it decomposes rapidly, transforming into compounds such as 2-chloroaniline, 2,6-dichloroaniline, 2,6-dichlorophenol (Bartels and Tumpling, 2007). Diclofenac is found in the ocean in its original form and as a metabolite 3'-OH-DCF and 4'-OH-DCF due to human metabolism (Vieno and Sillampaa, 2014), generating hydroxyl radicals, able to cause oxidative stress in marine species (Schmitt *et al.*, 2007). Occurrence of diclofenac was observed in environmental matrices of several countries (Lee *et al.*, 2005; Carvalho *et al.*, 2009) (Table 3). Previous studies also addressed toxicity on aquatic species when exposed to diclofenac concentrations (Table 4).

Table 3. Diclofenac concentrations found in marine environments.

Country	Concentration ($\mu g.L^{-1}$)	Environmental Source	Reference
Brazil	0.194	Coastal waters	Pereira *et al*., 2016.
Germany	2	Surface water	Bartels and Tumpling, 2007
France	1.5	Mediterranean Sea	Togola and Budzenski, 2008.
Greece	0.016	Mediterranean Sea	Alygizakis *et al.,* 2016.
Ireland	0.016	Marine Waters	McEneff *et al*., 2014.
Portugal	0.031	Arade estuary	Gonzalez *et al.*, 2015.
Canada	1.5	Effluents	Lee *et al.,* 2005.
Spain	0.031	Mediterranean Sea	Gros *et al.*, 2012; Biel-Maeso *et al*., 2018.
Singapore	0.003	Marine Waters	Rodríguez-Navas, 2013; Bayen *et al.*, 2013.
Taiwan	0.053	Marine Waters	Fang *et al*., 2012.
United Kingdom	0.195	Estuary	Thomas and Hilton, 2004; Nebot *et al.*, 2007.
Switzerland	2.4	Treatment station	Gonzalez *et al*., 2017.

Table 4. Toxicity effects in aquatic species to exposure of Diclofenac

Species	Concentration ($\mu g.L^{-1}$)	Parameters	Reference
Mytillus sp.	1000	Reduction of antioxidant enzymes; DNA damage.	Ericson *et al.,* 2010; Schimidt *et al.,* 2011, 2014; Gonzalez and Bebiano, 2014; Ribas *et al.,* 2016.
Mytillus galloprovincialis	0.25 to 25	Immunological changes; Genotoxic effects; enzyme induction oxidants; Lipid peroxidation; Bioaccumulation; granulocytes; Phagocytosis; Variation of genes in cell renewal.	Mezzellani *et al.*, 2018, 2016; Erickson *et al*., 2010.
Mytillus galloprovincialis	1000	Oxidative stress; Activation of immune responses; Energy imbalance, impacting growth and reproduction.	Schimidt *et al.*, 2011, 2014; Ericson *et al.,* 2010.
Oncohynchus mykiss	100 to 500	Cytological changes in liver and kidney.	Triebskorn, 2004.
Salmo trutta f. fario	≥ 10	Lesions in gills, liver and kidney.	Schwarz *et al*., 2017.
Salmo trutta f. fario	≥ 100	LC50	Ericson *et al.*, 2010; Schimidt *et al.*, 2014.

Since there were some previous studies showing the adverse effects of pharmaceuticals on marine species, the aim of the present study was to evaluate the ecotoxicity of the pharmaceuticals losartan and diclofenac, isolated and mixed through acute and chronic toxicity tests using marine organisms: *Artemia salina* and *Echinometra lucunter*.

2. MATERIALS AND METHODS

2.1 Exposure concentration selection

Pharmaceutical's diclofenac (CAS number 15307-79-6) and losartan (CAS number 124750-99-8) were purchased from Sigma-Aldrich (Steinheim, Germany). The stock solution (100 $mg.L^{-1}$) of both pharmaceuticals was diluted in seven nominal concentrations (1.56; 3.12; 6.25; 12.50; 25; 50 and 100 $mg.L^{-1}$) and used in the single exposure of each pharmaceutical. For the mixture exposure, a stock solution (50 $mg.L^{-1}$) of each pharmaceutical was prepared and diluted to reach the same seven concentrations above (50:50 v/v). Besides the pharmaceutical's concentrations, a control (negative control) and a DMSO control (solvent control) were performed. DMSO (0.001%) was used to dissolve the pharmaceuticals, which were posteriorly dissolved in filtered sea water. The concentrations were based on preliminary laboratory tests as well as the International Directive 93/67/EEC (EEC-Council of the European Community), amended in Regulation (EC) no. 1907/2006. The International Directive establishes the potential risks of pharmaceuticals in the environment through criteria for the identification of persistent and bioaccumulative substances in marine organisms in the order of 0.01 $mg.L^{-1}$.

2.2 Acute toxicity tests

The microcrustacean *Artemia salina* (Crustacea: Brachiopoda) was used in the acute toxicity test, due to its practicality in handling (Cavalcante *et al.*, 2000) and for having protocol standards established by ABNT NBR 16530 (2016). There are previous studies showing that *A. salina* is a good bioindicator to evaluate pharmaceuticals toxicity in the environment (Webb, 2001, Lestari *et al.,* 2017).

Seawater used in the tests was taken from the Laje de Santos (Santos, SP), 45km far away from the coast. In the laboratory, the seawater was autoclaved and filtered through a cellulose membrane with a porosity of 0.45 μm, used for dilution and handling of the cysts.

Nauplii of *A. salina* were obtained after hatched from dehydrated cysts. The cysts were placed in a glass beaker with seawater and incubated for 24 hours before the test. The nauplii were exposed to the concentrations of the pharmaceutical's losartan, diclofenac and their mixtures for 48 hours under controlled laboratory conditions (temperature 25 ± 2ºC, salinity 34, photoperiod 16:8 light: dark, absence of food and aeration). 10 nauplii were used in each replicate (3 per concentration) in glass test-tubes with 10 mL of test solution. After the test period, the organisms were observed in stereoscopic microscope and the endpoint analyzed was the survival of the nauplii (Meyer *et al.,* 1982; Veiga and Vital, 2002; ABNT, 2016). This analysis aimed the determination of the Lethal Concentration in 50% of organisms within 48 hours of exposure ($LC50_{48h}$).

2.3 Chronic toxicity tests

Sea urchins *Echinometra lucunter* (Echinodermata: Echinoidea) are found in tropical waters in the Atlantic Ocean. This specie is used as a tool for ecotoxicological tests due to the ecosystem relevance, sensitivity to contamination and worldwide use as biomonitors. The test followed the established protocol standards according to ABNT (2012) (Tavares, 2004; Santos and Flammang, 2005).

The organisms were collected on Palmas Island (Guarujá, SP) by divers. After collection, individuals were packed in isothermal boxes, then taken to the laboratory, where they were kept in tanks filled with seawater under controlled conditions until the beginning of the test (constant aeration, temperature 21± 2 ºC, salinity 35).

To perform the toxicity test, reconstituted water was prepared with distilled water and salt (Coral Pro salt, Red Sea®). The reconstituted water was filtered through a Millipore® cellulose membrane, with a porosity of 0.45 μm. The gametes were obtained by applying electrical impulses of 35 v or by injecting 0.5 M of KCl. To collect the oocytes, the females were placed with its aboral surface facing downwards in a container smaller than their diameter filled with dilution water. The spermatic fluid was collected from the gonopore using a Pasteur pipette and placed in a dry beaker, kept on ice.

For fertilization, it was prepared a solution in the proportion of 0.5 mL of sperm to 25 mL of dilution water. Then, a volume of 1 mL of the sperm solution was added to the beaker containing the oocytes. After 10 minutes of gentle agitation to enable the fertilization and with a rate of 80% of successful oocytes fertilized the test was set up. Approximately 300 eggs were placed in tubes of each concentration tested, in replicate (3 per concentration). The tubes were kept on a culture chamber for 42 hours at temperature 26 ± 2ºC, photoperiod (16: 8 light: dark) and salinity 35.

After the period of exposure, 10 μL of the control were removed and verified that at least 80% of the larvae reached the *pluteus* stage, according to the test's acceptability criteria. The assay was finalized by adding 0.5 mL of 40% formaldehyde-borax buffered in each replicate.

The first 100 larvae were analyzed under an optical microscope, using a Sedgewick-Rafter chamber to observe anomalies and/or delays in the development through its morphological aspect.

2.4 Statistical analysis

Data was submitted to analysis of variance to determine if the mean of the sample groups were different (ANOVA - $p < 0.05$), followed by Dunnett's test to determine the NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect Concentration). The statistic program GraphPad Prism® 5.01 was used. The IC_{50} (50% average inhibitory concentration of organisms) was calculated by linear interpolation for chronic bioassay.

3. RESULTS

3.1 Acute toxicity test

It was observed 100% of the survival rate to the exposed *A. salina* in the control. About the DMSO control, it was observed 97% of the survival rate. Both tests were in accordance with the standards stipulated by the protocols (ABNT, 2016). In both assays (isolated and mixed losartan and diclofenac) it was not observed significant acute effects compared to the controls ($p < 0.05$). Since the survival rates between the organisms exposed to the pharmaceuticals treatments and the controls were not significant, it was impossible to calculate the $LC50_{48h}$ (Figure 1a, 1b and 1c).

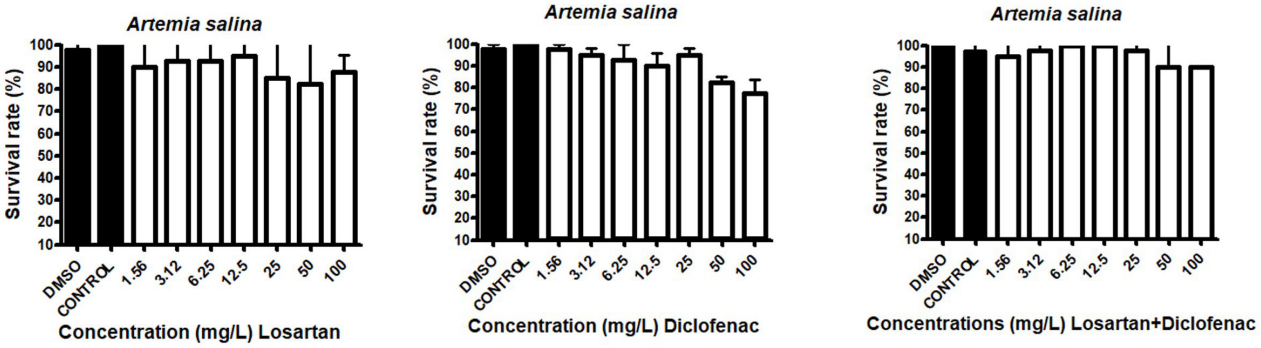

Figure 1. Mean and standard deviation of acute toxicity test with *Artemia salina* to exposure to different concentrations of losartan (a), diclofenac (b) and their mixture (c). DMSO is the solvent control, and CONTROL was the negative control (only seawater)

Statistically significant differences were not observed compared with the controls ($p < 0.05$).

3.2. Chronic toxicity test

In the assay performed to assess the chronic toxicity, the organisms exposed to the water control and DMSO control showed 96.75 and 97.50% normal embryo larval rates, respectively. It was not observed statical differences in the development of the organisms exposed to the losartan and the controls (Figure 2.a).

Embryo larval development of *E. lucunter* exposed to diclofenac were affected in the concentrations of 12.5, 25, 50 and 100 $mg.L^{-1}$. NOEC and LOEC were 6.25 and 12.5 $mg.L^{-}1$, respectively. Through the linear interpolation method, it was possible to estimate the IC_{50} = 62.15 $mg.L^{-1}$ (Figure 2.b).

Larvae of *E. lucunter* exposed to the mixture of the two pharmaceuticals showed chronic effects in the development when exposed to concentrations: 12.5, 25,50 and 100 $mg.L^{-1}$ when compared to the control. NOEC and LOEC were 6.25 and 12.5 $mg.L^{-1}$, respectively. These results did not allow the calculation of the IC50 (Figure 2.c).

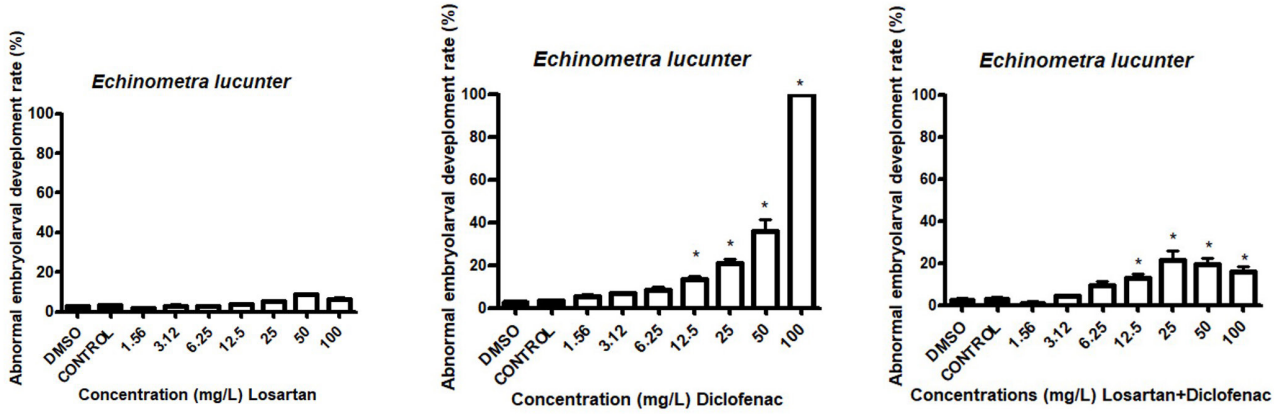

Figure 2. Mean and standard deviation of chronic toxicity test with *Echinometra lucunter* exposure to different concentrations of losartan (a), diclofenac (b) and their mixture (c). DMSO is the solvent control, and CONTROL was the negative control (only seawater)

* Statistically significant differences compared to the controls ($p < 0.05$).

4. DISCUSSION

In the present study, it was not observed the acute effects in nauplii of *A. salina* exposed to the pharmaceuticals and their mixture. Our results corroborate to the study performed by Fabbri *et al.* (2014) which showed no acute effects to organisms exposed to pharmaceuticals since the *A. salina* is a resistant organism in the natural environments. To observe the toxicity effect on filtering organisms, chronic tests are indicated where the organisms are exposed to a longer period (Fent *et al.*, 2006; Peake *et al.,* 2016). Andrade *et al.* (2013) used *A. salina* as a bioindicator and observed 83% of mortality of the microcrustacean exposed for 72 hours to concentrations of cellulose nano fibrils. In addition, Andrade *et al.* (2013) concludes that longer periods of exposure to contaminants interferes with basic movements, consequently in the metabolism of the organism.

In the chronic toxicity tests, losartan in concentrations until 100mg/L^{-1} did not demonstrate toxicity to sea urchin (*E. lucunter*) embryos. Cortez *et al.* (2018) observed alterations in the embryo larval development of the brown mussel *P. perna* exposed to losartan in the concentration up to 75 mg.L^{-1}. Through a battery of biomarkers, it was observed sublethal effects as an increase in the activity of the enzymes CYP450-*like* and in glutathione S-transferase in gills of the mussels exposed to concentrations to 0.03 and 3 µg.L^{-1}. The glutathione peroxidase activity also increased in the concentration of 3 µg.L^{-1} as well as cyto-genotoxic effects in hemocytes and gills in concentrations up to 0.03 µg.L^{-1}.

Previous studies demonstrated that marine organisms exposed to losartan increased the DNA damage and decrease the stability of the lysosomal membrane due to changes to the fluidity of the cell membrane. (Zoumpoulakis, 2003; Gonzalez *et al.*, 2015; Pereira *et al.,* 2016; Gros *et al*., 2017). Godoy *et al.* (2015) indicate the need to evaluate the biological effects of losartan on the marine biota through chronic tests since there is a lack of information about the adverse effects of this pharmaceutical on non-target organisms.

The chronic test performed exposing *E. lucunter* larvae to diclofenac showed no development of pluteus larvae in the concentration of 100 mg.L^{-1}. Diclofenac is found in effluents from sewage treatment plants, representing a risk to species in embryonic and juvenile stages, impacting in the survival rates, development and behavior (Meyer *et al.,* 2016).

Pourahmad *et al*. (2011) described that the diclofenac metabolites form oxygen-reactive species, being able to decrease the stability of membranes. Through the chronic assay with sea urchin (*E. lucunter*) embryos performed in this study, we observed effects in the development of larvae that may be caused by the activation of the antioxidant system. Guiloski et al. (2017) exposed the fish *Rhamdia quelen* for 28 days to the concentration of 20 µg.L^{-1} of diclofenac. They observed an increase in the activity of the Glutathione S-Transferase in the liver, which might activate oxidative stress (Antunes *et al*., 2010), increasing the formation of H_2O_2 (hydrogen peroxide) and increasing the catalase activity (Hite *et al.*, 1999).

The chronic toxicity test with the mixture of losartan and diclofenac, using *E. lucunter* embryos, showed the lowest effect in the concentration of 12.5 mg.L^{-1}. The toxicity of a mixture of pharmaceuticals is a result of the interaction of their constituents, which can be additive, synergistic, or antagonistic (James *et al.*, 2000; Panouilleres *et al.*, 2007; Ince *et al.,* 1999). Our results suggest that the mixture of the two studied pharmaceuticals might decrease the toxicity, since diclofenac showed higher chronic toxicity to *E. lucunter* embryolarval development when it was isolated than when it was mixed with losartan (Figures 2A, B and C). However, there is a need for further ecotoxicological studies to clarify the pathways of the pharmaceuticals in non-target organisms.

As a source of pharmaceuticals to the aquatic environment, the conventional system of sewage treatment is not efficient to degrade the molecules of pharmaceutical compounds, which are partially removed through primary and secondary processes (Ince, 1999; Ziylan, 2011; Yang *et al.*, 2017). For an effective process, it is necessary that the sewage treatment follow tertiary techniques, which allow filtration and adsorption technologies by activated carbon, with oxidative processes in the presence of ozonation and photolysis (Li *et al.*, 2014; Peake *et al.*, 2016) which can eliminate some pharmaceuticals from the sewage and minimize the impact on the aquatic environment that receives them.

Further studies using biomarkers as a tool are necessary to generate subsidies to establish safe environmental concentrations for non-target aquatic organisms and consequently review the environmental legislation, that currently does not contemplate these pharmaceuticals. Studies with organisms of other trophic levels are also necessary to understand the metabolism pathways of these pharmaceuticals and their mixture and the transfer of pharmaceuticals through food webs.

5.CONCLUSION

The data obtained on the occurrence of pharmaceuticals in oceans and estuarine ecosystems led to the investigation of toxic effects at biological levels and different life stages from the trophic chain, exposed to different concentrations of losartan, diclofenac and their mixture. Assays performed with *A. salina* did not show acute effects. However, chronic effects in the sea urchin embryos were observed in the treatments with diclofenac and the mixture of losartan and diclofenac.

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7. DISCLAIMER

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.

8. CREDIT AUTHOR STATEMENT

JAS was the Master that developed this study for her dissertation; FHP gave all the technical and writing support; ASBO: investigation, writing-reviewing and editing; MUC: investigation, RAGS: investigation, DMSA: investigation, CDSP: investigation, LAM: investigation, writing-reviewing and editing.

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