PYRETHRUM ENCAPSULATED IN NANOPARTICLES: TOXICITY STUDIES BASED ON GENOTOXIC AND HEMATOLOGICAL EFFECTS IN BULLFROG TADPOLES

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ABSTRACT

The environment receives a large amount of pesticides annually, about 2.7 kg/ha per year are used in crop production, having a negative impact on the environment and biodiversity, for example, physiological effects on non-target species. Advances in technology and nanocarrier systems for agrochemicals have led to new alternatives to minimize these impacts, such as nanopesticides, which are considered more efficient, safe and sustainable. However, it is important to evaluate the risk potential, action and toxicity of nanopesticides in aquatic and terrestrial organisms. This study aims to evaluate genotoxic and hematological biomarkers in bullfrog tadpoles (*Lithobates catesbeianus*) submitted to acute exposure (48h) to pyrethrum extract (PYR) and solid lipid nanoparticles loaded with PYR. Results showed increased number of leukocytes changed significantly during short-time exposure, specifically eosinophils in nanoparticle-exposed groups and basophil in PYR-exposed group. Hematological analysis showed that PYR encapsulated in nanoparticles significantly increased the erythrocyte number compared to the other exposed groups. Data from the comet assay indicated an increase in frequency of the classes that correspond to more severe DNA damage in exposed groups, being that the PYR-exposed group showed a high frequency of class 4 DNA damage. Moreover, erythrocyte nuclear abnormalities were triggered by short-time exposure in all treatments, which showed effects significantly higher than the control group. These results showed genotoxic responses in tadpoles, which could trigger cell death pathways. These analyses were interesting for applications in contamination in aquatic environments and biomonitoring, because will evaluate the toxicity in species and environmental contamination. However, to better understand the effects of nanopesticides and botanical insecticides on non-target organisms' further studies are needed in order to contribute to regulatory aspects of future uses for these systems.

KEYWORD: Nanotoxicology; Solid lipid nanoparticles; Botanical insecticides; Pyrethrum, Amphibian; Genotoxicity.

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1. INTRODUCTION

Agrochemicals can be found in water bodies in rural areas and have a direct impact on the niches occupied by amphibians. These species may have cardiac and physiological effects (COSTA et al., 2008; COSTA et al., 2015); histological and cellular (OLIVEIRA et al, 2016; PÉREZ-IGLESIAS et al., 2016; PÉREZ-IGLESIAS et al., 2019; ZANELLI et al., 2016;); biochemical (RICHARDS; KENDALL, 2002; VAN METER et al., 2019); and others. Despite the fact that large-scale use of agrochemicals in crops increases food production, about 2.7 kg/ha per year are used in crop production (ZHANG, 2018), and their residues either in crops or surrounding landscapes eventually reach toxic levels for aquatic and terrestrial organisms.

An alternative to minimizing the negative impact of these agrochemicals on non-target organisms is by using botanical insecticides, which are natural substances from plants with insecticidal properties, and considered effective and environmentally safer than their synthetic molecules, therefore reducing environmental contamination (ISMAN, 2008; McKENNA et al., 2013; SENTHIL-NATHAN, 2013; HIKAL et al., 2017). However, botanical insecticides are less stable in environmental conditions than synthetic pesticides, because those are photosensitive and rapid biodegradation (ISMAN, 2006). An example is a pyrethrum extract produced from flowers of chrysanthemum (*Chrysanthemum cinerariaefolium* and *Chrysanthemum cineum*; SCHLEIER; PETERSON, 2011) which is composed of pirethrin I and II, and jasmolin, it is lipophilic and photosensitive, and present toxicity to insects and aquatic organisms (COX, 2002; SCHLEIER; PETERSON, 2011; USEPA 2006). Nowadays, pyrethrum is the main botanical insecticide in use, representing three quarters of the global insecticide market (ISMAN, 2005).

Nanotechnology promotes the development of new compounds to be applied in crop protection, this can encapsulate actives and contribute to stabilizing botanical insecticides by providing modified release systems (KUMAR, 2000; RIEHEMANN et al., 2009; SILVA et al., 2012). Agricultural nanocarrier systems release active ingredients over time, act directly into the pest, and can reduce the amount of active and residues in the crops and environmental (CHEN; YADA, 2011), as well as improve the stability of the active compounds and protect them from degradation by light and temperature (BILIA et al. 2014). The use of nontoxic solvent, natural and biodegradable compounds in their formulation, help in the lowest toxicity (LU; OZCAN, 2015). Therefore, these systems are potentially more efficient, providing greater security for the environment and are safer for

non-target organisms (CHEN; YADA, 2011; GRILLO et al., 2014; KARERU et al., 2013; OLIVEIRA et al., 2015, SILVA et al., 2012). However, the legislation should be renewed and/or adapted to provide a more accurate risk assessment of nanomaterials (WALKER et al., 2017), as well as ensuring the development of a regulatory framework for nanopesticides (KOOKANA et al., 2014).

The nanocarriers are being used in several different applications by society, which increases the risk the environmental contamination and effects on biodiversity (BUNDSCHUH et al., 2018). Additionally, pyrethrins and their residues can be found in sediments, and in soil and water bodies (TANG et al., 2018). In this scenario, it is important to assess the potential risks, effects and toxicity of nanopesticides carrying pyrethrum. The nanotoxicological studies with pyrethrum extract encapsulated in solid lipid nanoparticles (SLN) on non-target animals (i.e. amphibian tadpoles) are important, because these can be exposed to this botanical insecticide and its carrier systems via runoff water.

The amphibians were chosen as the experimental models because their populations have been decreasing in recent years, mainly due to changes in their habitats and pollution generated by xenobiotics (BLAUSTEIN; KIESECKER, 2002; BEEBEE; GRIFFITHS, 2005; HAYES et al., 2010, WAKE; VREDENBURG, 2008). In the agricultural fields, chemical compounds should minimize their effects on non-target organisms, such as amphibians, and act selectively only on the target pest. The aims of this study were to analyze genotoxic and hematological biomarkers in order to provide a better understanding of the potential toxicological effects of nanopesticides in anuran tadpoles, providing new approaches for risk assessment in the environment for nanotechnological products

2. MATERIALS AND METHODS

2.1 Chemicals

The pyrethrum extract, Pestanal® (extract botanical insecticide, CAS 8003-34-7 - analytical standard), poly-vinyl alcohol (PVA, 30-70 kDa, CAS 9002-89-5 – hydrolyzed > 99%) and glyceryl tripalmitate (tripalmitin, CAS 555-44-2, purity ≥ 99%) was purchased from Sigma-Aldrich Chemical Co, and chloroform ($CHCl_3$, CAS 67-66-3 - purity ≥ 99%) was used for nanoparticles preparation. Acetone (CAS 67-64-1 - purity = 100%) was used as a solvent to prepare the pyrethrum solution.

122 *2.1.1 Solid Lipid Nanoparticles*

123 Solid lipid nanoparticles (SLN) containing pyrethrum extract were prepared
124 according to the emulsification/solvent evaporation method with some modifications
125 (VITORINO et al., 2011). Initially, 30 mL of aqueous phase was prepared containing
126 1.25% PVA and distilled water kept under magnetic stirring (100 rpm). In addition, the
127 organic phase was prepared with 250 mg of glyceryl tripalmitate and 5 mg of pyrethrum
128 extract (active ingredient – a.i.), which was dissolved in 5 mL of chloroform. The organic
129 phase was added to the aqueous phase and this mixture was sonicated for 5 min at 40 W
130 producing an emulsion. The emulsion was placed in an ULTRA-TURRAX™ homogenizer
131 at 14.000 rpm for 7 min. Next, the organic solvent was removed using a rotating
132 evaporator and the emulsion was concentrated up to 10 mL of the nanoparticles. The final
133 concentration of botanical insecticide was 0.05 $mg.mL^{-1}$. The solid lipid nanoparticles
134 (control) were also prepared without pyrethrum extract. The summary of the nanoparticle
135 characterization data is described in the supplementary material (Table 1S). Although
136 pyrethrum is photosensitive, once inside the nanoparticle it does not suffer degradation, as
137 proven by HPLC.
138
139 **2.2 Animal collection and acclimation in laboratory**
140 Newly hatched *Lithobates catesbeianus* (Shaw, 1802) tadpoles were acquired from
141 a frog farm in Santa Barbara d'Oeste, São Paulo State, Southeast Brazil (22°78'S,
142 47º40'W). In the laboratory, tadpoles were kept in 60 L aquariums equipped with
143 continuous aeration supply (1.2 $L.h^{-1}$) and dechlorinated water (> 6.0 mg $O_2.h^{-1}$), with a
144 constant temperature (25 ± 1 ^oC) and natural photoperiod (~12 h light: 12 h dark cycle)
145 during the acclimatization period (7 days). Animals were fed on organic spinach leaves,
146 which were withheld 48 h before the bioassay. The water was monitored daily to ensure
147 that the physical and chemical parameters (supplementary material – Table 2S) were kept
148 at acceptable levels similar to those found in most Brazilian inland waters (CONAMA
149 Resolution 357/2005, CETESB, 2009). During the acclimation and exposure periods,
150 ammonia measurements were performed daily. All procedures followed ASTM (2000)
151 guidelines. The experiments were previously approved by the University Ethics Committee
152 (Protocols nº 1472160516/2016 - CEUA/UFSCar), which follows Brazilian regulatory
153 laws.
154

155 **2.3 Determination of LC50**

156 The pyrethrum extract concentration used for ecotoxicological bioassays was
157 previously defined by determining LC50 (48 h) as there was no data providing information
158 about the effects of this pesticide for bullfrog tadpoles. The range of concentrations to
159 determine the LC50 was based on the concentration of permethrin, which is a synthetic
160 pyrethroid, used in a previous study with the same species (100 $\mu g.L^{-1}$) performed by
161 França et al. (2015).

162 Animals were tested in bottle systems according to the methodology developed by
163 Nunes et al. (2008). Each individual was placed into one bottle containing 1 liter of
164 dechlorinated water, in the absence of light. Thus, each tadpole represents a replica in the
165 test (N = 8 tadpoles per concentration) assayed in six pyrethrum-exposed groups: 130 $\mu g.L^{-1}$
166 , 170 $\mu g.L^{-1}$, 190 $\mu g.L^{-1}$, 220 $\mu g.L^{-1}$, 400 $\mu g.L^{-1}$, 580 $\mu g.L^{-1}$, and two control groups:
167 pyrethrum-free water and solvent control with acetone (580 $\mu g.L^{-1}$) diluted in water. The
168 LC50 of PYR was 400 $\mu g.L^{-1}$ (Supplementary material – Figure 3S). Ecotoxicological
169 bioassay was performed testing 1/5 of LC50 (80 $\mu g.L^{-1}$ concentration) for pyrethrum
170 extract, which was immediately prepared.

171

172 **2.4 Ecotoxicological experimental design**

173 One-hundred and forty-four tadpoles at Gosner (1960) developmental stage 25 were
174 randomly divided into six experimental groups assayed in triplicate: I) Control (CTL); II)
175 pyrethrum extract (PYR); III) Solid lipid nanoparticles (SLN); IV) pyrethrum extract
176 associated with solid lipid nanoparticles (SLN+PYR); V) Poly-vinyl alcohol (PVA); VI)
177 Acetone control (CTA).

178 Each experimental group had 24 individuals divided into three glass test aquariums
179 (N = 8 tadpoles per aquarium). Each test aquarium was filled with 8 L of well-aerated,
180 dechlorinated water (> 6.0 mg $O_2.L^{-1}$). During the bioassays, all glass test aquariums with
181 the animals were kept under controlled temperature (25 ± 1 °C), on a 12:12 h light: dark
182 cycle in the laboratory. To prevent external disturbances and photodegradation of tested
183 compounds all the aquariums were covered with black bags. Acute exposure to pyrethrum
184 extract and nanoparticles (NPs) was performed in a static system for 48 h.

185

186 **2.5 Experimental protocols**

187

2.5.1 Leukocyte and Erythrocyte measurements

At the end of bioassay, the tail vein was immersed in cold water (15 min) followed by anesthesia with lidocaine (50 $mg.g^{-1}$), 2 µL of blood were taken from each (N = 6 individuals from each experimental group). Blood samples were stored in heparinized microtubes containing saline solution (100 µL of formaldehyde citrate) for erythrocyte counting in a Neubauer chamber (number of cells x 2.5 x 10^3 mm^{-3}). The differential leukocyte count was performed using blood smear, approximately 10 µL were taken from each tadpole (N = 6 per group) by cardiac puncture, after was made fixation in pure cold methanol for 20 min (4°C) and then stained using the Instant Prov Kit. In addition, red blood cells (RBC) from 6 animals for each experimental group were counted by means of double blind count.

2.5.2 Erythrocytes nuclear abnormalities

An aliquot of 10 µL blood were taken from each tadpole (N = 6 per group) by cardiac puncture, was used to perform blood smear with a drop of blood for the micronuclei assay and the analysis of other erythrocytes nuclear abnormalities, following the procedures described by Lajmanovich et al. (2014) and Fenech (2000). Slides containing blood smear were immersed in a fixative solution, cold pure methanol (4 °C), for 20 min. Then, they were stained using the Rosenfeld method (1947), according to Ranzani-Paiva (2013). Afterwards, 1,000 cells were analyzed per animal in each experimental group (N = 6 individuals per experimental group).

2.5.3 Comet assay (Single cell gel electrophoresis assay)

Glass slides were previously prepared in agarose (1.5% in PBS pH 7.4, Ca^{2+} and Mg^{2+} free) and kept at room temperature. Tadpoles' blood was collected (10 µL per individual, N = 3 tadpoles per experimental group) and transferred to a microtube with 10 µL of PBS (phosphate buffer saline pH 7.4, Ca^{2+} and Mg^{2+} free). Thus, 10 µL of the diluted blood was mixed with 80 µL of the agarose low melting point (LMP; 0.5% in PBS pH 7.4, Ca^{2+} and Mg^{2+} free) and distributed on pre-prepared slides. These slides were immediately covered with a coverslip and refrigerated (4°C) for up to 20 minutes. Afterwards, the laminates were carefully removed so as not to disturb the material. Slides were immersed in a previously prepared lysis solution (NaCl 2.5 M, EDTA 100 mM, Tris 10 mM, N-lauroyl- sarcosine 1%) kept at 4°C for 1 hour. The slides were then washed 3 times in a buffer solution (PBS pH 7.4, Ca^{2+} and Mg^{2+} free) for 5 minutes each time, and

then they were submitted to the electrophoresis in a chamber with an alkaline buffer (NaOH 300 mM, 1 mM EDTA, pH > 13). Cell gel electrophoresis was carried out for 20 minutes, at 26 V and 300 mA. After this time, the slides were washed 3 times in a neutralization solution (Tris 0.4 M) for 5 minutes each one. The slides were kept overnight at room temperature. Staining was carried out by silver nitrate staining. To analyze the DNA damage, a photomicroscope (Leica Microscope- DM1000) was used, at 100x magnification, and classify the migration of DNA fragments form the nucleoid, visually resembling a comet tail. For statistical analyses, the number of cells with DNA tail were counted per experimental group and, additionally, the tail length was used for classify the damage class (class 0, 1, 2, 3 and 4) according to Tice et al. (2000) and Gedik et al. (1992). The obtained data were normalized for 50 cells in order to get a percentage of cells for the DNA damage classes per experimental group.

2.6 Statistical analysis

For statistical analysis, data were previously submitted to homogeneity of variances (Bartlett's test) and normality tests (Shapiro-Wilk and Kolmogorov-Smirnov tests). A one-way ANOVA test was applied followed by the parametric Tukey-Kramer multiple comparison test to compare the results obtained for different experimental groups. The Kruskal-Wallis test was performed for non-parametric data, followed by the Dunn's multiple comparison test. The significance level was 0.05. The program used for statistical analysis was the GraphPad Prism, version 5.0.

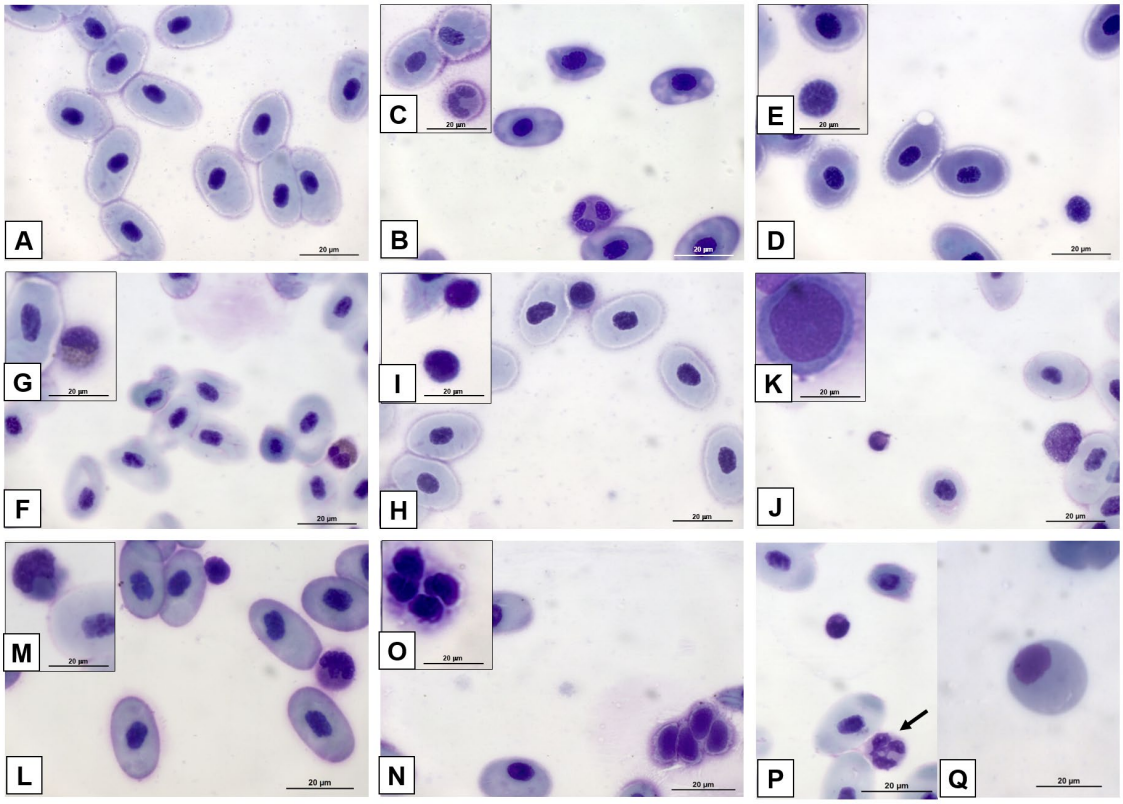
3. RESULTS AND DISCUSSION

3.1 Leukocyte and Erythrocyte measurements

Bullfrog tadpoles blood contains erythrocytes (RBC), leukocytes (WBC) and thrombocytes (platelets). The erythrocytes are nucleated in amphibian species and have an elliptic, flat and biconvex format; the leukocytes are divided into lymphocytes, monocytes, neutrophils, basophils and eosinophils (Figure 1).

Lymphocytes and monocytes are considered agranulocytes. Lymphocytes can be either small (Figure 1H-I) or large and their nucleus occupies the largest portion of the cytoplasm (Figure 1J-K). On the contrary, the nucleus of monocytes occupies the smallest area of the cytoplasm and can have different forms (Figure 1M-L); the rineform is the most frequent. Neutrophils, eosinophils and basophils are considered granulocytes. Neutrophils

256 have lobed nucleus and few visible granules in cytoplasm (Figure 1B-C); eosinophils have
257 both larger and more visible nucleus and cytoplasmic granules (Figure 1F-G); basophils
258 have small nuclei visible due to the large amount of cytoplasmic granules. (Figure 1D-E).
259 Thrombocytes are oval-shaped and aggregated cells, this were rarely found (Figure 1N-O)
260 and, for this reason, they were not counted nor included in the discussion. Non-terminally
261 differentiated cells, heterophils (immature neutrophils) and immature erythrocytes were
262 also found in smears (Figure 1P-Q), but not included in the count.



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264 Figure 1 – Blood cells of bullfrog tadpoles (*Lithobates catesbeianus)*. A) Normal
265 erythrocytes; B e C) Neutrophils; D-E) Basophils; F-G) Eosinophils; I-H) Small
266 lymphocytes; K-J) Large lymphocytes; M-L) Monocytes; O-N) Thrombocytes; P)
267 Heterophil (arrow) and Q) Immature erythrocytes Color: Instant-Prov Kit. Bars: 20 µm.
268

269 These cell types were already reported for amphibians in other studies (CLAVER;
270 QUAGLIA, 2009; ARIKAN; ÇIÇEK, 2014). Concerning the hematological biomarkers
271 evaluated in the present study, similar characteristics of blood cells were observed by
272 Heatley et al. (2009) in the Malaysian frog (*Megophrys nasuta*) and in the various species
273 of Turkish Herpetofauna (ARIKAN; ÇIÇEK, 2010). Variations in the shape of RBC
274 indicate that bullfrog tadpoles are undergoing a critical condition (DAS; MAHAPATRA,
275 2012).

For all hematological and genotoxic parameters, there was no significant difference between the controls (CTL) with solvent control (CTA, acetone) and polyvinyl alcohol (PVA, surfactant control), except in the comet assay, which had a significant difference between the PVA group and the CTL group.

3.1.1 Leukocytes

The number of lymphocytes (P = 0,0339); monocytes (P = 0,0001) and neutrophils (P = 0,0405) of the exposed groups was similar and had no significant difference among these groups. Eosinophils had a significant difference between nanoparticles groups and CTL (0,5 ± 0,1508, Mean ± SE; P = 0,0004) showing an increase in the SLN group (2,75 ± 0,4106, Mean ± SE) and SLN+PYR (2,35 ± 0,4144, Mean ± SE) compared to control. Basophils had a significant difference between PYR (16,58 ± 0,9, Mean ± SE) and CTL (9,167 ± 0,7671, Mean ± SE, P = 0,0001) showing an increase in the exposed group (Figure 2).

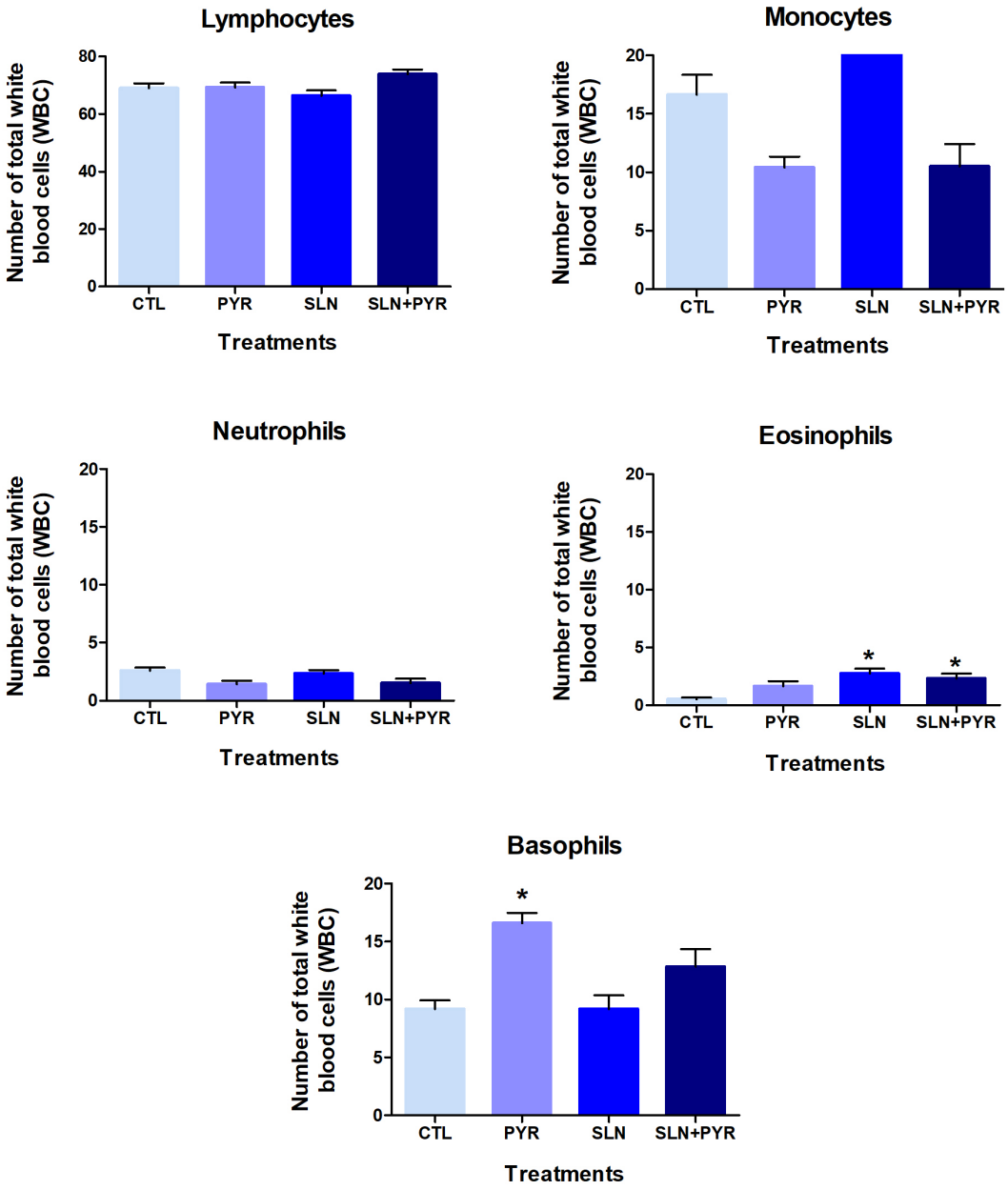


Figure 2 – Number of white blood cells of bullfrog tadpoles (*Lithobates catesbeianus*) from each experimental group. CTL: control, PYR: pyrethrum extract, SLN: solid lipid nanoparticles, SLN+PYR: nanoparticles associated to pyrethrum. The asterisks above horizontal bars denote a significant difference in relation to the CTL. Kruskal Wallis One-way ANOVA, followed by the Dunn's multiple comparison test. Note the differences in the scales of the graphics.

Lymphocytes were the most frequent type of leukocytes observed at the present study that and it is considered standard for amphibians (ROUF, 1969). In addition, eosinophils were more frequent in the SLN and SLN-PYR exposed groups than the other

303 ones, and basophils were more frequent in the PYR-exposed group than the other
304 experimental groups.
305 Solid lipid nanoparticles are produced with natural products such as solid lipids and
306 surfactants. It should also be mentioned that the lipid external layer of nanoparticles keep
307 in its solid phase at room temperature (BATTAGLIA et al., 2014), probably it keeps this
308 property even inside organisms when absorbed for them. These nanoparticles are
309 considered to have an excellent physical stability, spherical format, good release profile of
310 active ingredient, and to be a non-toxic carrier system (NASERI et al., 2015). In fact,
311 studies indicate that the exposure to xenobiotics or environmental stressors (CHEN,
312 ROBERT, 2011) and solid lipid nanoparticles (WINTER et al., 2016) can activate animals'
313 immune systems. Additionally, the physicochemical proprieties of nanoparticles can
314 stimulate and/or suppress the immune responses (DOBROVOLSKAIA, 2007; ZOLNIK et
315 al., 2010), and the SLN enter de body can induce inflammatory processes (KONONENKO
316 et al., 2015) and cell death in organisms (WINTER et al, 2016).
317 Eosinophils are effector multifunctional cells that migrate toward several mediators
318 released at parasitic infections, inflammatory sites and allergy (LUNA-GOMES et al.,
319 2013, DAVOINE; LACY, 2014). These immunomodulatory cells participate both in innate
320 and adaptive immune response via expression of various receptors and secretion of a
321 variety of mediators (HOGAN et al., 2008, BREEDVELD et al., 2017). SLN induced
322 hematological effects in Swiss albino mice, which probably leads to inflammatory
323 response (WINTER et al., 2016). In the present study, SLNs-exposed groups probably
324 triggered an inflammatory response, activating the eosinophil recruitment and activation in
325 the test-organism. Based on study performed by Oliver et al. (2000) demonstrating that
326 solid lipid nanoparticles interact with macrophage-like cells, we suggest that this here-
327 obtained response occurred because the SLP interacted with macrophage present in
328 circulating blood, after their absorption by the tadpoles. Macrophages produce cytokines as
329 Interleukin-8 (DUQUE; DESCOTEAUX, 2014), which in turn is a mediator of eosinophil
330 chemotaxis (ERGER; CASALE, 1995). Similarly, another type of organic nanoparticle
331 associated to the herbicide clomazone also resulted in eosinophil activation in exposed
332 bullfrog tadpoles (OLIVEIRA et al., 2016).
333 Basophils were more frequent in the PYR-exposed group. Basophils play a role in
334 allergic processes and inflammatory disorders (BREEDVELD et al., 2017). Probably, the
335 pyrethrum extracted from a botanical source, which has high lipophilia, could be interact
336 with amphipathic molecules present in tadpole’s blood. Thus, this interaction is recognized

by the organism as a foreign substance triggering an immune response that induced the increase of basophils number in the blood of tadpoles. A higher number of granulocytes was already correlated with increased environmental stress in vertebrates (AGIUS; ROBERTS, 2003). Basophilia has been described in different species green frogs living in the urban environment near to the industrial and it could be regarded as specific forms of antitoxic responses, according the study performed by Romanova and Egorikhina (2006).

Another possibility for increase basophils in the PYR-exposed group is the interaction of pyrethrins, which compose the pyrethrum extract, with some serum blood protein of tadpole, so that this conjugate (pesticide plus protein) could plays a role of antigen and, consequently, antigenic molecules can be recognized and has the potential to active the immune system (CHIPINDA et al., 2011). This hypothesis was based on capacity of pyrethroids bind to a protein (hapten) and, consequently, to raise antibodies against the conjugate (protein plus pesticide), triggering an immune response. This mechanism is the basic principle of the immunoassays for measuring the residues of pyrethroids in environmental samples (AHN et al., 2011). Antigens can interact with IgE antibodies present in the surface membrane of basophils and triggers IgE-dependent allergic inflammation (MIYAKE; KARASUYAMA, 2017).

3.1.2 Erythrocytes

Figure 3 shows an increase in the erythrocytes in SLN+PYR (140833 ± 19284, Mean ± SE) in comparison to other experimental groups CTL (86667 ± 8115, Mean ± SE), PYR (47273 ± 5997, Mean ± SE) and SLN (46818 ± 7863, Mean ± SE). There were no significant differences between the exposed groups (P = 0.0001).

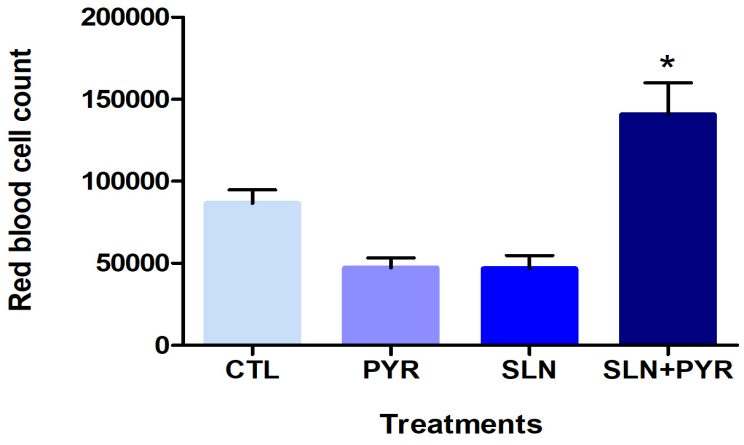


Figure 3 – Number of red blood cells of bullfrog tadpoles (*Lithobates catesbeianus*). CTL: control, PYR: pyrethrum extract, SLN: solid lipid nanoparticles, SLN+PYR: nanoparticles associated to pyrethrum. The asterisk above horizontal bars denotes a significant difference between SLN+PYR and the CTL. One-way ANOVA, followed by the Tukey's multiple comparison test.

The increase in the number of erythrocytes or other parameters in RBC can also be altered in organisms exposed to xenobiotics. Erythropoiesis is directly stimulated in tadpoles and adults when exposed to xenobiotics (BARNI et al., 2007). Although there are no studies with amphibians so far, Raheem (2018) described that rabbit immunized with AgNPs trigger in erythrocytose and Winter et al. (2016) observed hemolysis lower than 5% in mice submitted to intravenal application of several solid lipid nanoparticles. Thus, here is not discarded the possibility of hemolysis that could be proved at future studies, which potentially could induce erythropoiesis in tadpoles in order to compensate this adverse effect. This explains the mitosis in erythroid cells observed in this study (Figure 4J-K). In *Lithobates catesbeianus* all life stages naturally present mitotic cell in circulating blood (MANIATIS; INGRAM, 1971).

Physiologically, the increase in RBC frequency in SLN+PYR group could intensify the oxygen supply of the organisms that were exposed to this xenobiotic, consequently enhancing the oxygen transport capacity (GATTEN; BROOKS, 1969). The increase in red blood cells and oxygen supply may be the indirect mechanism of increased ATP (*adenosine* triphosphate) production for xenobiotics's detoxification (BARNI et al.; 2007).

3.2 Erythrocytes nuclear abnormalities

Erythrocytes are commonly nucleated and their nuclei have an elliptic or circular shape, but a decentralized nucleus with a circular shape can also be found (Figure 4E). Erythrocytes with nuclear abnormalities (ENA) in bullfrog tadpoles are shown in Figure 4. Rineform and lobed shape nucleus were also found in erythrocytes (Figure 4E, 4F and 4I). Pycnotic cells (Figure 4H) were rarely found, but mitosis cell division was frequently observed (Figure 4J-K).

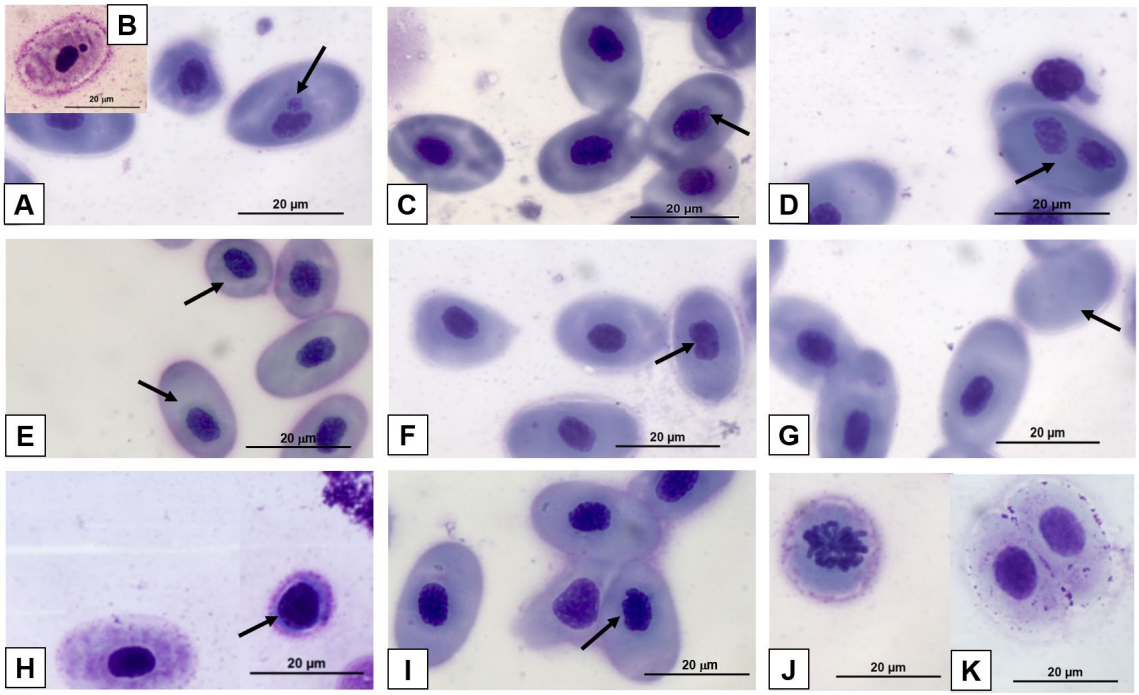
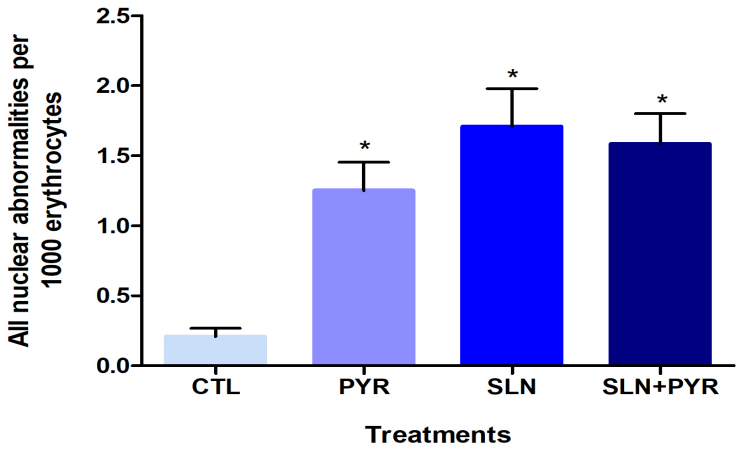


Figure 4 – Red blood cells characterization of bullfrog tadpoles (*Lithobates catesbeianus)* A and B) Micronuclei; C) Bud nucleus; D) Binucleate cell; E) Espherical Cell and decentralized nuclei; F) Rineform nucleus; G) Anucleated cell; H) Pycnotic cell; I) Lobed nucleus; J-K) Mitotic cell division. Color: A, C-K) Instant-Prov Kit and B) Rosenfeld method. Bars: 20 µm.

The number of erythrocyte nuclear abnormalities (ENA) increased in all the exposed groups (PYR, SLN and SLN+PYR) in relation to the control (0,2083 ± 0,05924, Mean ± SE, P = 0.0001; Figure 5). ENA were more frequent in SLN (1,708 ± 0,2712, Mean ± SE), SNL+PYR (1,583 ± 0,2185, Mean ± SE) and PYR (1,250 ± 0,2030, Mean ± SE) groups than in the control group. These abnormalities are considered genotoxic damage in most studies due to problems in cell division process (SERRANO-GARCIA; MONTERO-MONTOYA, 2001). The increase in micronuclei frequency observed for bullfrog tadpoles can be related to pre-metamorphic phase of tadpoles, and/or to a rate of mitosis in erythrocytes, and/or the xenobiotic exposure level (BARNI et al., 2007). Thus, ENA are indicative of the cellular response from the organism, which results in the removal of cells damaged by the xenobiotics.



411

412 Figure 5 – Erythrocytes nuclear abnormalities found in the red blood cells of bullfrog
413 tadpoles (*Lithobates catesbeianus*) from the experimental groups. CTL: control, PYR:
414 pyrethrum extract, SLN: solid lipid nanoparticles, SLN+PYR: nanoparticles associated to
415 pyrethrum. The asterisks above horizontal bars denote a significant difference in relation to
416 the control. Kruskal Wallis One-way ANOVA, followed by the Dunn's multiple
417 comparison test.
418

419 To the best of our knowledge, there are no studies evaluating the effect of solid
420 lipid nanoparticles in amphibians, however the increase of ENA in the exposed-groups was
421 also observed by Vignardi et al. (2015) in the marine fish, *Trachinotus carolinus*, when
422 exposed to titanium dioxide nanoparticles. The genotoxicity of nanoparticles was observed
423 by Chen and Von Mikecz (2005) using fluorochrome-labeled SiO_2 nanoparticles in
424 epithelial cell culture and showed that these nanoparticles enter inside the cell nucleus. The
425 genotoxicity of pyrethroids demonstrated by the increased occurrence of micronuclei was
426 observed in the freshwater fish, *Channa punctatus*, exposed to cypermethrin (ANSARI et
427 al., 2011) and by Campana et al. (1999) for other species of fish (*Cheirodon interruptus*
428 *interrupus*) exposed to lambda-cyhalothrin.

429 Hematological parameters are accurate biomarkers that measure stress and
430 physiological disturbances in aquatic organisms exposed to nanoparticles (SHALUEI et al.,
431 2013). SLNs prepared with tripalmitin (lipid) and polysorbate or lecithin (surfactants) did
432 not result in hemolytic effects nor in the disturbance of the erythrocyte membranes
433 (PIZZOL et al., 2014). In the present study with tadpoles, SLNs did not result in hemolytic
434 alterations in the RBCs, thus, it is important to mention that SLSs were prepared with
435 organic compounds, which decreases the toxicity and has better bioavailability and
436 stability compared to other NPs (SURENDER; DEEPIKA, 2016). However, the pyrethrum
437 and nanoparticles triggered ENA in tadpoles, showing genotoxic damage. Toxicity and

438 biological interaction of nanoparticles are influenced by several factors, such as size,
439 shape, composition, aggregation, surface area and organism species (ALBANESE et al.,
440 2012; GATOO et al., 2014; SHARIFIA et al., 2012), being important to evaluate these
441 properties for better determination of toxicity.

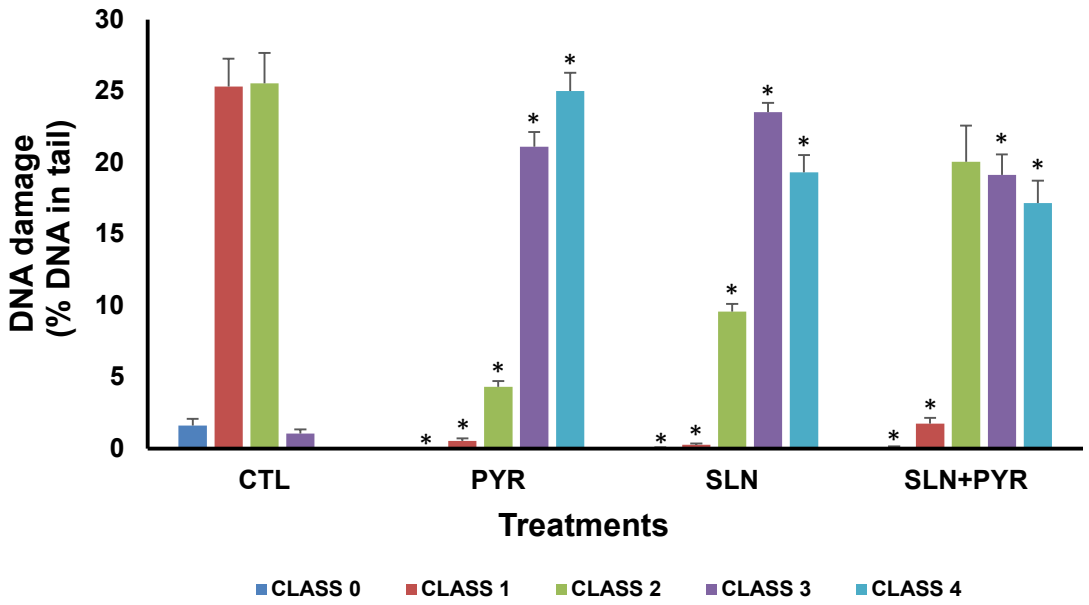
442 The types of erythrocyte nuclear abnormalities (Figure 5) that we found were also
443 described by Das and Mahapatra (2014) for adults of tree frog (*Polypedates teraiensis*) and
444 by Lajmanovich et al. (2014) for *Rhinella arenarum* (common toad) tadpoles. Cytogenetic
445 and genotoxic effects can be analyzed by the micronucleus test, which is widely used for
446 detecting clastogenic (chromosome breakage) or aneugenic (chromosome loss) damages
447 induced by the chemical or physical agents and, therefore, can be used for environmental
448 monitoring (SERRANO-GARCIA; MONTERO-MONTOYA, 2001; POLLO et al., 2015;
449 UDROIU, 2006).

450

451 **3.3 Comet assay**

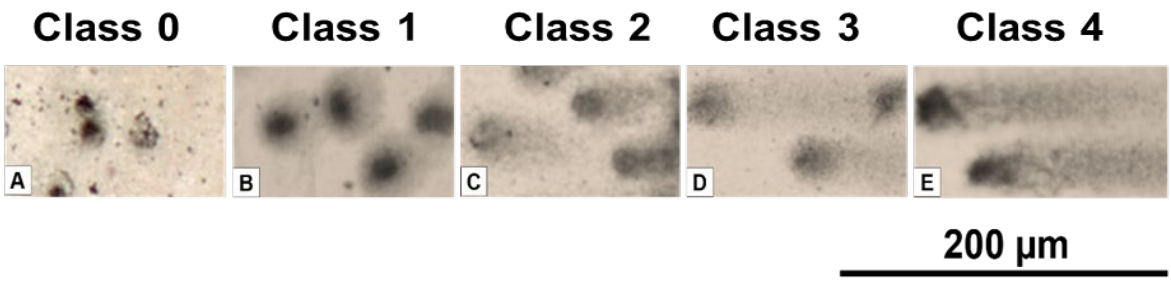
452 Comet assay or single cell gel electrophoresis assay (SCGE) showed an increase in
453 the damage of class 3 (P = 0.0001) and 4 (P = 0.0001) for exposed groups (PYR, SLN and
454 SLN+PYR) in comparison with the control group. However, no differences between the
455 exposed groups were observed (Figure 6).

456 The most frequent damage classes in the control group were class 1 (P = 0.0001)
457 and class 2 (P = 0.0001), cases with no damage were also frequent (P = 0.0001); but for
458 class 2, no significant difference was observed between CTL and SLN+PYR. On the other
459 hand, the most frequent damage classes in the exposed groups were classes 3 and 4, and
460 the PYR group had the most class 4 damage, and the SLN group had the most class 3
461 damage (Figure 7).



462

463 Figure 6 - Comet assay and DNA damage in the bullfrog tadpoles (*Lithobates*
464 *catesbeianus)* of the experimental groups showed significant differences in the group of the
465 pyrethrum extract, nanoparticles and pyrethrum nanoparticles (*) with the control group.
466 CTL: control, PYR: pyrethrum extract, SLN: solid lipid nanoparticles, SLN+PYR:
467 nanoparticles associated to pyrethrum. Statistical analysis represents the number of cells
468 with DNA tail length. Kruskal Wallis One-way ANOVA, followed by the Dunn's multiple
469 comparison test (Mean ± SE).
470



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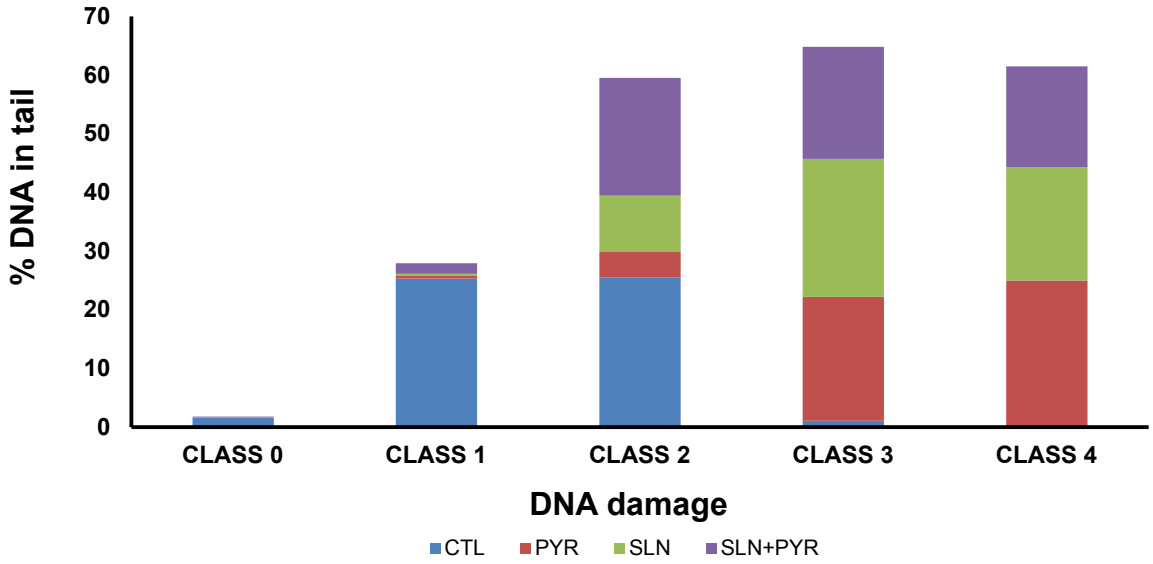

Figure 7 - Comet assay and DNA damage in the bullfrog tadpoles (*Lithobates catesbeianus)* of the experimental groups. Graphical analysis represents the number of cells with DNA tail and the classification of the DNA damage. CTL: control, PYR: pyrethrum extract, SLN: solid lipid nanoparticles, SLN+PYR: nanoparticles associated to pyrethrum. A) Class 0; B) Class 1; C) Class 2; D) Class 3 and E) Class 4.

Comet assay has been used in various species, different tissues and isolated cells to evaluate xenobiotic exposures and to understand some diseases (SINGH, 2016). Moreover, single strand-breaks in DNA were expected as an effect of nanoparticles (KARLSSON, 2010; KAIN et al., 2012; RAHMAN et al., 2017), as well as for pyrethrum extract (CABAGNA et al., 2006) and for nanoencapsulated pyrethrum (SLN+PYR) treatments. Nanoparticles associated to pyrethrum had a lower genotoxic and mutagenic effect in the comet assay when compared to the other groups – SLN and PYR, these results show that the botanical insecticide, once encapsulated, becomes less toxic to the organism than in its free form.

An effect that was not expected was the damage observed in the PVA treatment group, i.e., the surfactant used in SLN preparation, therefore there is no report about its toxicity (DEMERLIS; SCHONEKER, 2003; KELLY et al., 2003; MENON et al., 2012). However, the values observed for PVA and SLN treatments were not different, thus, the toxic effect of SLN may also be induced by an additional effect of PVA, as the PVA is a component in its synthesis, as a dispersive agent (MAHMOUDI et al., 2009). De according Scholer et al. (2002), the lipid matrix with your constituintes or the concentration of active in nanoparticle, can determine the citotoxicity in organisms

Nanoparticles and botanical insecticide cause mutagenicity in the erythrocytes of bullfrog tadpoles (*L. castesbeianus*), causing damage during cell division that result in the formation of micronuclei and other erythrocyte nuclear abnormalities and DNA damage. However, it has been shown that TiO_2–NP have effects on the erythrocyte DNA of the marine fish (*T. carolinus*), causing a significant increase at the tail lengths and at % of Tail DNA of the comets of all groups exposed to nanoparticles (VIGNARDI et al., 2015). Mutagenic and genotoxic damage induced in exposed groups can potentially lead to necrosis (MAHAYE et al., 2017), which leads to an inflammatory response potentially mediated by eosinophils (BREEDVELD et al., 2017), which were recruited by proinflammatory cytokines from macrophages (CHANG, 2010) after the probable nanoparticle endocytosis (OLIVER et al., 2000).

In this context, Doktorovova et al. (2014) highlighted the relevance of further studies to understand the possible genotoxic action of SLN, mainly with *in vivo* assays and ecotoxicology analysis in order to verify the safety of nanoparticles in the environment. The micronucleus test and evaluation of other nuclear abnormalities, as well as the comet assay, showed to be sensitive and accurate biomarkers of early effects induced by the SLN and botanical insecticide on bullfrog tadpoles.

Indeed, it has been demonstrated that bullfrogs are particularly sensitive to environmental pollution, being therefore, used as bioindicators (PARMAR et al., 2016; SIMON et al., 2011; VENTURINO et al., 2003), as well as, hematological analysis and changes in blood parameters are used in some species and are representatives in the study of contamination in aquatic environments (CABAGNA et al., 2005; SHALUEI et al., 2013; ZHELEV et al., 2015). Therefore, our results indicate that these biomarkers can be used in the biomonitoring and assessment of contaminated areas in the vicinity of the amphibian habitat.

4. CONCLUSION

Bullfrog tadpoles are suitable model organisms in the toxicity study of both nanopesticides and botanical insecticide. Hematological parameters, genotoxic and mutagenic analyses were accurate biomarkers of acute toxicological effects to evaluate the tadpole's exposure to SLN and pyrethrum extract, either isolated or encapsulated.

This work is pioneer in the analysis of the effects of solid lipid nanoparticles on anuran amphibians, and it brings novel findings regarding botanical insecticide and solid lipid nanoparticles toxicity, which will and contributing to regulatory frameworks and

future legislation. Finally, it is important to study both the safety and efficiency of SLNs in non-target aquatic organisms.

CONFLICT OF INTEREST

The authors declare there are no conflicts of interest in the present study.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIALS

Table 1S – Characterization of SLN and SLN+PYR in function of time (mean of 0 – 120 days).

PARAMETERS	SLN	SLN+PYR
z-average Diameter (nm)	324	258
Polidispersity index (PdI)	0,19	0,11
Zeta Potential (mV)	-11	-10
pH	5,3	5,6
Encapsulate efficiency (%)	-	*≥ 99*

Fonte: The author, 2017.

Table 2S - Physical-chemical parameters of the water (Means ± 1 E.P.M.) of the bioassay with bullfrog tadpoles (*Lithobates catesbeianus*) in the different experimental groups.

Groups	Temperature °C	PH	Total Hardness (mg/L^{-1} $CaCO_3$)	CO_2	Ammonia
CONTROL	19,9 ± 0,1	7,5 ± 0	50 - 150[1]	4,7 ± 0,6	0,6 ± 0,2
PYRETHRUM	19,9 ± 0,1	7,5 ± 0,1	50 - 150	3,4 ± 0,9	0,6 ± 0,2
SLN[2]	19,8 ± 0,1	7,6 ± 0	50 - 150	2,8 ± 0,5	0,5 ± 0,1
SLN+PYR[3]	19,7 ± 0,1	7,5 ± 0	50 - 150	3,4 ± 1,5	0,5 ± 0,2
ACETONE	19,9 ± 0,1	7,6 ± 0	50 - 150	2,9 ± 0,6	0,5 ± 0,1
PVA[4]	19,8 ± 0,1	7,5 ± 0	50 - 150	3,5 ± 0,6	0,5 ± 0,1

Legend: [1]Evidencing the 50-150 soft water; [2]SLN - Solid Lipid Nanoparticle; [3]SLN+PIR - Solid Lipid Nanoparticle associated to pyrethrum; [4]PVA - Poly-vinyl alcohol.

Fonte: The author, 2017.

Figure 3S – Determination of concentration to pyrethrum botanical insecticide after 48h exposure.