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Cytotoxicity, hemolysis and *in vivo* acute toxicity of 2-hydroxy-3-anilino-1,4-naphthoquinone derivatives

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ABSTRACT

The 1,4-naphthoquinones, important members of the family of quinones are used as both crude extracts and as compound manipulated by the pharmaceutical industry. They have gained great emphasis by presenting different pharmacological properties as antibacterial, antiviral, antiprotozoal and anthelmintic, and has antitumor activity. Our aim was to evaluate the cytotoxicity, hemolytic activity and *in vivo* acute toxicity of three derivatives of 2-hydroxy-1,4-naphthoquinones. The cell viability *in vitro* against RAW Cell Line displayed IC_{50} ranging of 483.5–2044.8 μM, whereas in primary culture tests using murine macrophages, IC_{50} were 315.8–1408.0 μM for naphthoquinones derivatives **4a** and **4c** respectively, besides no hemolysis was observed at the dose tested. The *in vivo* acute toxicity assays exhibited a significant safety margin indicated by a lack of systemic and behavioral toxicity up to 300 mg/kg, and at a dose of 1000 mg/kg the derivatives not triggering signs of toxicity although the compound **4a** have promoted hepatic steatosis and hyperemia in kidney tissue. Thereby, these modifications decrease the toxicity of the tested derivatives naphthoquinones, providing a high potential for the development of news drugs.

1. Introduction

The 1,4-naphthoquinones are widely distributed in nature and may be used as crude extracts or as compounds manipulated by the pharmaceutical industry [18,17]. These compounds have diverse pharmacological properties such as antimicrobial [46,49], antiviral [8], antiprotozoals [32,33,35,45,10] and anthelmintic [19], besides of antitumoral activity [37].

There are also already naphthoquinones reports against *P. falciparum in vitro* [24,15,19,12,44] and *in vivo* [35]; and the antiplasmodial activity of benzo [a] phenazine synthesized from 1,2-naphthoquinone and beta-lapachone lapachol [3].

The atovaquone, a hydroxy-naphthoquinone which inhibits the electron transport chain and pyrimidine biosynthesis in *Plasmodium falciparum* [48], it was used as a prophylactic agent for travelers [40]. It causes few side effects, the most common symptoms are rash, fever, vomiting, diarrhea, abdominal pain, headache and, occasionally, transaminase and amylase levels are abnormal [2].

Despite several synthetic naphthoquinone derivatives, such as the 2-acetoxy and 2-ethoxy-1,4-variants widely used in industry, the mechanisms involved in cytotoxicity of the quinone derivatives are still largely unknown [47]. The cytotoxic effects of naphthoquinoidal compounds such as menadione might be due to oxidative stress and arylation of cellular thiols [39]. Hepatic biotransformation of napththoquinone derivates can lead to cell injury through several mechanisms unknown yet [14]. Studies shown that the 2-hydroxy-1,4-naphthoquinone is a substance highly toxic, causing both hemolytic anaemia and renal tubular necrosis in rodent [26] and non-human primates models [22]

Previous studies by our group demonstrated antimalarial activity in three novel naphthoquinoidal derivatives against rodent malaria caused by *P. berghei* infected erythrocytes in mice [35]. According to Resolution CNS 251/97, pre-clinical research is the first step in the study on the development of new drugs and should promote sufficient information as the possible therapeutic applications and preview some risks with its use as toxicity to support the conduct of research in humans [20]. Thus, in this study, we evalu-

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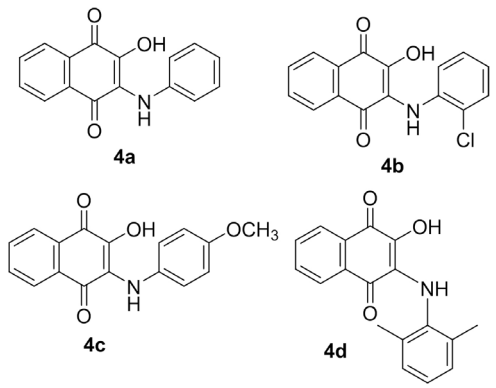

Fig. 1. Chemical structure of the derivatives of 2-hydroxy-3-anilino-1,4-naphthoquinone. 2-hydroxy-3-anilino-1,4-naphthoquinone (**4a**), 2- hydroxy-3-(2-chloro-aniline)-1,4-naphthoquinone (**4b**), 2-hydroxy-3-(4-methoxy-aniline)-1,4-naphthoquinone (**4c**) and 2-hydroxy-3-(2,6-dimethyl-aniline)-1,4-naphthoquinone (**4d**).

ated the toxic potential of three derivatives naphthoquinones using models in vitro and in vivo.

2. Materials and methods

2.1. Compounds

The tested 2-hydroxy-1,4-naphthoquinones derivatives were synthesized according previously report [35], which are: 2-hydroxy-3-anilino-1,4-naphthoquinone, 2-hydroxy-3-(2-chloro-aniline)-1,4-naphthoquinone, 2-hydroxy-3-(4-methoxy-aniline)-1,4-naphthoquinone and 2-hydroxy-3-(2,6-dimethyl-aniline)-1,4-naphthoquinone, designated **4a**–**4d**, respectively. (Fig. 1). The compound **4b** showed problems with solubility thereby preventing the continuity of its use in this study. Compounds **4a, 4c** and **4d** were selected for the tests to be soluble in the solution with a maximum of 1% DMSO and have satisfactory results in the screening tests showing antimalarial activity when tested in a murine model-*Plasmodium berguei* (Table 1).

2.2. Cytotoxicity assay

Cell viability was assessed by colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] assay, as described by Mosmann [23], with modifications. The cells used were murine macrophages obtained by washing the peritoneal cavity from Swiss mice and Cell Line RAW 264.7. The compounds to be tested were diluted with 0.1% DMSO in culture medium in serial dilution to obtain seven concentrations ranging 1000–15.6 μg/mL (1:2; 7× dilution), corresponding to 3773–58.9 μM for the **4a** derivative; 3390–53.0 μM for derivative **4c** and 3413–53.3 μM for **4d**. For tests, RAW 264.7 line cells (1×10^4 cells per well) and macrophages from primary culture cells (1×10^6 cells per well) were distributed into 96 well microplates. After 48 h incubation at 37 °C, 20 μl of MTT solution (5 mg/mL) were added to each well and, after 3 h, the supernatant was removed and 200 μl of DMSO were added. The optical densities of the plates were read by a microplate spectrophotometer (Thermo Plate ®) at 570 nm. The IC_{50} for the primary cell culture and RAW cells was determined using GraphPad PRISM software ®. The test was performed in three repeats.

2.3. Hemolysis assay

The test was performed as suggested by Rabelo et al. [34]. Compounds were tested at two concentrations each: **4a** (100 and 500 mM), **4c** (50 and 250 mM), and **4d** (200 and 1000 mM). References to 100% and 0% hemolysis were made by incubating a suspension of red cells with Triton X-100 1% (v/v) and 0.9% saline, respectively. The values tested were experimentally determined from IC_{50} obtained in a test antiplasmodial, corresponding to 10 and 50 times more than the respective IC_{50}. The hemolysis percentage was calculated on the positive control group. Assays were performed in triplicates and repeated twice.

2.4. Acute toxicity

The acute toxicity was performed according protocol OECD [30]. Groups of three female Swiss mice of 8–12 weeks weighing around 29.0 ± 2 g were randomly separated into the following groups: group I, **4a** compound; group II, **4c** compound; group III **4d** compound and group IV, untreated control. The compounds were diluted in DMSO (maximum final concentration of 4%) and administered orally (*per gavage*), 200 μl single dose. The negative control group receiving DMSO 4%.

As recommended, the initial dose tested was 300 mg/kg body weight, dose for starting the test if there is no information on the toxicity of the compound to be tested. As was observed no mortality or toxicity signs, the test was repeated with a higher dose (1000 mg/kg body weight). Body weight of the animals was checked on the day of administration of the compounds and on the 15th day after administration, and the weight variation was calculated.

After treatment, the animals were observed dialy for 14 days. The signs observed were piloerection, changes in the eyes and mucous membranes; physical changes associated with central nervous, autonomic, cardiovascular and respiratory systems; pattern of behavior and somatomotor activity, direct attention to tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma. After 14 days, the animals were autopsied for some hispathological analysis. For dose 1000 mg/kg, the absence or presence of inflammation and its activity were determined in livers, spleen and kidneys of each study group. The specimens were fixed in 10% formaldehyde, dehydrated and embedded in paraffin. Sections of 5 μm thickness were obtained for haematoxylin-eosin staining (H&E Easypath) and examined by light microscopy (40×, Olympus BX50); additionaly, the tissues were stained with periodic acid Schiff (PAS) to indirectly to demonstrate area affected by steatosis due to dye has an affinity for glycogen in the liver sections and not by lipids. Furthermore, the presence of degenerative lesions were examined:

Table 1
Antimalarial activity of three novel naphthoquinoidal derivatives measured by reduction of parasitaemia by *Plasmodium berghei* and survival of mice treated as compared to untreated mice control.

Compound	Parasitemia		Parasitemia inhibition (%)		Mean Survival (Days)
	Day 5	Day 5	Day 5	Day 7	
4a	0.7	2.2	53.3	18.5	32.5 ± 6.6
4c	0.5	2.0	66.6	25.9	26.0 ± 4.0
4d	1.5	2,7	0	0	26.7 ± 2.5
Vehicle (control)	1.5	2.7	0	0	20 ± 2.7

Compounds previously tested; adapted from [35].

Table 2
IC_{50} (μM) of RAW 264.7 cells and murine resident peritoneal macrophages (RPMϕ) exposured to concentration serial dilution of naphthoquinones derivatives.

Compounds	RAW 264.7Cells	RPMϕ[a]
2-hydroxy-3-anilino-1,4-naphthoquinone (**4a**)	483,5 ± 195.40	315,8 ± 31.30
2-hydroxy-3-(4-methoxy-aniline)-1,4-naphthoquinone (**4c**)	714,9 ± 182.95	532,6 ± 103.24
2-hydroxy-3-(2,6-dimethyl-aniline)-1,4-naphthoquinone (**4d**)	2044,8 ± 93.99	1408,0 ± 52.49

Results show the mean of IC_{50} values ± SD (concentration required to inhibit cell growth by 50%) in micromolar. Data represent the means of three independent experiments, with each concentration tested in triplicate.
[a] Resident peritoneal macrophages.

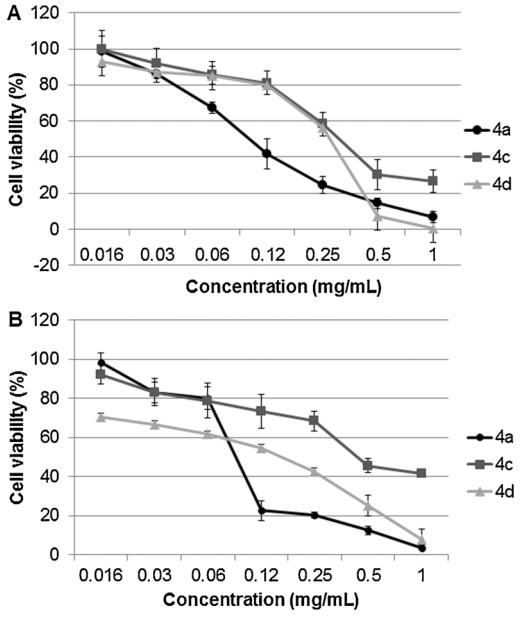

Fig. 2. Dose–response curves of RAW 264.7 cells (A) and Resident peritoneal macrophages (B), after treatment for 24 h with 2-hydroxy-1,4-naphthoquinones derivatives. The concentrations ranging between 0.016 to 1,0 mg/mL. Results are expressed in percentage of control. Three independent experiments are performed in triplicate.

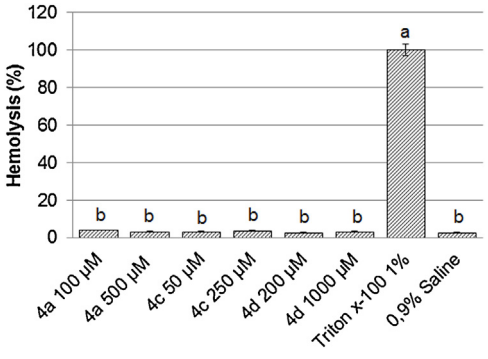

Fig. 3. Percent hemolysis in vitro naphthoquinone of the 4a, 4c e 4d derivatives. The values tested there were 10 and 50 times higher than the IC_{50} obtained in antiplasmodial testing. Data are expressed as mean ± standard deviation. Was used ANOVA followed by Tukey's test. The average followed by different letters are statistically different ($p < 0.01$).

vacuolar, micro and macro- vesicular steatosis and the presence or absence of fibrosis and necrosis [43,13].

The animal tests were approved by the Animal Ethics Committee (CEUA/UFRN) under the protocol number 046/2013.

2.5. Statistical analyses

The IC_{50} was estimated by linear interpolation, in comparison with untreated controls, using software HN-NonLin V1.1 [29]. For the acute toxicity test, the results were expressed in mean ± standard deviation. The change of data between the treated groups and controls was carried out by applying the Simple Analysis of Variance (ANOVA) followed by *Tukey* test (95% confidence interval), using the statistical program *Assistat* 7.7 beta [42]. The results were considered statistically significant for *p*-value ≤ 0.05.

3. Results

The IC_{50} values for 2-hydroxy-1,4-naphthoquinones derivatives ranging from 483.5 to 2044.8 μM for RAW 264.7 cells and from 315.8 to 1408.0 μM for macrophages from primary culture cells. **4d** compound presented lower toxicity (Detailed data on Table 2). The viabilities of cells exposed to 2-hydroxy-1,4-naphthoquinones derivatives were declined with the increase of concentration (Fig. 2). None of the compounds caused hemolysis at the doses tested (Fig. 3).

Table 3
Signs of acute toxicity and mortality derivatives naphthoquinones **4a**, **4c** and **4d**.

Parameters observed	Observations	
	300 mg/kg	1000 mg/kg
Skin/Fur	W.C.	W.C.
Eyes and Mucous Membranes	W.C.	W.C.
Cardiac/Respiratory Signs	W.C.	W.C.
Behaviour Pattern	W.C.	W.C.
Somatomotor Activity	W.C.	W.C.
Salivation	N.O.	N.O.
Tremors	N.O.	N.O.
Convulsions	N.O.	N.O.
Lethargy	N.O.	N.O.
Sleep	N.O.	N.O.
Coma	N.O.	N.O.
Mortality	N.O.	N.O.
Necropsy	W.C. macroscopic	W.C. macroscopic

W.C.: Without Change; N. O.: Not observed.

At the doses tested, none of the compounds caused alteration of behavior or have led to symptoms related to the central nervous system, autonomic, circulatory and/or respiratory during the period tested as summarized in Table 3.

During animal necropsy observed no macroscopic change of organs for both compounds in the two tested doses as well as for the untreated control.

The weight of the animals decreased in a dose-dependent manner in animals treated with the naphthoquinones derivatives, but at a dose of 1000 mg/kg there was no significant difference between the treated groups and the untreated control and to dose of 300 mg/kg only derivative **4c** gained less weight than the other groups (Fig. 4).

The Fig. 5 displays the weight of the organs of the animals treated at dose of 1000 mg/kg. Only the group average **4c** liver weight was higher compared to untreated control. Nor spleen or kidneys of any groups were statistically different from the control group.

None injury was observed in the liver from animals of the group control, hepatocytes with cytoplasm, nucleus, nucleolus presented

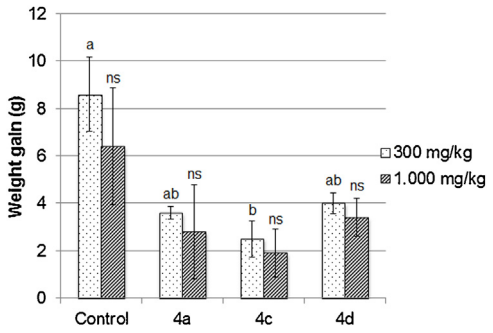

Fig. 4. Weight gain of mice treated with naphthoquinone derivatives **4a**, **4c** and **4d** and the untreated control. The change in weight was dose-dependent and dose of 1000 mg/kg there was no significant difference between groups. Data are expressed as mean ± standard deviation (3 animals). Was used ANOVA followed by Tukey's test. The average followed by different letters are statistically different ($p < 0.05$).

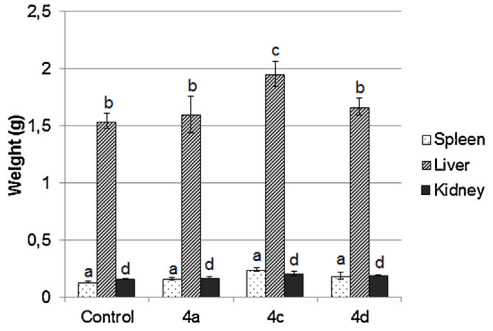

Fig. 5. Weight of spleen, kidney and liver of mice treated with naphthoquinone derivatives orally administered (dose 1.000 mg/kg) and untreated control. Data are expressed as mean ± standard deviation. Was used ANOVA followed by Tukey's test. The average followed by different letters are statistically different ($p < 0.01$).

normal aspects and intact central vein. However, in the treated groups with the compounds **4a**, **4c** and **4d** an inflammatory infiltrate and congested blood vessels were observed. In the group treated with **4a** compound was also observed hemorrhagic foci and steatosis (Fig. 6). Using PAS staining, can be observed the circumscription of injury due to steatosis (Fig. 7).

Only the group treated with the compound **4a** showed signs of nephrotoxicity, which was observed by the presence of tissue hyperemia (Fig. 8). No microscopic changes were observed in the spleen of the animals treated and untreated animals (data not shown).

4. Discussion

The naphthoquinone derivatives are toxic and several studies have shown that the structure changes in the molecule have reduced its cytotoxic effect, and improve biological activity. Davanço et al. [9], evaluated a prodrug primaquine and when compared with the primaquine, it was less cytotoxic to BGM and HepG2 cells, caused less hemolysis of G6PD deficient red blood cells and caused less alteration in the biochemical parameters. In their study Oliveira et al. [31] produced a derivative of vanillin molecule from its condensation with resorcinol and absence of cytotoxicity observed in murine macrophage cells derived compound, while the vanillin presented an IC50 of 645 μg/mL. This toxicity can be explained in two ways: by one-electron reduction, forming semiquinones that self-oxidize quinone, with production of active oxygen species, or by reaction with cellular nucleophiles, due to its characteristic electrophilic [26,18]. This is because the 1,4-naphthoquinones can catalyze redox cycling to produce reactive oxygen species, generate a highly oxidative environment with increased levels of hydrogen peroxide and oxidized glutathione, and react with tissue nucleophiles to modify proteins covalently [28,18,6,12].

Ours results in cytotoxicity assays show that the derivatives have low toxicity to the two cell types tested when compared to other studies. Salustiano et al. [38] tested pentacyclic 1,4-naphthoquinones on peripheral blood mononuclear cells (PBMCs) and found the IC_{50} ranging from 8.56 to 23.50 μM; Kishore et al. [17] tested seven compounds on PBMCs and the compounds 8-Fluoro-5-hydroxy-7-methyl-1,4-naphthoquinone (188,7 μM) and 2,5-Dihydroxy-7-methyl-1,4-naphthoquinone (54 μM) were the least toxic, whilst the other compounds including 5-Hydroxy-7-methyl-1,4-naphthoquinone (7-MJ) (18.4 μM) were more toxic. In a study of six naphthoquinones compounds to HL-7702, Guo et al. [14] observed that 2-hydroxy-1,4-naphthoquinone was less toxic. The low toxicity of the compounds can be explained by low liposolubility of 2-hydroxy-1,4-naphthoquinone which ensures that its cell penetration is relatively poor [14].

Another good method for cytotoxicity evaluation is hemolysis, which is characterized by the erythrocyte rupture with release of hemoglobin. This, as will be free in plasma causes damage to various vital organs such as the liver, kidney and heart. Thus, it is necessary to observe the hemolytic activity in the screening of biological and toxicological activities of plant extracts and derivatives [5,9]. Furthermore, the lysis of red blood cells prevents direct intravenous administration of desired agents and often increases the toxicity of these agents when administered by other routes [7]. Many studies of naphthoquinones derivatives reported that these cause hemolytic anaemia *in vivo* [21,4,25,26,28]. Hemolysis induced naphthoquinones has been attributed to the formation of reactive oxygen species from the redox-cycling process induced by naphthoquinones [28]. None of the derivatives naphthoquinones tested showed hemolytic activity at concentrations tested, indicating that the scaffold of naphthoquinoidal compounds used in our work is less toxic than those previously described in the literature.

The animal body weight is an important factor to evaluate the toxicity of a substance [16]. In our study, for the dose to 1000 mg/kg there was no significant difference in weight gain between the treated and the untreated group. However, at the dose of 300 mg/kg, the compound **4c** whose radical contains a methoxy group, presented less weight than the untreated group, but not statistically different from the other treatment groups.

Changes in organ weight have been accepts as indicators of test-induced changes, which are often associated with treatment-related effects [41]. Regarding the weight change of organs, again only **4c** group presented changes, whose liver had greater weight gain compared to the other groups, although not being the group most hepatotoxicity by histological analysis of organs.

In acute toxicity tests all derivatives exhibited a significant margin of safety, proven by the absence of behavioral and systemic toxicity in the tested dose of 300 mg/kg, ie, there were no adverse effects related to animals. Unable to perform the assay at a concentration of 2000 mg/kg because the compounds are non-polar in nature, it is not possible dilution in water in high concentrations. The test was then conducted with a dose of 1000 mg/kg, which were not observed signs of toxicity. Thus, one may suggest the classification of compounds in category 4 of GHS (Globally Harmonized System of Classification and Labelling of Chemicals) [30]. A necropsy of the animals at the two doses tested showed macroscopic aspects of normal organs.

The liver, kidney, and spleen were removed for histopathological analysis because they are among the organs primarily affected by metabolic reaction caused by toxicant [11]. The liver is the main target organ affected where exposed to toxin substances, after being absorbed in intestines and metabolized to other compounds [36]. In our study, the findings revealed less severe morphologi-

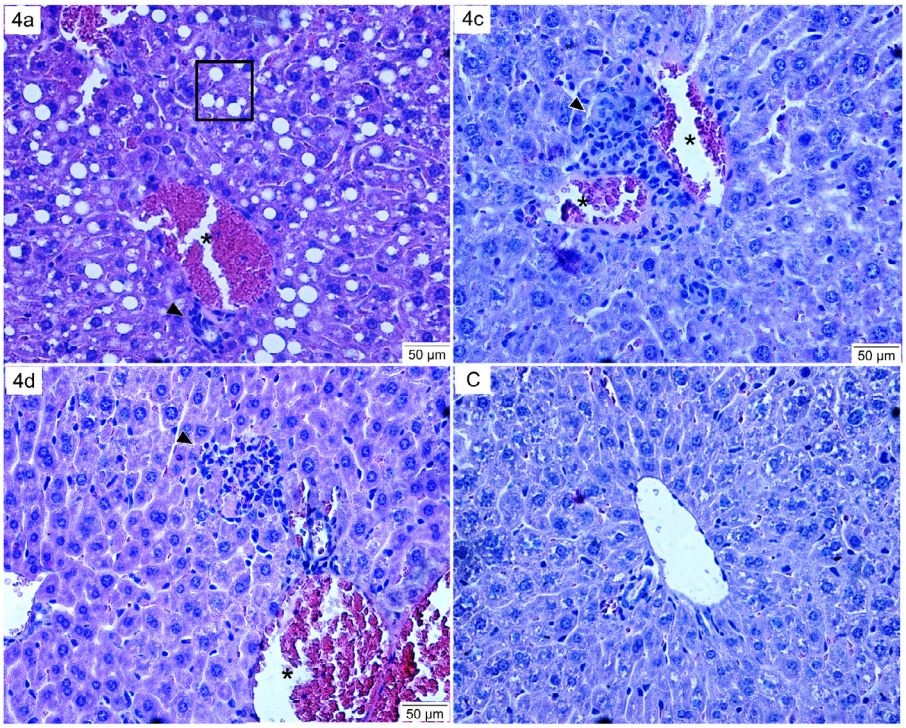

Fig. 6. Analysis representative histological sections of the livers of mice treated with derivatives naphthoquinones orally administered (dose 1.000 mg/kg) and untreated control. The arrowheads indicate inflammatory infiltrates. Asterisks indicate congested vessels. The square indicates steatosis. (Hematoxylin & Eosin, magnification 400×).

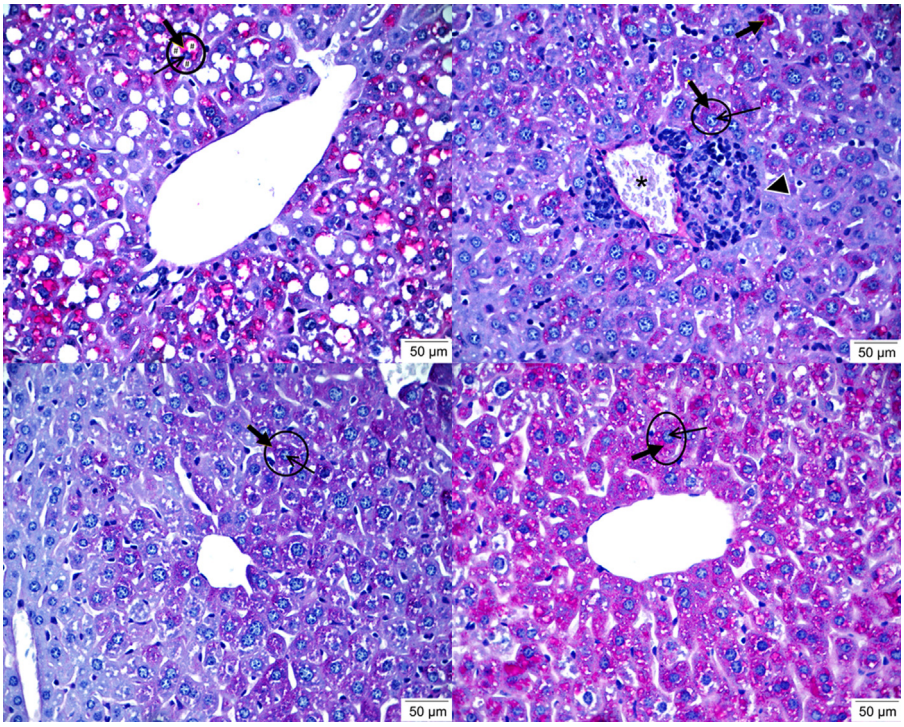

Fig. 7. Histological sections stained, with periodic acid–schiff (PAS) method, of the livers of mice treated with derivatives naphthoquinones orally administered (dose 1.000 mg/kg) and untreated control. In the image, the circles stand one hepatocyte. Black arrows point the nucleus of hepatocytes and large arrows show the glycogen. While accumulation of lipids doesn't show a positive reaction the method; hashtags (**4a**) show the lipid droplets indicating steatosis. The asterisk shows a congested vessel and the arrow head indicates inflammatory infiltrate.

cal changes in liver of mice treated, being observed only points of inflammatory infiltrate and congested vessels. Derivative **4a** was the one who presents steatosis, proving to be more hepatotoxic than the other compounds.

Second Munday et al. [28], beyond the hemolytic activity, many naphthoquinone derivatives can cause necrosis of tubular epithelial cells, the substituents at C-3 of position 3 of the 2-hydroxy and 2-amino-1,4-naphthoquinone reduces or eliminates

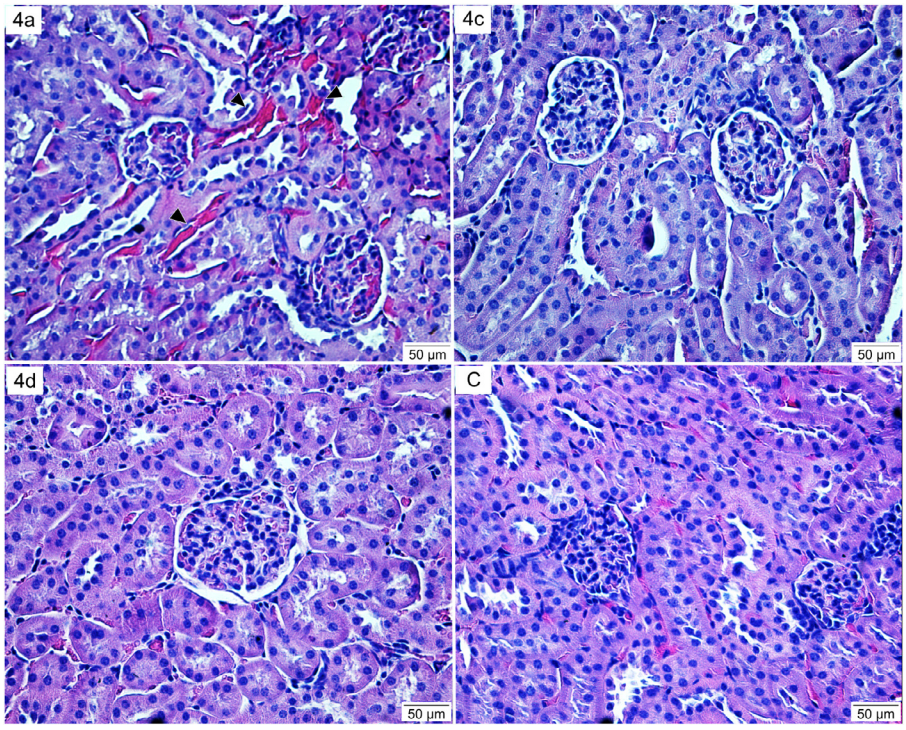

Fig. 8. Histological analysis of representative sections of the kidney of mice treated with derivatives naphthoquinones orally administered (dose 1.000 mg/kg) and untreated control. The arrowheads indicate bleeding tissue (Hematoxylin & Eosin, magnification 400×).

the nephrotoxicity, though the mechanism of how this occurs remains unknown. Contrary to what was observed in our study, in which only the **4a** group exhibited signs of kidney damage, proven by observation of tissue hyperemia, several studies have shown kidney damage, with tubular necrosis in rats treated with derivatives of hydroxy derivatives such as 2-hydroxy-1,4-naphthoquinone [25], 2-hydroxy-3-alkyl-1,4-naphthoquinone [26] and 2-amino-1,4-naphthoquinone [27]. Furthermore, no changes in the renal tissue of the treated groups with a naphthoquinone derivative was observed, the glomeruli and capsules appeared normal and the Bowman's space are also marked clearly.

The hematopoietic system is very sensitive to toxic compounds and serves as an important index of the physiological and pathological status in both animals and humans [1]. Histological analysis of the spleen showed an apparently normal tissue, with morphologically distinct compartments, white and red pulp distinguishable.

Compared with the results found by Munday et al. [25] with 2-hydroxy-1-4-naphthoquinone and by Munday et al. [28] with 2-hydroxynaphthoquinone derivatives, which showed severe hemolytic anaemia, reflected by splenic enlargement, besides the renal tubular necrosis, our compounds have reduced toxicity, can be a result of the anilino group at C-3 of naftoquinoidal ring.

5. Conclusion

In our work, the 2-hydroxy-3-anilino-1,4-naphthoquinone derivatives do not show severe toxicity *in vitro* and *in vivo* models, none clinical serious sign were observed in treated animals. The only sign of toxicity observed for all products was hepatotoxicity, which may be explained by the extremely high dose. Thus, the chemical structure of these compounds is closed related to the toxicity of naphthoquinones which are compounds having known toxicity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxrep.2016.09.007.

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