TOXICITY ASSESSMENT OF NANOEMULSION CONTAINING THE ESSENTIAL OIL OF ROSMARINUS OFFICINALIS L. IN ZEBRAFISH

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Abstract - The essential oil of Rosmarinus officinalis (EORO) has been used for medicinal purposes around the world, which encouraged research toward the elucidation of its biological activities. Currently, a methodology was developed to encapsulate it in the form of a nanoemulsion (NEORO), which managed to improve the pharmacological activities of EORO using lower doses. However, no toxicological evaluation were performed, and hence, this study aimed to evaluate the toxicity of NEORO in zebrafish (Danio rerio), obtaining the LD_{50} value and analyzing histopathologic alterations of liver, kidney, and intestine. Zebrafishes were treated with crescent doses of NEORO (1, 2, 3, 4 and 5 mg/kg), and the LD_{50} value was 2.4 mg/kg. Histologically, kidney and intestine were more altered, while the liver remained relatively normal, despite being accountable for a significant part of toxic compounds metabolism in zebrafish.

Index Terms - Essential Oil, Nanoemulsion, Rosmarinus Officinalis, Zebrafish.

I. INTRODUCTION

Rosmarinus officinalis L. is a shrub-type plant of Lamiaceae family [1], used for medicinal purposes in several ways, including using its essential oil (EORO). Studies performed with EORO report biological activities such as antioxidant, anti-inflammatory, anti-mutagenic, anti-microbial and anti-depressive due to the terpenes of its composition [2], [3], [4], [5].

Due to the intrinsic lipophilic nature of essential oils, encapsulation systems are currently being developed to improve the permeability of these compounds in biological membranes [6]. Nanoemulsions are formulation systems obtained by encapsulation of lipophilic substances into nanometric droplets, increasing its solubility [7].

Duarte et al. (2005) described a method for obtention of a nanoemulsion from EORO (NEORO) [8]. In biological assays, NEORO managed to improve the bioavailability of EORO and exerted its anti-inflammatory and analgesic with considerably lower doses [9], [3].

However, until now, no toxicity study was performed using NEORO, and hence, this research aimed to evaluate its toxicity in zebrafish, through LD_{50} determination and histopathological analysis.

II. MATERIAL AND METHODS

Animals

In this research were used 75 wild-type zebrafishes boughs from the company Aqua New Aquariums (PE, Brazil), kept in the Zebrafish Facility of State University of Amapá – UNIFAP, Amapá, Brazil. Animals were kept under the conditions described in [3]. This research was approved by the Ethics Committee of UNIFAP under the registry number 0021/2015.

DL_{50} Assessment

Animals (n = 75) were kept fasten for 24 h, then randomly divided into 5 groups with 15 animals per group. Each group received a different dose of NEORO (1, 2, 3, 4 and 5 mg/kg). The mortality was observed for statistical analysis.

NEORO Administration and Group Formation

The treatments with NEORO and vehicle (Tween 20 + water) were performed orally according to the method described by [3]. The groups were divided as follows: Group C (2 mg/kg), F (3 mg/kg), I (4 mg/kg), J (control – 5% Tween 20 + 95% water), M (1 mg/kg), P (5 mg/kg). Groups M and P were not selected for discussion of the results because they presented the minimum and maximum levels of tissue alterations.

Histopathological Analysis

Slide preparation, microscopic analysis and tissue alteration index calculation were performed using the techniques described by [10], [11]. The Histological Alteration Index (HAI) was appraised from the tissue alterations (levels I, II, III) in the liver, kidney and intestine; where HAI values ranging from 0 to 10 indicates normal tissue, from 11 to 20 means moderately altered, from 21 to 50 means moderately to severely injured, and > 100 means irreversibly injured [12], [13].

Statistical Analysis

DL_{50} was calculated through probit analysis using the software GraphPad Prism 6.0. The difference between

groups was appraised through ANOVA followed by the post hoc non-parametric test of Kruskal Wallis; $p < 0.05$ was considered statistically significant.

III. RESULTS

Among the group treated with NEORO at 2 mg/kg occurred death in 40% of animals, while the group treated with NEORO at 3 mg/kg had death in 60% of animals and 4 mg/kg induced 80% of mortality, as shown in Fig. 1. No death occurred in the control group, which indicates no toxicity induced by the surfactant alone; the DL_{50} value was calculated as 2.4 mg/kg.

Histopathology assessment of liver in group C (2 mg/kg) reveals level II (loss of cell outline) and level II (hyperemia, nuclei atrophy) tissue alterations (Fig. 2). Total tissue alteration was 25% and HAI was 4.2, indicating that the hepatic tissue remained normal after the treatment (Tables I and II). The group F (3 mg/kg) had similar level I and II alterations (Fig. 2), and the HAI was calculated as 4.6, indicating the normal function of the liver (Table II). Total tissue alteration was 41.6% (Table I). In the group treated with NEORO at 4 mg/kg was detected level III alteration, necrosis (Fig. 2). Total alteration tissue alteration was 50%, and HAI was 26.4, indicating moderate to severe tissue alterations (Table I and II). The control group had no tissue alterations (Fig. 2; Tables I and II).

In the kidney, the group treated with NEORO at 2 mg/kg had hypertrophy of tubular cells (level I alteration), cytoplasmic degeneration of tubular cell, hyperemia and tubular degeneration (level II alterations), totalizing an HAI of 6.2 (Fig. 3, Table II). This group had 33.3% of total tissue alteration (Table I). In the animals from groups F (3 mg/kg) and I (4 mg/kg) was detected necrosis (level III). These groups had an HAI of 28.0 and 30.0, respectively (moderate to severe alteration) and 50% of tissue alteration. No tissue alteration was detected in the control group (Fig. 3; Tables I and II).

Assessment of intestinal tissue showed level I (villi atrophy, leukocyte infiltration, hyperplasia of goblet cells, muscular layer degeneration) and II (lamina propria hemorrhage) alterations in the group C (2 mg/kg) (Fig. 4). The HAI value was 2.8, and total tissue alteration was 27.7% (Tables I and II). In the intestine of animals from group F (3 mg/kg) was observed tissue necrosis (level III) (Fig. 4); this group had an HAI of 23.2 and 44.4% of total tissue alteration in the intestine (Tables I and II).
In the same way, animals from group I (4 mg/kg) had intestinal necrosis with high HAI value (31.2) and total tissue alteration (66.6%). In the control group was observed level I alterations (villi vases dilation, leukocyte infiltration), an HAI value of 0.6 and 0.1% of tissue alteration (Fig. 4; Tables I and II).

Table I: Percentage of histological alterations observed in the organs of zebrafish caused by NEORO at doses 2, 3 and 4 mg/kg.

Group/tissue	Stage I	Stage II	Stage III	Total	%
Liver					
C - 2 mg/kg	1/5	2/6	0/1	3/12	25,0
F - 3 mg/kg	3/5	2/6	0/1	5/12	41,6
I - 4 mg/kg	2/5	3/1	1/1	6/12	50,0
J – Control	0/5	0/6	0/1	0/12	0,0
Intestine					
C - 2 mg/kg	4/11	1/6	0/1	5/18	27,7
F - 3 mg/kg	6/11	1/6	1/1	8/18	44,4
I - 4 mg/kg	6/11	5/6	0/1	12/18	66,6
J - Control	0/11	0/6	0/1	0/18	0,0
Kidney					
C - 2 mg/kg	1/4	3/7	0/1	4/12	33,3
F - 3 mg/kg	1/4	4/7	1/1	6/12	50,0
I - 4 mg/kg	0/4	5/7	1/1	6/12	50,0
J – Control	0/4	0/7	0/1	0/12	0,0

The percentage was calculated from the total number of each group (n = 5) According described by [12], [13].

Table II: Average Histologic Alteration Index (HAI) in the organs of zebrafishes exposed to different doses of NEORO in triplets (n = animals/group). Rep = Replicate, C (2 mg/kg), F (3 mg/kg), I (4 mg/kg), J (control).

	Kidney			
Replic.	**C**	**F**	**I**	**J**
1	6.5	27.5	30.4	0.1
2	6.4	28.2	30.0	0.0
3	5.7	29.0	29.7	0.0
M ± SD	6.2±0.43	28±0.75	30.0±0.35	0.0±0.6
	Liver			
Replic.	**C**	**F**	**I**	**J**
1	4.6	4.5	27.0	0.0
2	3.7	4.2	25.7	0.0
3	4.4	5.1	26.5	0.0
M ± SD	4.2±0.47	4.6±0.46	26.4±0.66	0.0±0.0
	Intestine			
Replic.	**C**	**F**	**I**	**J**
1	3.0	23.3	32.2	0.0
2	2.8	22.8	30.8	0.0
3	2.6	23.5	30.6	0.0
M ± SD	2.8±0.20	23±0.36	31.2±0.87	0.0±0.0

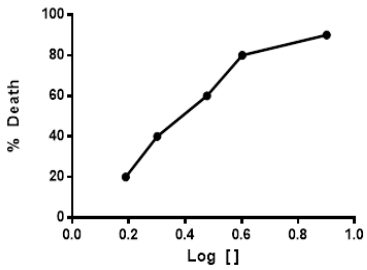


Fig. 1: Mortality percentage in function of crescent doses of NEORO (1 mg/kg, 2 mg/kg, 3 mg/kg, 4 mg/kg e 5 mg/kg) in zebrafish (n = 15 animals/group). DL_{50} = 2.4 mg/kg.

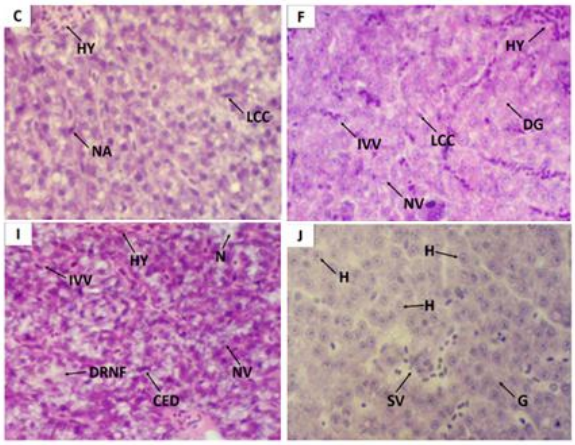


Fig. 2: Longitudinal sections of liver of zebrafish after the treatment with NEORO; x400, H&E. C: 2 mg/kg NA – nuclear atrophy, LCC – loss of cell outline, HY – hyperemia; F: 3 mg/kg DG – decreased glycogen, NV – nuclear vacuolization, IVV increased vessel volume; I: 4 mg/kg DRNF – decreased relative frequency of nuclei, CED – cell rupture, N – necrosis; J: control H – hepatocytes, SV – sinuous vessels, G – Glycogen.

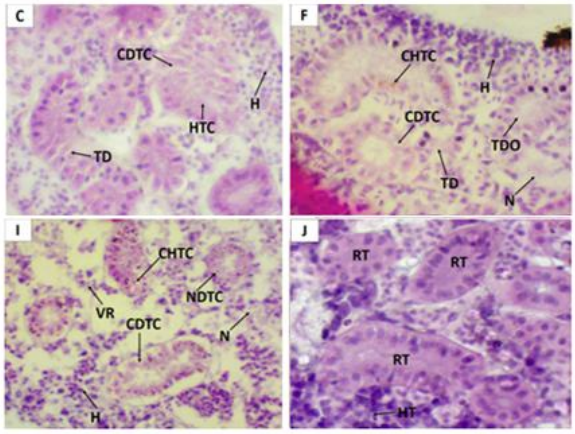


Fig. 3: Longitudinal sections of kidney of zebrafish after the treatment with NEORO; x400, H&E. C: 2 mg/kg CDTC – cytoplasmic degeneration of tubular cells, HTC – tubular cells hypertrophy, TD – tubular degeneration, H – hyperemia; F: 3 mg/kg CHTC – hyaline degeneration of tubular cells, TDO – tubular disorganization, N – necrosis; I: 4 mg/kg VR – Vessel rupture, NDTC nuclear degeneration of tubular cells; J: control RT – renal tubules, HT hematopoietic tissue.

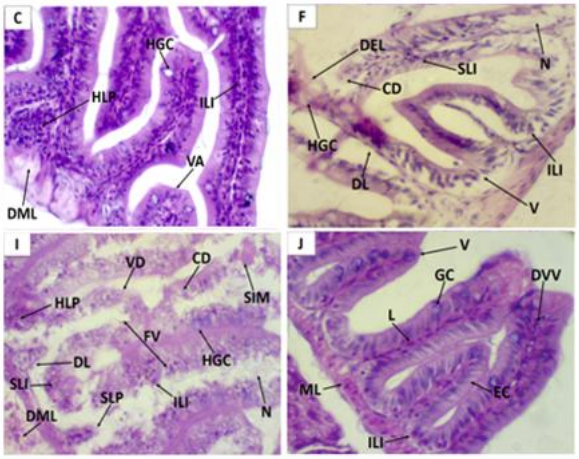


Fig. 4: Longitudinal sections of intestine of zebrafish after the treatment with NEORO; x400, H&E. C: 2 mg/kg HLP – lamina propria hemorrhage, VA – villi atrophy, LI – leukocyte infiltration, HGC – hyperplasia of goblet cells, DML – muscular layer degeneration; F: 3 mg/kg CD – cellular degeneration, DL – lamina propria detachment, DEL – villi epithelial displacement, SLI – stromal lymphocytic infiltration, V – enterocytes vacuolization, N – necrosis; I: 4 mg/kg FV – villi fusion, SLP – lamina propria shortening, VD – villi degeneration, SIM – intestinal mucosa peeling; J: control L – lamina propria, ML – muscular layer, GC – goblet cells, CE – enterocytes, V – villi, LI – leukocyte infiltration, DVV – vessel dilation.

DISCUSSION

In zebrafish, the toxicity can be assessed through several parameters, including mortality [14]. According to Bilberg et al. (2012), higher concentrations of nanoparticles induce higher mortality indexes. Which is in accordance to what was observed in this research [15].

The liver of zebrafish has similarities with mammals' regarding the main physiological processes. Including drugs metabolic pathways with the action of cytochrome P450. Therefore, its histopathology can be compared to hepatic injuries of mammals [16].

Vacuolization was one of the more frequently tissue alteration observed. It can be related to the action of chemical agents that can impair the normal function of the organ [3]. Other hepatic alterations observed in the hepatic tissue that are considered defense mechanisms were cellular degeneration and hyperemia, which occur to increase the blood flow, avoiding hypoxia [13], [11]. The hepatic necrosis observed in animals treated with NEORO at 4 mg/kg can be induced by high doses of chemical agents.

Zebrafishes' kidney is one of the most affected organs by toxic compounds [11]. Hypertrophy of tubular cells occurs due to drying of epithelial cells, causing the increased quantity of eosinophilic granules. Hyaline degeneration can be related to re-absorption of proteic substances excessively synthetized by glomeruli [13]. According to Carvalho et al. (2017), tubular alterations observed in the kidneys of zebrafish can be induced by metabolic dysfunction caused by toxic substances [11]. These alterations can often induce necrosis, explaining the necrosis formation in animals treated with the highest doses (3 and 4 mg/kg).

Exposure to toxic compounds leads to intestinal mucosa damage, affecting intestinal physiology and causing histological alteration [13]. It was observed leukocyte infiltration in the mucous layer; this can be explained by the occurrence of inflammation caused by toxicity [11], [17].

Displacements of intestinal mucosa were observed in animals treated with the highest doses. This alteration is commonly observed in the intestine of fishes exposed to toxic agents [17]. The vacuolization is frequent after exposure to toxic agents and often precedes necrosis [13], this explains the occurrence of intestinal necrosis in animals treated with the highest doses.

CONCLUSION

The exposure of zebrafish to crescent doses of NEORO allowed calculating the LD_{50} – 2.4 mg/kg. NEORO was efficient in the evaluation of toxicity, with evidence its capacity to improve the bioavailability of EORO. Most altered organs were intestine and kidney, which suggests that liver metabolization was more active, resulting in reduced damage in this organ. The data support previous toxicity patterns previously observed for nanoformulations.

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