Clinical and morphophysiological hepatic alterations caused by the ingestion of food with added ricinoleic acid esters a bioactive with acaricidal potential

Alterações hepáticas clínicas e morfofisiológicas causadas pela ingestão de alimentos com adição de ésteres ricos em ácido ricinoleico um bioativo com potencial acaricida

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ABSTRACT
The present study investigated the effects of castor oil ricinoleic acid through analysis of blood biochemical parameters and the liver morphology of female rabbits (animal model). The results of Bioassay 1 and Bioassay 2 showed that the reference indexes of the hepatic enzymes alkaline phosphatase, gamma-glutamyl transferase, and alanine aminotransferase were not significantly altered. However, the hepatic enzyme aspartate aminotransferase of the rabbits tested in Bioassay 2 presented alterations, suggesting a hepatotoxic effect of the esters. The morphological and histological data showed that the liver of the rabbits fed with the ester-added food presented the following alterations: a) disorganization of the hepatic cords; b) an increase in intercellular spaces; c) hepatocytes with barely evident cell limits; and d) hepatocytes with irregular and pyknotic nuclei, in most cases displaced to the periphery of the cell due to extensive vacuolation. The histochemical analysis showed that the hepatocytes of the rabbits fed with commercial food with added esters had a decrease in lipid content and an increase in the levels of stored cytoplasmic glycogen.

Keywords: ricinoleic acid esters, hepatic enzymes, morphophysiology, ticks, *Rhipicephalus sanguineus*.

RESUMO
O presente estudo investigou os efeitos do ácido ricinoleico do óleo de mamona através da análise dos parâmetros bioquímicos do sangue e da morfologia hepática das coelhas fêmeas (modelo animal). Os resultados do Bioensaio 1 e do Bioensaio 2 mostraram que os índices de referência das enzimas hepáticas fosfatase alcalina, gama-glutamil transferase e alanina-aminotransferase não foram significativamente alterados. Entretanto, a enzima hepática aspartato aminotransferase dos coelhos testados no Bioensaio 2 apresentou alterações, sugerindo um efeito hepatotóxico dos ésteres. Os dados morfológicos e histológicos mostraram que o fígado dos coelhos alimentados com o alimento adicionado de ésteres apresentou as seguintes alterações: a) desorganização das cordas hepáticas; b) aumento dos espaços intercelulares; c) hepatócitos com limites celulares pouco evidentes; e d) hepatócitos com núcleos irregulares e piknóticos, na maioria dos casos deslocados para a periferia da célula devido à vacuolação extensiva. A análise histoquímica mostrou que os hepatócitos dos coelhos alimentados com alimentos comerciais com ésteres adicionados tiveram uma diminuição no conteúdo lipídico e um aumento nos níveis de glicogênio citoplasmático armazenado.

Palavras-chave: ésteres de ácido ricinoleico, enzimas hepáticas, morfofisiologia, carrapatos, *Rhipicephalus sanguineus*

1 INTRODUCTION

Ticks are arthropods of significant veterinary importance, as their hematophagous habits affect a wide range of host animals, including humans. These ectoparasites are

widely distributed in tropical and subtropical regions and are also present in urban areas (LABRUNA, 2004) mainly through dogs and cats, the most popular household pets and preferred hosts of *R. sanguineus s. l.* (dog brown tick).

In addition to causing direct spoliation in hosts, ticks can transmit several pathogens while feeding, such as protozoa, viruses, and bacteria. Examples of these pathogens are: *Coxiella burnetii, Ehrlichia canis, Hepatozoon canis, Rickettsia conorii, R. rickettsia,* and *Leishmania infantum,* the etiologic agent of visceral leishmaniasis. In this context, and considering the significant economic losses caused by these ectoparasites, an efficient control method is needed to control infestations, which affect not only animal hosts, but also food products and byproducts (beef, leather, milk) as well as the environment (CAMARGO et al., 2014).

The literature reports the application of several synthetic chemical acaricides based of arsenic, organochlorine, organophosphate, carbamate, pyrethroids, ivermectin, and formamidine, among others (LEAL et al., 2003). Although such chemicals have been proven efficient, their toxic residues cause serious damage to the environment and public health, contaminating water streams, the soil, and animals. Still according to the literature, hundreds of synthetic acaricides are generated and inappropriately disposed of on a yearly basis (MARTHE et al., 2010).

In this scenario, the urgent need to develop safe and efficient strategies to control ticks is evident. The use of bioactives extracted from different parts of plants, as well as essential oils, or even from animals, has raised sanitary and economic interest, as these chemicals are natural and easily biodegraded (BORGES, 2011).

Several compounds containing bioactives have been tested, and the esters from castor oil ricinoleic acid (*Ricinus communis*) have been proven efficient against ticks (ARNOSTI et al., 2011 a, b; SAMPIERI et al., 2013a; 2015; CAMARGO-MATHIAS, 2018; SODELLI, 2019). These bioactives are capable of affecting the morphology and, consequently, the physiology of important organs associated with the feeding process (e.g., the salivary glands), impairing the generation of new individuals (ARNOSTI et al., 2011 b).

Additionally, the bioassays demonstrated that the esters have the potential to reduce reproductive rates by interfering in the vitellogenesis (ARNOSTI et al., 2011 b). It is important to highlight that, according to the literature, esters do not interfere in the hosts' clinical parameters (ARNOSTI et al., 2011 b), which was demonstrated by studies using rabbits infested with R. sanguineus and fed with ester-added commercial food —

the animals presented neither physical nor behavioral alterations. The animals tested even presented weight gain throughout the experiment (SAMPIERI et al., 2013 a, 2015).

Despite the promising reports regarding the use of ricinoleic acid esters such as acaricide, the literature lacks data confirming that these bioactives affect the morphology of the internal organs of vertebrate hosts, mainly the liver — a vital organ that is responsible for the metabolic pathways of detoxification processes.

Thus, from the perspective of using castor oil ricinoleic acid esters (R. communis) as an efficient and safe method for controling ticks and being of significant economic importance, the objective of the present study is to evaluate the biochemical parameters of blood (hepatic enzymes alkaline phosphatase APH, gamma-glutamyl transferase Ɣ-GT, alanine aminotransferase ALT and aspartate aminotransferase AST) and to analyze the histophysiology of the liver of rabbits (animal host model) fed with commercial food with added esters, aiming to provide information on the effects that exposure to esters cause on the organs of such vertebrates.

2 MATERIAL AND METHODS

2.1 MATERIAL

Each bioassay included seven two-month-old female rabbits of the Genetic Group of the Botucatu, provided by UNESP/Botucatu, SP, Brazil. The animals weighed 3 to 3.5 Kg and had no previous contact with ticks or acaricides. For adaptation, the female rabbits were kept for 15 days in individual galvanized cages under controlled temperature and photoperioded, receiving water and commercial food ad libitum.

The esters were produced and kindly provided by the deceased Professor Dr. Gilberto Orivaldo Chierice of USP - São Carlos, SP, Brazil.

The process of adding esters to commercial food was performed in the Laboratory of Fish Health and Nutrition, Animal Nutrition Department, School of Veterinary Medicine and Animal Science, UNESP/Botucatu (SP), Brazil, under the supervision of Dr. Pedro Luiz Pucci Figueiredo de Carvalho, a co-worker in this study.

All the procedures were approved by the Ethics Committee on Animal Use, UNESP Campus Rio Claro, SP, Brazil; certificate number 14/2017.

2.2 METHODS

2.2.1 The following procedures were developed

- Bioassay 1: seven rabbits received 140g of commercial repalletized food without the addition of esters on a daily basis for 60 days (experiment control).

-Bioassay 2: seven rabbits received 140g of commercial repalletized food with added esters in the proportion of 5.0g esters per kg of conventional food (experiment tests).

2.2.2 Clinical tests

Blood samples were collected (2mL) from the jugular vein of all the rabbits using a 3 mL syringe with a 25x8 sterile needle on days 0, 15, 30, 45 and 60.

The blood was kept in dry Vacutainer® with separation gel. The samples were centrifuged at 5000 rpm/5min and the serum was separated for the analysis of the hepatic enzymes alanine aminotransferase (ALT), (found in the cytoplasm of the hepatocytes), aspartate aminotransferase (AST), (found in the mitochondria of the liver cells), gamma-glutamyl transferase (γ-GT), (hepatic enzymes found in the liver cells and biliary ducts), alkaline phosphatase (APH), (found mainly in the liver and biliary ducts, but also present in the kidney, midgut, pancreas, and spleen cells). The experiment was performed using BIOPLUS® (multiparameter semiautomatic analyzer for colorimetric and enzymatic biochemical assays), and BIOLAB® kits (according to the manufacturer's recommendations) and were conducted in the Clinical Analysis Laboratory of the Integrated Faculties of Limeira, Limeira - SP, Brazil, under the technical supervision of the biomedical scientist Ms Luís Fernando Sodelli - CRBM nº 4.099.

2.3 HISTOLOGY

After the 60 days established in the experimental protocol, the rabbits were euthanized with an intraperitoneal injection of ketamine xylazine (300 mg/kg and 30 mg/kg, respectively) by veterinarian Letícia Maria Graballos Ferraz Hebling (CRMV nº 5.412) in the Animal Facilities of the General and Applied Biology Department of UNESP, Rio Claro, SP, Brazil.

Fragments of the liver were collected (0.4 cm) and immediately fixed and taken in for morphohistological analysis in the Histology Laboratory of the General and Applied Biology Department – UNESP, Rio Claro, SP, Brazil.

The liver fragments were fixed in paraformaldehyde (for routine histology, aqueous Harris-eosin hematoxylin, and for protein detection, bromophenol blue) at 4ºC for 72 hours in Bouin's aquous solution at 4ºC for 5 days (for PAS reaction, Schiff periodic acid to detect polysaccharides) and in neutral-buffered formalin 10% (pH 7- 7.4) at 4ºC for 1 hour and 30 minutes (to detect acid phosphatase activity).

After dehydrated in graded ethanol series, the liver fragments were embedded in Leica resin for 24 hours and included in plastic molds containing historesin and polymerizer (Leica Historesin Kit®). The blocks containing the material were sectioned (3 μm thickness) using a Leica RM2265 microtome (Leica®) and mounted on glass slides for further staining according to each technique. Following the staining process, the slides were dried on wooden test tube racks at room temperature, immersed in xylol and covered with Entellan and cover slips.

The permanent slides were analyzed and photographed using a bright field microscope Leica DM750 (Leica®) at the Histology Laboratory of the General and Applied Biology Department at UNESP Rio Claro, SP, Brazil.

2.4 RESULTS

2.4.1 Clinical Analyses

The biochemical analyses showed no significant difference between Bioassays 1 and 2 regarding the enzymatic parameters APH (Table 1), γ-GT (Table 2), and ALT (Table 3). However, significant difference was found for the enzyme AST (Table 4) for the individuals exposed to the esters (Bioassay 2) in comparison with the individuals not exposed (Bioassay 1).

Table 1 Alkaline Phosphatase (APH)

		T 0	T 15	T 30	T 45	T 60	Reference Value
Bioassay 1	Rabbit 1	277	301	295	288	313	250 – 600 U/L
	Rabbit 2	322	306	312	317	299	250 – 600 U/L
	Rabbit 3	305	321	307	291	316	250 – 600 U/L
	Rabbit 4	336	327	310	328	309	250 – 600 U/L
	Rabbit 5	297	317	322	285	288	250 – 600 U/L
	Rabbit 6	286	314	309	311	292	250 – 600 U/L
	Rabbit 7	302	279	314	297	326	250 – 600 U/L
Bioassay 2	Rabbit 1	314	312	331	299	311	250 – 600 U/L
	Rabbit 2	342	296	354	336	315	250 – 600 U/L
	Rabbit 3	288	306	318	294	341	250 – 600 U/L
	Rabbit 4	326	296	287	314	337	250 – 600 U/L
	Rabbit 5	342	336	327	319	295	250 – 600 U/L
	Rabbit 6	278	357	278	305	289	250 – 600 U/L

	Rabbit 7	269	321	332	298	314	250 – 600 U/L

Bioassay 1 = control group
Bioassay 2 = test group

T = time in days
U/L = units per liter

Table 2 Gamma- Glutamyl Transferase (Ɣ-GT)

		T 0	T 15	T 30	T 45	T 60	Reference Value
Bioassay 1	Rabbit 1	15	21	25	28	31	12,5 – 61 U/L
	Rabbit 2	22	26	18	17	28	12,5 – 61 U/L
	Rabbit 3	25	21	30	21	16	12,5 – 61 U/L
	Rabbit 4	26	23	21	28	30	12,5 – 61 U/L
	Rabbit 5	20	17	23	25	18	12,5 – 61 U/L
	Rabbit 6	19	21	23	18	16	12,5 – 61 U/L
	Rabbit 7	20	17	14	18	23	12,5 – 61 U/L
Bioassay 2	Rabbit 1	30	22	26	21	19	12,5 – 61 U/L
	Rabbit 2	26	18	20	22	21	12,5 – 61 U/L
	Rabbit 3	27	30	31	28	22	12,5 – 61 U/L
	Rabbit 4	17	19	24	21	17	12,5 – 61 U/L
	Rabbit 5	23	28	30	24	19	12,5 – 61 U/L
	Rabbit 6	27	16	19	25	28	12,5 – 61 U/L
	Rabbit 7	15	21	22	19	23	12,5 – 61 U/L

Bioassay 1 = control group
Bioassay 2 = test group

T = time in days
U/L = units per liter

Table 3 Alanine Aminotransferase (ALT)

		T 0	T 15	T 30	T 45	T 60	Reference Value
Bioassay 1	Rabbit 1	47	55	61	49	57	34 - 123 U/L
	Rabbit 2	48	47	53	50	46	34 - 123 U/L
	Rabbit 3	54	62	47	51	54	34 - 123 U/L
	Rabbit 4	46	47	51	60	52	34 - 123 U/L
	Rabbit 5	54	62	47	51	59	34 - 123 U/L
	Rabbit 6	52	56	60	61	58	34 - 123 U/L
	Rabbit 7	49	51	55	63	47	34 - 123 U/L
Bioassay 2	Rabbit 1	39	51	66	44	51	34 - 123 U/L
	Rabbit 2	56	46	49	44	60	34 - 123 U/L
	Rabbit 3	47	39	55	53	48	34 - 123 U/L
	Rabbit 4	58	51	60	48	46	34 - 123 U/L
	Rabbit 5	54	47	60	61	55	34 - 123 U/L
	Rabbit 6	55	53	44	49	52	34 - 123 U/L
	Rabbit 7	62	51	46	57	46	34 - 123 U/L

Bioassay 1 = control group
Bioassay 2 = test group

T = time in days
U/L = units per liter

Table 4 Aspartate Aminotransferase (AST)

		T 0	T 15	T 30	T 45	T 60	Reference Value
Bioassay 1	Rabbit 1	37	44	35	38	41	18 - 56 U/L
	Rabbit 2	24	35	28	33	29	18 - 56 U/L
	Rabbit 3	31	27	29	36	28	18 - 56 U/L
	Rabbit 4	35	27	29	34	30	18 - 56 U/L
	Rabbit 5	41	33	37	42	44	18 - 56 U/L
	Rabbit 6	35	25	31	41	29	18 - 56 U/L
	Rabbit 7	26	39	28	34	40	18 - 56 U/L
Bioassay 2	Rabbit 1	36	50	158	177	254	18 - 56 U/L
	Rabbit 2	39	47	166	223	272	18 - 56 U/L
	Rabbit 3	46	49	153	205	298	18 - 56 U/L
	Rabbit 4	28	59	179	238	301	18 - 56 U/L
	Rabbit 5	33	51	164	193	274	18 - 56 U/L
	Rabbit 6	41	56	187	227	299	18 - 56 U/L
	Rabbit 7	28	61	197	209	258	18 - 56 U/L

Bioassay 1 = control group
Bioassay 2 = test group

T = time in days
U/L = units per liter

2.5 CONTROL RABBITS

2.5.1 Histology

The hepatic morphology analysis (histology, cytology and histochemistry) of the rabbits that were not exposed to esters showed that the liver was intact (Figs. 1 B-C), as expected. The hepatocytes were distributed into cords regularly organized and with intact morphology, i.e., cubic cells with one or two round-shaped nuclei (Fig. 1 C), also presenting intact morphology. The Kupffer cells (hepatic macrophages) were scattered among the hepatic cords and showed elongated and intact nuclei (Fig. 1 C).

2.5.2 Histochemistry

The control rabbits were tested for total proteins and neutral polysaccharides, and the results showed no alterations in the tissue or in the hepatic cells (Figs. 1 F-G, J-K).

The cytoplasm of the hepatocytes was vacuolated (unstained round-shaped spaces), (Fig. 1 G), likely representing the sites with an accumulation of lipid drops, which could not be identified due to the specificity of the technique applied (not suitable for lipid detection).

The test to detect the presence of polysaccharides showed that the glycogen, which is stored in the liver, was distributed in the cytoplasm of the hepatocytes, mainly in the periphery (Fig. 1 K) in the form of fine granulation.

2.5.3 Cytochemistry

The cytochemical test demonstrated the absence of acid phosphatase in the liver of the control rabbits, indicating low hydrolytic activity in the hepatic tissue (Figs. 1 N-O).

2.6 RABBITS EXPOSED TO ESTERS

2.6.1 Histology

The analysis of the liver of the rabbits exposed to esters showed a disorganization in the arrangement of hepatocytes in the hepatic cords (Figs. 1 D-E). Additionally, cell limit loss was observed, most likely due to the toxicity of the bioactive. Extensive vacuolation was verified not only in the cytoplasm, but also in the spaces between the cells (Figs. 1 E-F). The nuclei of these cells were very irregular, i.e., loss of original morphology (Fig. 1 E), and presented intense pyknosis (Fig. 1 E) followed by the dislocation of the organelle from the center to the periphery of the cell, most likely due to the massive number of vacuoles.

2.6.2 Histochemistry

The total protein test of the rabbits exposed to esters (Figs. 1 H-I), showed less intense staining in comparison with the ones not exposed to the bioactive.

The presence of round-shaped spaces in the cytoplasm of the hepatocytes, previously occupied by lipid drops, also appeared to have decreased, as unstained regions (vacuoles) were less frequent in this case (Fig. 1 I).

The results of the glycogen test showed that, in the exposed rabbits, this element had its distribution altered throughout the hepatic tissue. The glycogen presented a rough granulation and was observed both in intracellular and extracellular regions, and, in the latter case, also deposited in the tissue spaces (Fig. 1 M).

2.6.3 Cytochemistry

The acid phosphatase activity test confirmed the presence of the enzyme in the hepatocytes of the rabbits exposed to esters, as the cytoplasmic staining indicated intense hydrolytic activity of the liver cells in this group (Figs. 1 P-Q).

Figure 1

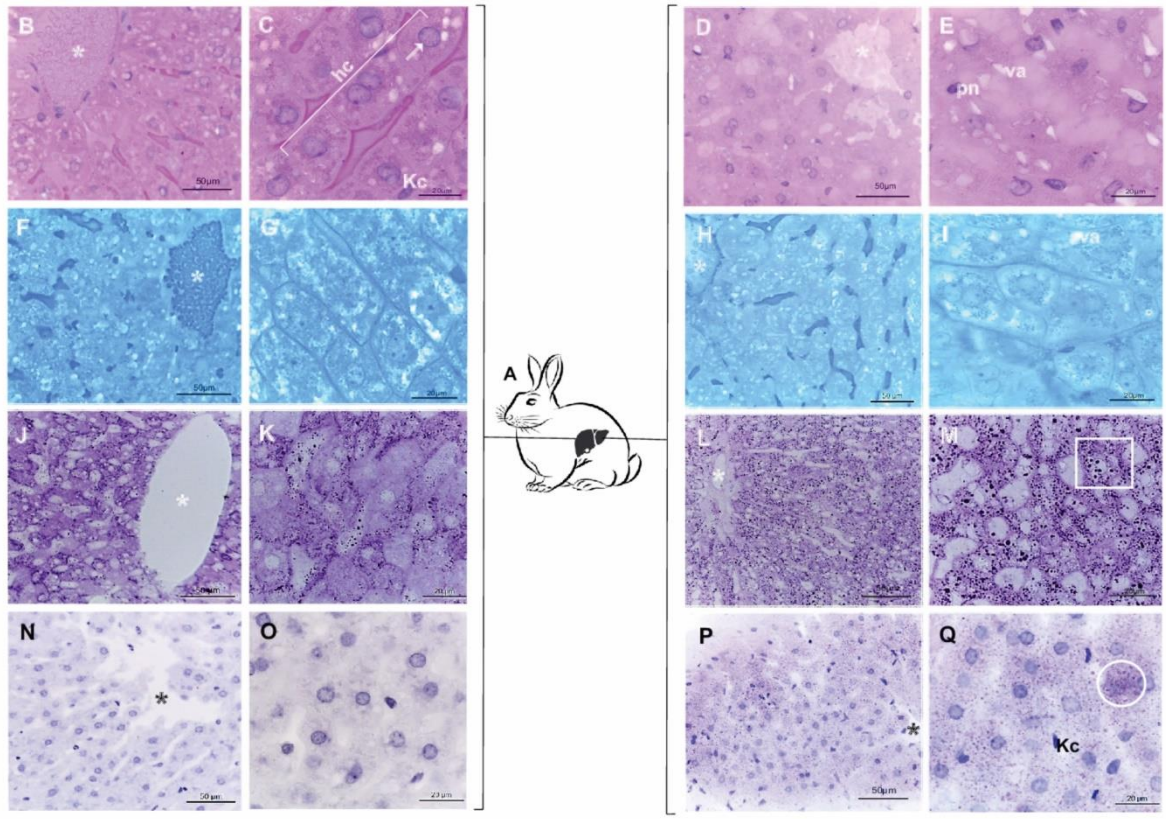

Figure 1: Schematic representation showing the position of the liver (A) and histological sections (B-Q) of the liver. B-C: Bioassay 1: histological sections of the liver stained with hematoxylin and eosin (HE). D-E: Bioassay 2: histological sections of the liver stained with hematoxylin and eosin (HE). F-G: Bioassay 1: Total proteins of the liver stained with bromophenol blue. H-I: Bioassay 2: Total proteins of the liver stained with bromophenol blue. J-K: Bioassay 1: polysaccharides of the liver – PAS staining. L-M: Bioassay 2: polysaccharides of the liver – PAS staining. N-O: Bioassay 1: acid phosphatase activity in the liver.P-Q: Bioassay 2: acid phosphatase activity in the liver.

3 DISCUSSION

Over the last decades, the literature has reported the biological properties of castor oil ricinoleic acid, which has been used in several economic sectors in the composition of paints, enamels, pigments, disinfectants, low-temperature lubricating oils, glues, nylon, polymers and plastics (MESSETTI, 2010).

These bioactives have been recently proven to contain excellent acaricides, and some authors regard them as an efficient strategy to control ticks (ARNOSTI et al., 2011a, b; SAMPIERI et al., 2013a, 2015; CAMARGO-MATHIAS, 2018). Although the acaricidal potential of esters has already been demonstrated, there are still some research

gaps regarding nontarget organisms (hosts) responses when exposed to the chemical. Such demand motivated the present study, which aimed to contribute more information on the use of esters as an efficient control strategy that is both sustainable and safe for the hosts and other nontarget organisms.

Considering the relevance of the data, the objective of the present study was to evaluate the response of the host rabbits when fed with commercial food with added castor oil ricinoleic acid esters (laboratory experiments), which would provenly control R. sanguineus s. l. infestations. For this purpose, histological, histochemical and cytochemical techniques were applied to the hepatic tissue of the tested animals, and the blood biochemical parameters were analyzed as well.

In contrast with previous studies (ARNOSTI, 2011; SAMPIERI et al., 2013a), the results revealed that the rabbits exposed to esters (Bioassay 2) had their histological and cellular structure altered, which was confirmed by a disarrangement in the shape and organization of hepatic cords. Such alterations triggered a complete disorganization of the blood circulation pathways in the organ, directly interfering in its physiology.

The morphophysiological modifications in the liver of the rabbits exposed to esters were strongly corroborated by the alteration of the enzyme AST (aspartate aminotransferase), detected through the analysis of the blood samples collected. According to the literature, AST levels increase when the animal presents any type of hepatic pathology, or even in the presence of necrosis processes. Approximately 80% of the enzyme AST is present in the mitochondria, and, in association with ALT (alanine aminotransferase), indicate alterations and signalize the onset of hepatic diseases. It is important to highlight that in the case of mild hepatocellular damage, the enzyme ALT predominates in the blood serum, while in more severe lesions a release of AST (mitochondrial) occurs. Therefore, the results of the present study regarding the AST levels of the rabbits exposed to esters suggest that morphological damage (with consequent physiological alterations in the organ) were intense, reinforcing the hepatotoxic potential of these bioactives.

The histological and histochemical analyses confirmed the hepatoxicity of the esters on the hepatic tissue of the treated rabbits, showing both tissue and cell alterations. This data was corroborated by the presence of hepatocytes undergoing a cell death process and tissue necrosis, which was demonstrated by strong acid phosphatase staining in the cytoplasm of the hepatocytes. This effect most likely occurred due to the intense

hydrolytic activity in the cytoplasm of the hepatocytes, which favored the degeneration of the cells, also triggering tissue disorganization.

Although there are still few studies in the literature regarding the effects of natural acaricides on different tissues of tick-infested hosts, the present study corroborates with Cunha et al. (2017), who studied the effects of bioactives on mice and found that they would be hepatotoxic to the hosts, causing damage to the cells, triggering death processes in vital organs (e.g., the liver) and eventually the death of the individual.

With specific regard to rabbits exposed to ricinoleic acid esters, the results of the present study showed an increase of polysaccharides in the cytoplasm of the hepatocytes, demonstrated by the strong and extensive staining of different regions containing glycogen deposits. This suggests that the esters provided through feeding stimulate glycogenesis in the hepatocytes, signaling that the most suitable pathway to obtain glucose would be via lipid degradation (not detected in this study; only the spaces where they would be stored in the cytoplasm of the hepatocytes were observed), rather than glycogen degradation, which would result in the release of energy to synthetize ATP. Therefore, the esters are most likely acting as regulators of the glycogenesis pathway in the hepatocytes, possibly with the participation of peroxisomes — cell organelles that would have the function of oxidating fatty acids, releasing acetyl coenzyme-A, an essential molecule for the synthesis of ATP during cellular respiration processes (ALBERTS et al., 2017).

Thus, these findings demonstrate that the castor oil ricinoleic acid altered the morphophysiology of the hepatic tissue of the exposed rabbits. Therefore, despite being an efficient acaricide against R. sanguineus s.l. ticks, the chemical is hepatotoxic to the hosts. Such data indicate that this natural alternative to control ticks still must be classified as not completely safe, although some previous studies oppose this statement (ARNOSTI et al., 2011a; SAMPIERI et al., 2012a), as their authors reported that esters would be beneficial to hosts (cattle), mainly for having caused the animals to gain weight during the experiments.

The results of the present study demonstrated that these bioactives, in the conditions and concentrations used here, caused hepatoxicity, which would prevent esters from being recommended for safe use. Thus, for these bioactives to be considered as a viable alternative to control tick infestations, further studies are needed, mainly ones including in vitro cytotoxicity tests.

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