



Latex proteins from *Calotropis procera*: Toxicity and immunological tolerance revisited



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ABSTRACT

Many thousands of plants are disseminated worldwide in traditional and folk medicines based on the belief that their leaves, roots, seeds, bark or secretions, when adequately handled, can treat, alleviate or ameliorate numerous disease symptoms. *Calotropis procera* (Apocynaceae) is a popular medicinal plant and the claims of this shrub's phytomedicinal properties have been scientifically validated. In this study, further prospects towards the *in vivo* toxicity and oral immunological tolerance of phytomodulatory proteins isolated from the latex of *C. procera* are reported. Acute toxicity was determined in mice by oral and intraperitoneal administration of latex proteins (LP) and was followed behavioral, hematological and histological analyses. Oral immunological tolerance to LP was assessed by intraperitoneal immunization in mice that had received LP orally before. Animals given 5000 mg/kg orally exhibited only discrete behavioral alterations and augmentation of monocytes. Death was not notified 14 days after exposure. However, all animals receiving LP 150 mg/kg by i.p. died in 1 h. Death (20%) was documented when LP (75 mg/kg) was given in the peritoneum and signs of harmful effects were observed in the survivors (80%). Oral immunological tolerance was observed in animals previously given LP orally, when they were further immunized/challenged with peritoneal exposure to different doses of LP. This was confirmed by the lowering of IgE and IgG in the serum, IL-4 and IFN- γ in spleen homogenates and the absence of anaphylaxis signs. It is therefore concluded that LP exhibited quite discrete adverse effects when orally administered at higher concentrations and this route of administration did not stimulate adverse immunological reactions. Instead it was observed immunological tolerance. The present study contributes very important information concerning the safe use of *C. procera* as a phytotherapeutic agent.

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1. Introduction

Regardless of the numerous medicines available for the treatment of human illnesses, especially those addressed to chronic diseases, the discovery of new drugs providing better clinical results and reduced side effects challenges the pharmaceutical

industry worldwide. The concept of highly purified chemicals associated with efficacy or monofunctional drugs has been reviewed to explore the benefits of new therapies encompassing natural sources belonging to traditional medicines [1]. As a result, phytomedicine is once again in focus. This time, however, it is not seen only as a source of new chemicals. The prime aim is no longer to search for new chemicals and activities or to revalidate documented pharmacological activities to confirm potentialities to the market. Rather, it is intended to establish the safety of the use of phytomedicines and their potential for practical use in different molecular correlated sicknesses, and also to ensure better

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homeostasis for patients during therapies. Indeed, the bio-prospecting of natural sources for a wide range of ailments, infections and syndromes are daily reported [2]. However, adverse effects of these materials are quite neglected and finding further toxicological evaluation is quite rare.

The plant *Calotropis procera* (Apocynaceae) is a shrub, well established in tropical regions, and is native to India. There, the recognized status of this medicinal plant, historically documented by its popular use, permitted its inclusion in the worldwide-recognized pharmacopoeia compendium Ayurveda [3]. In the last 20 years many of the claimed pharmacological properties related to this plant have been scientifically confirmed by Indian researchers. The anti-inflammatory activity of the latex of *C. procera* was first reported by Kumar and Bazu [4], followed by its analgesic activity [5], and later its antidiarrheal action [6]. After characterizing its activities on conceptual models, the efficacy of this phytomedicine was successfully examined in more reliable models, such as on gastrointestinal smooth muscles [7] and its antioxidant and protective effects on diabetic rats [8], among others. These authors also reported sequential studies that drew attention to the proinflammatory activity of the latex, suggesting that adverse compounds were also present in the plant [9]. In the second half of the 2000s, we joined efforts with the Indians to better understand the pharmacological potentialities and toxicological limitations of the latex of *C. procera*. By separating soluble proteins from rubber and other chemical constituents of the whole latex, we preliminarily demonstrated that the latex proteins displayed anti-inflammation activity by i.v. administration while the complementary fraction retained pro-inflammatory activity [10]. Investigating a chemically well-defined source (LP) we later successfully demonstrated the anti-tumoral [11], anti-arthritis [12,13] and coagulation homeostasis [14] properties of latex proteins of *C. procera*. However, outstanding results were achieved when we established the ability of the latex protein fraction to prevent septic shock in animals lethally infected with pathogenic bacteria [15], and to eliminate all clinically measurable adverse effects of oral and intestinal mucositis induced in experimental animals given 5-fluorouracil and irinotecan, respectively [16,17]. In all these cases, the ability of LP in inhibiting inflammation by modulating important inflammatory mediators was mechanistically demonstrated. Because of this, LP preliminarily met important prerequisites sought in modern medicines, such as to be potentially advantageous in application to more than one clinical purpose. However since LP is composed of proteins the possible toxic or allergenic effects of these must to be adequately addressed. The current understanding about the safety use of LP is limited to our own studies and therefore there is an urgent need to advance this understanding regarding the toxicological and immunological knowledge of this phytomedicine. This was the purpose of this study.

2. Materials and methods

2.1. Plant material and latex processing

Fresh latex was collected in tubes (1:1, v/v in distilled water) from the aerial parts of *C. procera* (Ait.) R.Br. growing in the vicinity of Fortaleza, Brazil. The plant voucher (sample specimen no. 32663) used for identification of the botanical material collected is deposited at Prisco Bezerra Herbarium of the Federal University of Ceará, Brazil. After the latex was collected, it was processed as described before by Alencar et al. [18]. Briefly, the mixture was centrifuged at 5000 g (10 °C for 10 min) to partially precipitate the rubber, following dialysis against distilled water (1:10, v: v) at 8 °C for 60 h using dialysis membranes with a cutoff of 8000 Da. After being centrifuged under the same conditions, a cleaned

supernatant rich in proteins and devoid of rubber was obtained. These soluble proteins (LP) were lyophilized and used in the study. LP was also subjected to fractionation by ion exchange chromatography with a CM Sepharose fast-flow column previously equilibrated with 50 mM of acetate buffer (pH 5.0) following the protocol proposed by Ramos et al. [19]. Three protein fractions, named LP-PI, LP-P II and LP-P III, were obtained, lyophilized and then stored at 25 °C. LP and the fractions LP-PI and LP-P II were used in the study. LP-P III was not included. The rationality to exclude LP-P III of the study is because (1) there is not data reporting pharmacological activity of this sample; (2) LP-P III comprises residual proteolytic activity found in LP-P II and therefore is nearly similar to LP-P II in terms of chemistry and activity; (3) almost all of the pharmacological effects reported in LP were later confirmed in LP-PI or LP-P II but not in LP-P III or only marginally observed, confirming the similarities with LP-P II. The use of LP-P II-IAA but not LP-P II was justified by the previous observation that LP-P II injected by route i.p. at 5 mg/kg induced high mortality in animals with macroscopy damage and hemorrhages seen in the peritoneal cavity of the animals. These experimental procedures performed in parallel guided us to exclude the evaluation of LP-P II of the present study since it was established its strong adverse effect in other ongoing studies. LP and LP-P II were treated with 30 mM iodoacetamide (IAA) to inhibit the endogenous proteolytic activity reported previously by Ramos et al. [20]. When treated with IAA, LP-P II completely lost the proteolytic activity since a covalent bond is irreversibly formed between the proteins and IAA. After completing the reaction, LP-P II (now LP-P II IAA) was exhaustively dialyzed with distilled water for 72 h and all remaining free IAA was washed out in the dialyses water. All the samples were tested in the acute toxicity experiment. Only LP was assayed orally in the tolerance experiment. LP and the cited protein subfractions (LP-PI; LP-P II and LP-P III) were extensively studied in terms of their biochemical and pharmacological characterization aspects [11,16,21]. Briefly, cysteine proteases, chitinases, peroxidases, germin and osmotin have been identified as the major proteins present in LP. LP-PI is abundantly formed by chitinases [21], LP-P II comprises strong proteolytic activity of cysteine proteinases [20] and minority osmotin [22]; LP-P III is composed of the residual proteolytic activity found in P II. The other cited proteins were previously investigated only in LP.

2.2. Animals

Adult (8–12 weeks) female Swiss (26 ± 5 g) mice (*Mus musculus*) were obtained from the Central Animal House of the Federal University of Ceará (UFC), Brazil. Female BALB/c (20 ± 3 g) mice were purchased from the University of Fortaleza, Brazil. The former were used in acute toxicity tests and the latter were used in oral tolerance experiments, respectively. The animals were housed in plastic cages under controlled laboratory conditions (temperature: 25 ± 3 °C, humidity: $55 \pm 10\%$, light/dark cycle: 12/12 h) and provided with water and food *ad libitum* (commercial sterile diet; Purina, Paulínia, SP, Brazil). To assay passive cutaneous anaphylaxis (PCA) reactions, 6-month-old female Swiss mice (38 ± 3 g) were used and maintained under similar conditions as above. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institute of Health (NIH 1985), and were approved by the Ethical Committee of the Federal University of Ceará (protocol n°8/2016).

2.3. Acute toxicity

For the determination of acute toxicity, the acute toxic class method Organization for Economic Co-operation and Development

[23], with modifications, was followed. Toxicity assays were performed via two routes, oral (p.o) and intraperitoneal (i.p). For the oral test, the animals were fasted 4 h prior to a single LP dose of 5000 or 2000 mg/kg/body weight (bw) ($n = 5$ females/group) and control group was administered with sterile saline. For the i.p test ($n = 5$ females/group), the dose (at different concentrations of LP) was given in a stepwise procedure with the initial dose being selected as the dose expected to produce mortality in some animals (LP 300 mg/kg/bw), followed by lower doses (LP 150–15 mg/kg/bw) until achievement of the classification of the test substance within a Global Harmonized System (GHS) toxicity class.

To investigate some macroscopic biological aspects of the toxicity displayed by the tested samples given i.p, five experimental groups were established ($n = 5$ females): control group (treated with saline, i.p) and test groups (treated with LP 75 mg/kg/bw; LP 75 inhibited with iodoacetamide (IAA); and LP fractions (PI 75 and PII 75 IAA), i.p). The IAA was used to inhibit the action of proteases, which are at higher concentrations in LP-PII fraction and could account for toxicity per route i.p.

After the acute administration, the animals were observed closely for the first 8 h and thereafter daily up to 14 days. Records on physiological parameters (water and food consumption, urine and excreted production and body weight) were made daily. In addition, the animals were also observed for general health and clinical signs of toxicity following the criteria used by Brito [24]. At the end of the observation period, the animals were anesthetized for bleeding and the blood samples recovered were further used for the determination of biochemical and hematological parameters. Next, the animals were sacrificed by ketamine/xylazine overdose ($>100/10$ mg/kg, s.c, União Química, São Paulo, Brazil). The vital organs such as liver, kidney, spleen, heart and lung were weighed and placed in 10% formaldehyde for histological analysis. The liver was frozen at -80°C until use.

2.4. Cellular and biochemical analyses of blood

The blood samples were collected from the retro-orbital plexus under anesthesia with a combination of ketamine (60 mg/kg) $\pm$ xylazine (8 mg/kg) and divided into two aliquots. One was anticoagulated for the hematological examination and the residual was allowed to clot to recover serum and use it in the biochemical analysis. Cell counting was analyzed with an optical microscope. The non-heparinized blood was allowed to coagulate and centrifuged at $5000 \times g$ for 10 min after collection. The serum was analyzed for biochemical parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (Cre) and urea according to the manufacturer's instructions for the kits used (LABTEST, São Paulo, ref.: 52; 53; 35 and 27, respectively).

Peroxidation of lipids was measured in the liver in terms of malondialdehyde (MDA) concentration, following by the thio-barbituric acid method similar to the work of Agar et al. [25]. The standard curve was obtained by using 1,1,3,3-tetramethoxypropane. The results were expressed as nmol/g hepatic tissue.

2.5. Oral tolerance

To induce oral tolerance, different concentrations of LP (2.5, 5 and 10 mg/kg/bw) in 200 μl of sterile saline were given to the mice (BALB/c) by gavage every day for 10 days. An additional booster was given on the 21st day following the same conditions. The mice of the control groups received only saline, following the same protocol by oral route. A positive control with ovalbumin (OVA) was used. Seven days after completing the oral booster schedule (day 28), all of the mice were immunized with an intraperitoneal injection of

10 μg of LP or OVA mixed with 1 mg of aluminum hydroxide in 0.15 M NaCl (saline) solution, according to the groups. Fourteen days later (day 42), the mice received an i.p challenge dose of 10 μg of the antigen (LP or OVA) in saline solution. At day 49 the mice were bled by the retro-orbital plexus and the blood samples were used for hematological and immunological assays. After this the animals were injected subcutaneously (s.c.) (50 μg in 50 μl saline of desired samples) for DTH determination as described later. The experimental protocol proposed here was motivated by the previously described model reported by Ramos et al. [26] and intended to progress the former study. The studies of Simioni et al. and Kim et al. [27,28] were taken as reference for other aspects.

After the immunization (day 28) and i.p. challenge (day 42), symptoms of anaphylaxis and body rectal temperature were assessed. In addition, delayed-type hypersensitivity (DTH) reaction was evaluated at the end (49–50 days) of the oral tolerance experiment.

At the end of the experiment (day 50), the mice were sacrificed and the lymphoid organs such as spleen and thymus were weighed. The small intestine (jejunum) was placed in 10% formaldehyde for further histological analysis.

2.6. Anaphylactic symptoms

Immediately after the immunization (day 28) and challenge (day 42) of the mice, anaphylactic symptoms were scored according to the method described earlier by Kumar et al. [29]. The score system proposed by these authors covers a range of notes from 0 to 5: mice without symptoms (Score 0); scratching and rubbing around the snout and head (Score 1); puffiness around the eyes and snout, piloerection, diarrhea, and reduced activity or standing still with an increased respiratory rate (Score 2); wheezing, labored respiration, and cyanosis around the mouth (Score 3); symptoms as in n°3 with loss of consciousness, tremors and/or convulsion (Score 4); and death (Score 5). Rectal temperature was monitored 20 min after immunization or challenge using a digital rectal thermometer (Brasmed, Brazil).

2.7. Delayed-type hypersensitivity reaction (DTH)

At day 49 of the oral tolerance protocol, a recall challenge with 50 μg LP in 50 μl sterile saline in the right hind foot-pad and 50 μl saline in the left hind foot-pad of mice were given and DTH reactions were measured according to Kumar et al. [29]. Swellings of foot-pads were measured before and 24 h after the immunization using a micro-caliper (0–25 mm digital caliper, Forbes).

2.8. Immunological assays

Immunoglobulin levels IgG, IgA and IgE in the sera of the mice submitted to different treatments were determined by a standard enzyme-linked immunosorbent assay (ELISA). Specific IgG and IgA levels were estimated according to the protocol performed earlier by Ramos et al. [26]. In brief, assays were developed in 96-well microplates and antigen (LP) was prepared at concentrations of 8 $\mu\text{g}/\text{ml}$ to detect (IgG) and 31 $\mu\text{g}/\text{ml}$ to detect (IgA). The OVA was 10 μg per well for both IgG and IgA. Sera of 49 days (oral tolerance protocol) were diluted in the ratio of 1:10,000 (IgG), 1: 5000 (IgA) and secondary antibodies conjugated to phosphatase alkaline were diluted to 1:2000. Alkaline phosphatase reaction using the substrate p-nitrophenyl phosphate disodium (5 mg/ml) was stopped by adding of 50 μl of 4N NaOH, and absorbance was determined at 492 nm. To the determine total IgE levels, the sera of treated mice were evaluated by a commercially available enzyme-linked immunosorbant assay (ELISA) kit following the manufacturer's

instructions (Mouse IgE ELISA kit, ref. RAB0799, Sigma Aldrich-Saint Louis, MO, USA). All the measurements were made in triplicate.

The immunoglobulin levels of IgG1 were measured by PCA in 6-month-old Swiss mice with a 2 h latent period for skin sensitization, as cited in Melo et al. [30]. The PCA reactions were elicited by intravenous injections of the antigens via the retro-orbital plexus. The antigens (LP or OVA) were prepared in 0.5% Evans blue in sterile saline to correspond to 250 µg in 250 µl.

2.9. Cytokine measurement

The spleens of mice were homogenized and processed as previously described by Safieh-Garabedian et al. [31]. The measurement of interleukin IL-4, interferon (IFN)-γ and IL-10 concentrations was performed using mouse ELISA Kits IL4 and interferon (IFN)-γ (ref. RAB0299 and RAB0224, respectively (Sigma-Aldrich, Saint Louis, MO, USA) and DuoSet® ELISA Kits IL10 (ref. M1000B -R&D Systems, Inc., Minneapolis, USA). The steps were carried out according to the manufacturer's instructions. All measurements were performed in triplicate.

2.10. Histopathological analyses

Sections of liver, kidney, spleen, heart, lung and segments of small intestine (jejunum) were fixed in 10% buffered formalin. Posteriorly, the samples were dehydrated in absolute ethanol, immersed in xylene to eliminate debris and embedded in paraffin. Slides of 5 mm thickness were cut and the sections were stained with hematoxylin-eosin (H&E). Histopathological analysis consisted of the observation of tissue integrity, searching for injuries, such as degeneration, necrosis, apoptosis and infiltration of leukocytes, which could indicate signs of toxicity. The histological description of each sample examined was elaborated after examination of ten independent slices corresponding to at least two distinct initial materials.

2.11. Data analyses

Data are expressed as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was followed by Bonferroni's test, and Student's T-test, followed by Mann–Whitney. Statistical significance was accepted when $p < 0.05$. All data were analyzed using GraphPad Prism software version 5.0 (Graph-Pad Software, Inc., LaJolla, CA, USA).

3. Results

Oral acute toxicity was investigated in Swiss mice. The higher doses of LP (5000 and 2000 mg/kg/bw) did not adversely affect the animals in terms of survival. Death was not observed until day 14. However slight behavioral alterations were observed all within the first 24 h, i.e. hypothermia and abdominal contortions and legs stretching. At day 14 all animals seemed healthy and had gained weight similar to the control group. Macro physiological parameters accompanied during the experiments suggested that experimental and control animals performed similarly. The summary of these data are given in the [Supplementary Material Tables S1–S3](#) and [Fig. S1](#). It was documented increment of monocytes in the blood of animals given 2000 or 5000 mg/kg. This effect was also observed in the studies of Oliveira et al. [11] that showed *in vivo* tumor growth inhibition, when mice were given LP per oral. Increment on monocytes count suggests LP indeed triggers immune stimulation. In more recent studies LP has been termed as phytomodulatory due the ability to activate the immune-

inflammatory response [15].

When LP was given by intraperitoneal route (i.p.) to Swiss mice, doses of 300 mg/kg/bw provoked 100% death within 30 min. Immediately after injection it lethargy was observed, followed by intense convulsions and tremors, and then death. The examination of the peritoneal cavity revealed ruddiness, hemorrhages and evident tissue damage. Reducing the dose to 150 mg/kg/bw death persisted within 1 h. At 75 mg/kg/bw i.p. LP, 80% survival was observed until day 14. Animals lost body weight until day 4 followed by recovery comparable to the controls ([Fig. 1](#)). This profile was accompanied by adverse behavioral signs ([Table S4](#)). The overall performance of the animals in terms of macro physiological parameters is given in [Table 1](#). At this moment it was thought that the endogenous proteolytic activity of LP was involved in the adverse effects documented. To investigate this possibility, LP (75 mg/kg/bw) was inhibited with IAA, which fully blocks the activity of the cysteine proteinases existing in LP, as recognized before in studies of Ramos et al. [20]. In parallel, two lower doses of LP were tested. As expected, LP given at 35 and 15 mg/kg/bw did not lead to the animals' death. However, the animals still showed slightly diminished general activity. Discrete alterations in liver weight and monocyte counts in blood were seen after examination ([Tables S4–S6](#)). Monocytes were reduced in animals treated by i.p. and thus this result is opposite to that observed to animals given LP per oral that showed increment of monocytes. Since both treatments were different in terms of via of administration and the doses were drastically different it is difficult to comparatively argue both profiles, although LP is recognized as pro-inflammatory when injected by i.p. [15]. Animals given LP IAA at 75 mg/kg/bw grew similarly to the control animals and exhibited comparable performance to almost all parameters investigated. This sample did not repeat the adverse effects attributed to LP. This data suggested the adverse effects observed with LP could be the consequences of the proteolytic activity. When the subfractions LP-PI and LP-P II IAA were assayed i.p at 75 mg/kg/bw, LP-PI induced very slight piloerection and accelerated respiration in the first 4 h and LP-P II IAA only induced respiratory fatigue ([Table S4](#)). All animals survived until the end of the experiment.

The histological analysis of liver, kidney and spleen of animals (day 15) treated (i.p) with LP at 75 mg/kg/bw did not indicate signs of toxicity or tissue injuries ([Figs. S2–S4](#)). A similar profile was observed in animals given LP IAA at the same dose, with the exception of kidney, where discrete retraction on the Bowman's capsule was observed and rare and isolated foci of necrosis were seen ([Fig. 2](#)).

Discrete to moderate hydropic degeneration was perceived in the liver of animals given LP-PI (75 mg/kg/bw) ([Fig. 3](#)). Alterations to kidney, spleen, lung and heart were not observed for this treatment. LP-P II IAA (75 mg/kg/bw) did not induce detectable toxicity in any of the all tissues examined. Protocols performed with lower doses (35 or 15 mg/kg/bw) of LP indicated very discrete hydropic degeneration and vascular congestion (35 mg/kg/bw) and total absence of adverse signs (15 mg/kg/bw) in all histological evaluations. Heart and lung were histologically conserved in all groups treated with 75 mg/kg/bw. Some histological slides representatives are shown ([Figs. S5 and S6](#)).

Oral immunological tolerance was investigated in BALB/c mice. The earlier study of Ramos et al. [26] indicated that animals receiving LP orally did not respond immunologically. However, the authors reported that subcutaneous administration of LP induced appreciable immunological response, including strong PCA reaction, which is a recognized allergenic response. Since LP has been shown to have great potentialities as a phytomedicine, it is urgent that a better understanding be found regarding the possible adverse effects upon its use. Here the possible oral immunological

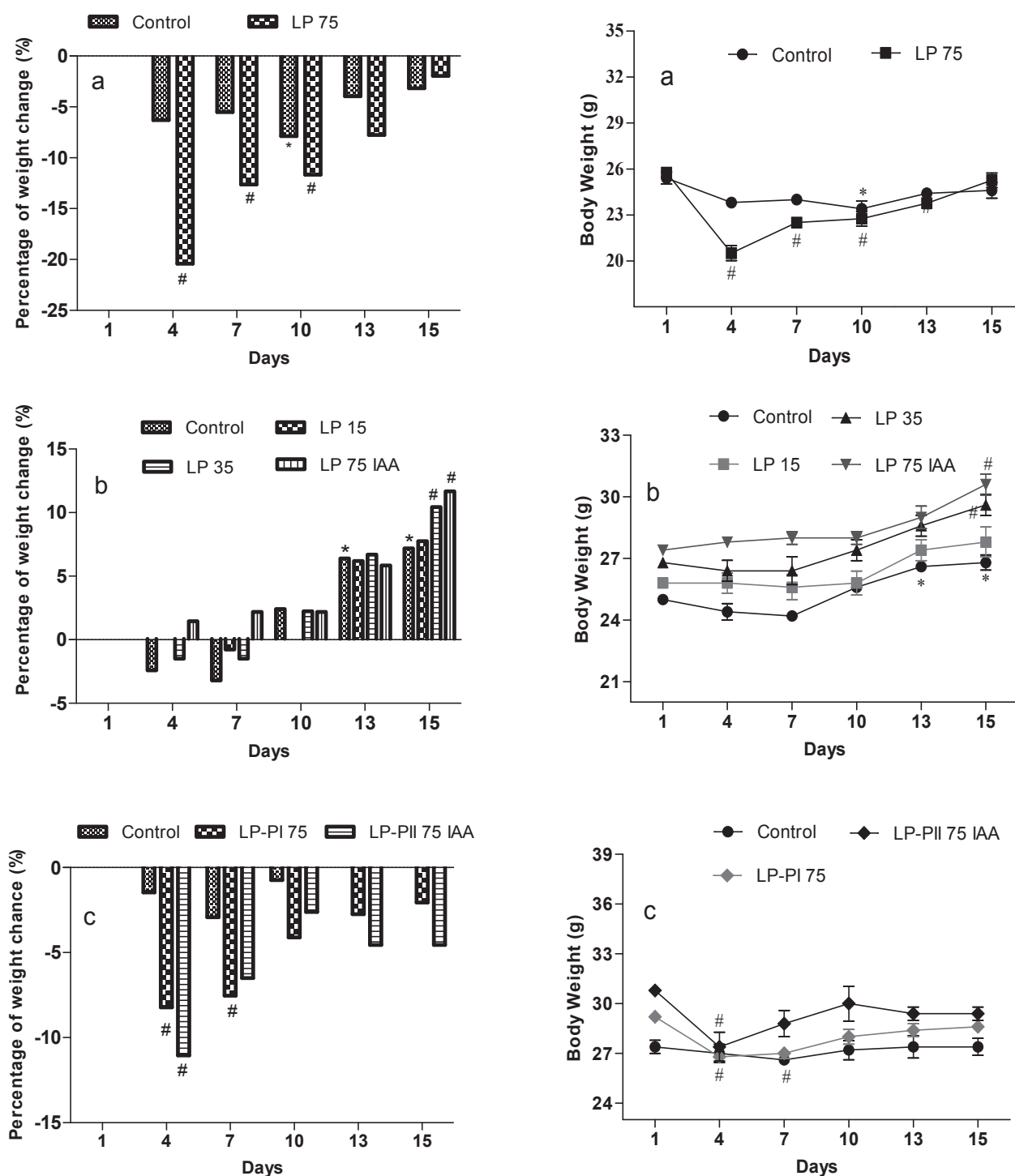


Fig. 1. Body weights in mice treated intraperitoneally with LP (a and b) or LP-PI/PII (c) in acute toxicity. Results are expressed in percentage of body weight variation annotated in the indicated days (right) and body weight progress (left). Independent controls were used in each set of experiments. IAA (iodoacetamide). LP-P II IAA represents the sample free of proteolytic activity. The values are presented as means $\pm$ SEM (5 mice/group). ($p < 0.05$ – ANOVA – Bonferroni's test. Control (*) and LP 15, 35, 75, 75 IAA, LP-PI and PII IAA (#) in relation to the 1st day).

tolerance for LP was tested following the classical protocol found in the literature [27,28]. The general scheme of immunization/tolerance performed is recorded in Fig. 4. Treatment of animals with LP did not alter body temperature, and body weight loss was not perceived. Similarly, key organs, such as spleen and thymus, overall exhibited comparable aspect and weight. The general profile of the animals is documented in the supplementary material

(Figs. S7–S9). The data suggested the absence of differences among the groups.

The count of monocytes on all groups compared at the beginning and the end of the experiment were all statistically similar between the control (LP; i.p.) and the groups (LP oral followed i.p) (Table S7). This was the criterion we used to examine the data. According to the literature, variances for monocytes in BALB/c

Table 1

Physiological parameters in mice treated intraperitoneally with LP and fractions inhibited with iodoacetamide (LP 75, LP 15–75 IAA, LP-PI and LP-PII IAA -75).

Groups	Parameters				
	Excreted (g/day)	Pellets (n°/day)	Urine (n°/day)	Water (ml/day)	Food (g/day)
Control	2.15 ± 0.31	98.20 ± 8.92	2.45 ± 0.46	6.54 ± 0.54	4.30 ± 0.65
LP 75	1.95 ± 0.89*	90.59 ± 26.94*	2.73 ± 0.58	6.64 ± 1.67*	3.91 ± 1.66*
Control	2.75 ± 0.34	101.60 ± 10.41	2.43 ± 0.43	7.69 ± 0.86	4.77 ± 0.86
LP 15	2.94 ± 0.57	99.87 ± 12.82	2.44 ± 0.47	7.31 ± 0.95	4.99 ± 1.12
LP 35	3.20 ± 0.68	79.04 ± 13.13*	2.81 ± 0.35	8.77 ± 0.99	5.17 ± 1.10
LP 75 IAA	3.63 ± 0.84*	86.27 ± 17.16*	2.67 ± 0.49	9.71 ± 1.42	5.63 ± 1.27*
Control	1.87 ± 0.33	86.53 ± 10.57	2.81 ± 0.29	7.70 ± 1.50	4.40 ± 0.80
LP-PI 75	1.82 ± 0.42	83.96 ± 16.75*	2.94 ± 0.29	7.83 ± 1.70	4.51 ± 1.15
LP-PII 75 IAA	1.96 ± 0.64*	85.87 ± 23.85*	3.10 ± 0.27	7.63 ± 1.80	4.63 ± 1.29*

Physiological parameters: production of excretas, pellets and n° humid wells of urine and consumption of water and food. The values are presented as means ± SEM (5 mice/group). ($p < 0.05$ ANOVA - Bonferroni's test compared with the control until the 6th day of treatment (*).

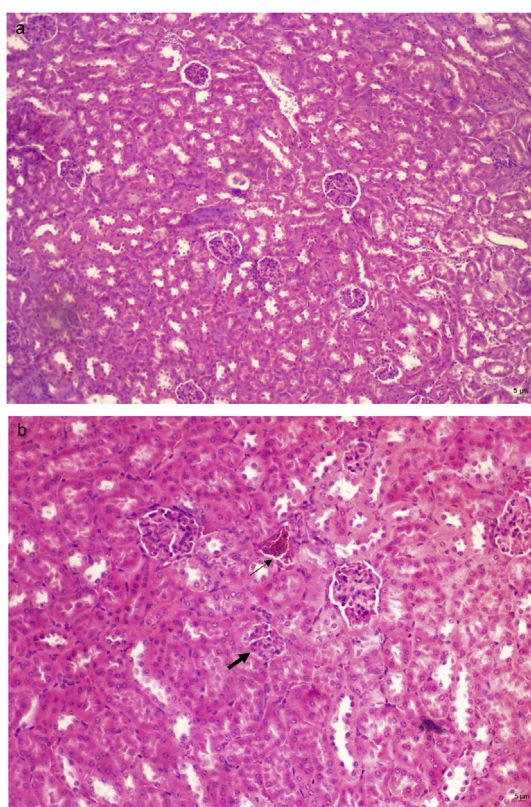


Fig. 2. Effect of LP (75 IAA, i.p.) on the microstructures of kidney of mice. LP 75 IAA group: alteration of the glomerular structure, necrosis process (thin arrow) and Bowman's capsule retraction (thick arrow).

between 0 and 6 are clinically normal [32]. Therefore the data were considered unaltered among the groups, without statistical significance by this criterion used and by the statistical analysis performed.

The data shown in Figs. 5 and 6 suggest that an oral immunological tolerance may have been achieved in animals that have been formerly treated orally and then immunized/challenged by i.p. administration of LP. The serum level of IgE, the pivotal marker of allergy, was increased in animals immunized with LP and in the control (OVA), indicating an allergenic response in both cases (Fig. 5a). Animals first given LP, orally, and thus immunized/challenged by being given it intraperitoneally, exhibited diminished IgE levels. This also occurred in animals given OVA. Secondarily, the IgG

level was similar to that of the control (LP) when animals were treated orally at 10 mg/kg. Lower doses still induced IgG response (Fig. 5b). The tolerance protocol with LP at different concentrations did not modify the profile of IgA and IgG1 among the LP groups that were later immunized/challenged with 10 µg LP (Fig. 5c and d). Although IgA is not the key response that characterizes allergy, it helps to understand the overall response upon allergenic antigens. Data of Fig. 5c suggested that LP induced IgA production whether compared to the control OVA that did not produce response. Comparing among LP treatments, the data indicate that both treatment; by i.p. only; or by oral and later by i.p. were similar, thus LP increases IgA response which indicates protection of mucosa. While increasing of IgE is high associated with allergy, in the case of IgA it can be motivated by different events and thus we cannot speculate. However we can remember that IgA produced in mucosal response is an important regulatory factor for possible cases of allergy since its production inhibits anaphylactic responses induced by allergic antigens [33]. Therefore increment of IgA levels in response to LP treatments were not exacerbated and could be beneficial to some aspects contributing to the tolerance of LP observed.

Further measurements of inflammatory mediators involved in allergic reactions such IL-10, IL-4 and (IFN)-γ agreed with this understanding. Notable reduction of IL-4 and (IFN)-γ levels in the spleen homogenates of animals immunized/challenged with LP suggested tolerance (Fig. 6a and b). This is also expected when immunological tolerance occurs [27,34]. The level of IL-10 was similar within the groups (Fig. 6c). The oral immunological tolerance was also suggested according to the data recorded for hypersensitivity reaction (Fig. 7, a–c). LP (10 mg/kg) did not produce symptoms of anaphylaxis after immunization (day 28) or challenge (day 42). DTH was, however, observed in animals given LP at 10 mg/kg. This was characterized by increased paw edema, seen after 24 h (Fig. 7 c).

Histological examination of intestinal slides (jejunum) representative of animals submitted to the tolerance protocol, showed only a slight increment of mucous cells (5.0 and 10 mg/kg LP) and mucus (10 mg/kg LP). Tissue integrity was similar among all groups (Fig. 8).

4. Discussion

The pharmacological properties currently documented for the shrub *Calotropis procera* indubitably validate its folk and traditional uses, mainly observed in Western Asia and North African countries. Although different parts of the plant are used for different purposes, the latex produced by the laticifers found in the plant

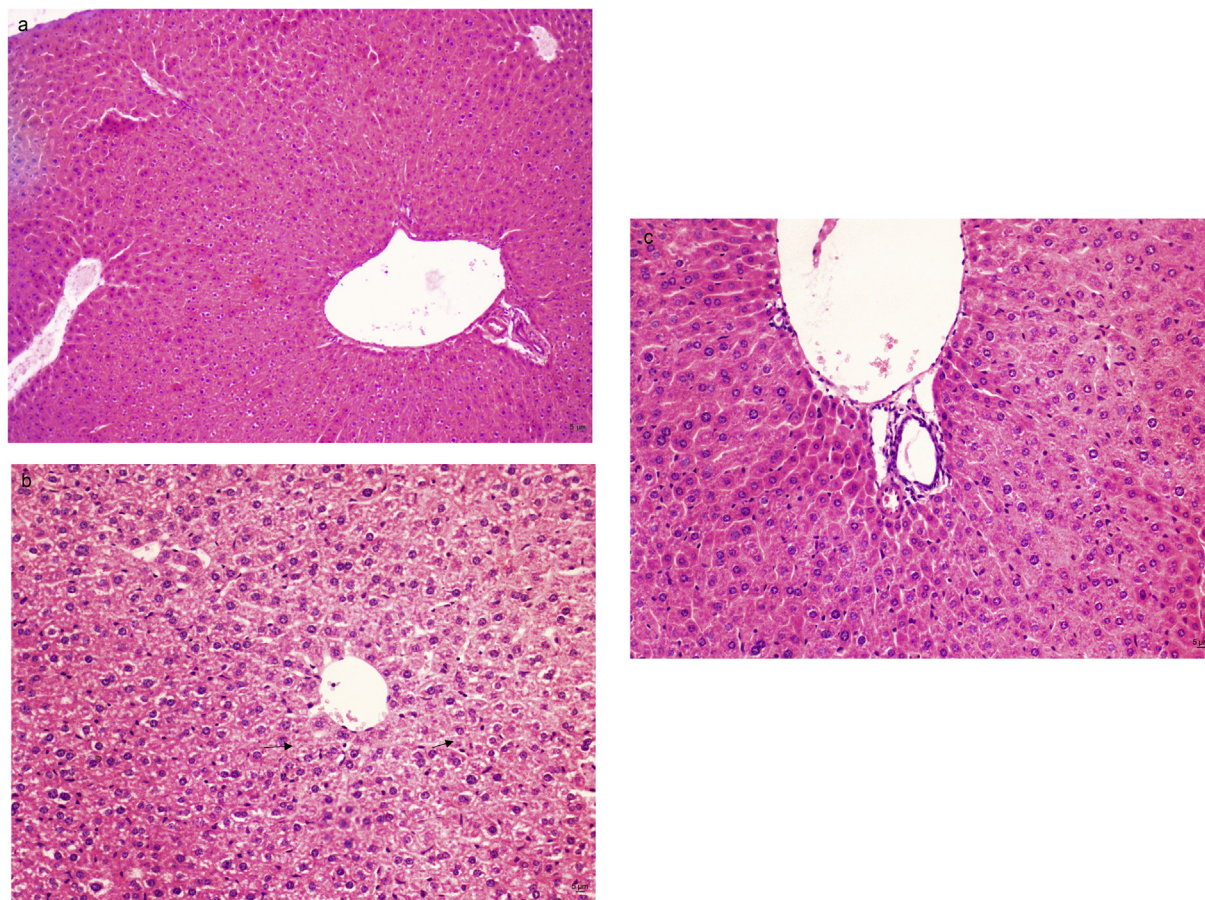


Fig. 3. Effect of LP-PI/PII (i.p) on the microstructures of liver of mice. (H&E stain, 10 and 20 $\times$) (Bar = 5 μ m). a: control group: no changes; b: LP-PI 75 group: mild to moderate foci of hydrophobic degeneration lobular center (black arrows); c: LP-PII 75 IAA: no changes.

segments seems to be the common source of the active compounds. The first challenge to confirm the potential of *C. procera* as a safe phytomedicine is to better understand its intrinsic toxicology. Earlier toxicological studies on goats reported the death of animals after receiving latex of *C. procera* orally or by intravenous or intraperitoneal routes [35]. Recently, Ahmed and co-workers [36] described important toxic effects of ethanolic leaf extracts and latex of *C. procera* on animals after oral gavage, with severe damage to heart and testes and the most dramatic literature reports the ingestion of *Calotropis* used in suicide [37]. Indeed, the literature devoted to reporting the diversity of the toxicological aspects of the plant and of its latex continues to grow [38]. In our preceding studies with the latex of *C. procera* we determined that the rubber fraction of the latex comprises over 90% of the dry matter of the latex, and the soluble protein fraction is lower than 3%. We have no estimate for the content of other compounds such as secondary metabolites that are present in the latex. Many chemical properties and activities of latex proteins, however, have been described in the literature [11,16,21]. Hence, we suppose that rubber may account, at least in part, for some adverse effects reported with latex. Efficient removal of the rubber content is not a laborious task in the case of *C. procera* latex, and thus this should not be an impairment to fully exploring the pharmacological uses claimed for this plant. After collecting, dialyses and centrifugation, the laticifer proteins can be recovered and cleaned of rubber and other latex components [10]. While the toxicology of the whole latex has been addressed to a limited degree, and to some extent, that of organic fractions, the toxicology of latex proteins remains quite obscure. Regardless of

the progressive purification of latex proteins and the description of their molecular and functional properties, data recording toxicology are still unavailable. This is a conflicting situation since a robust literature has pointed to proteins of latex as important candidates for displaying the diverse pharmacological properties currently known in latex. This includes suppression of tumor growth, anti-arthritic and anti-septic effects and preventing oral and intestinal-induced mucositis [11–13,15–17]. In addition, two important questions must be addressed. First, worldwide referenced experimental conventions that are performed, in order to prospect for *in vivo* pharmacological responses of natural drugs, are based in acute response. The animals tested are sacrificed a few hours after treatment in order to measure biochemical and physiological parameters, among others. Second, even though the folk or herbal uses are known in terms of health purposes and efficacy, frequently these therapies are empirical, without a defined time-course, daily dosages and with no specific frequency of use. Therefore, in this report our current efforts were addressed to giving new insights into the potential of the laticifer proteins extracted from the latex of *Calotropis procera* as phytotherapeutics, taking into account all of our previous experience with the biochemical and pharmacological properties of the sample, named LP.

The latex of *C. procera* possesses strong proteolytic activity consistently documented [20]. Our personal observations for more than a decade indicate this activity is perennial and quite constant in all samples tested after lyophilization. The data presented in this study and other unpublished observations permit us to suggest that

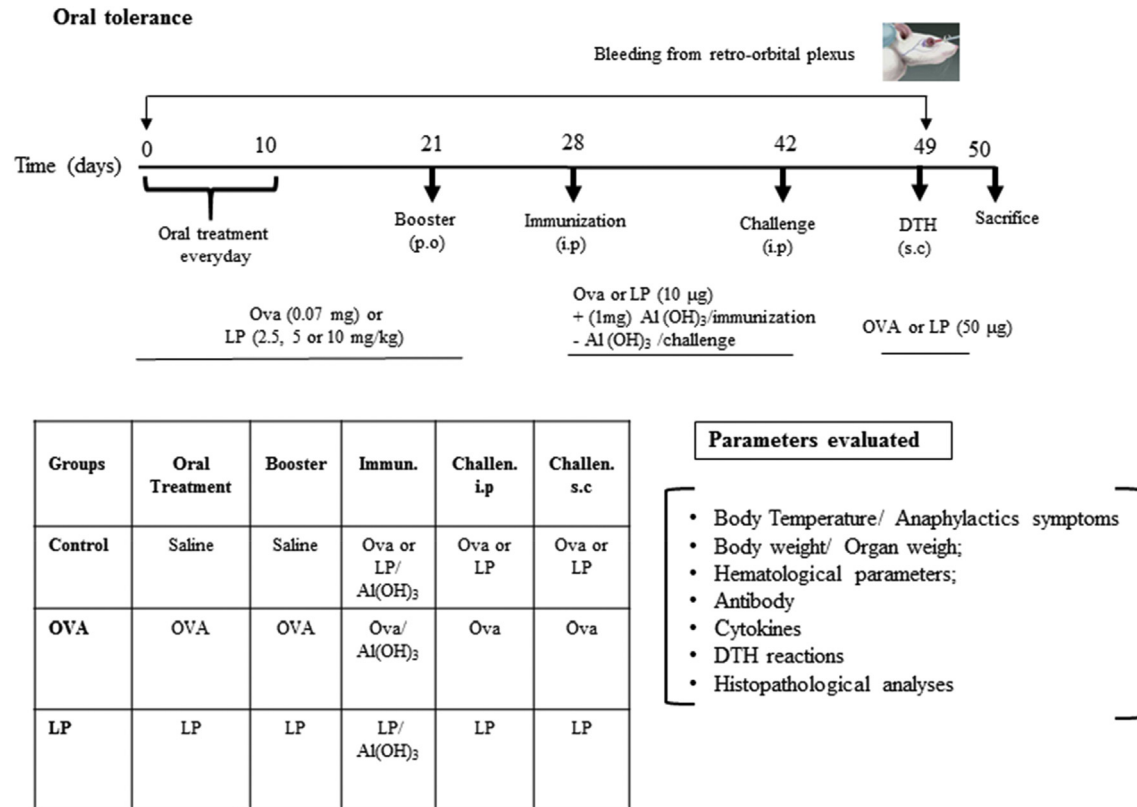


Fig. 4. Experimental design: Schedule of the oral tolerance protocol with parameters evaluated during the experiment. The stating point to define the experimental design was the study of Ramos et al. [26]. Further, the experimental design was elaborated following the strategies reported by Simioni et al. [27]; Kim et al. [28] and Kumar et al. [29] with adaptations to the hypothesis of the study.

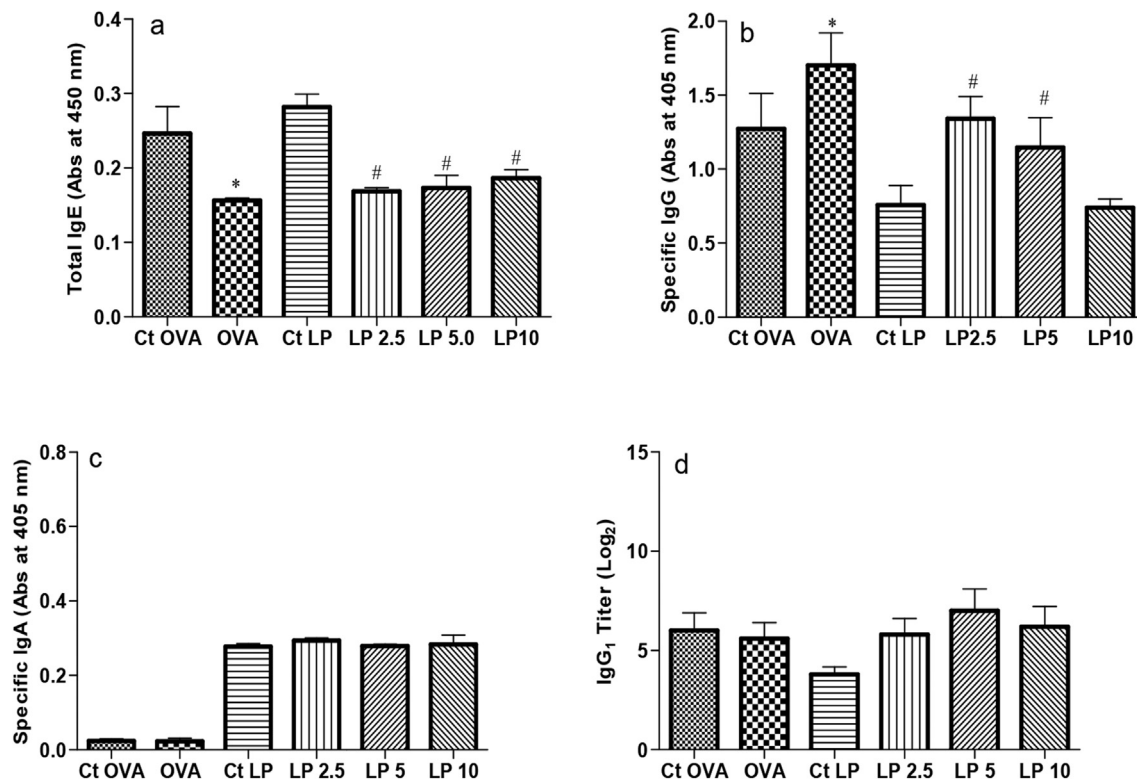


Fig. 5. Immunoglobulin level of treated mice in control OVA, OVA, control LP and LP (2.5, 5 and 10 mg/kg/bw) groups. a. IgE, b. IgG, c. IgA, d. IgG₁. The data are expressed as the mean $\pm$ SEM. ($p < 0.05$) ANOVA – Bonferroni's test: Comparisons between Ct OVA and OVA (*) and comparisons between Ct LP and LP (2.5–10) (#).

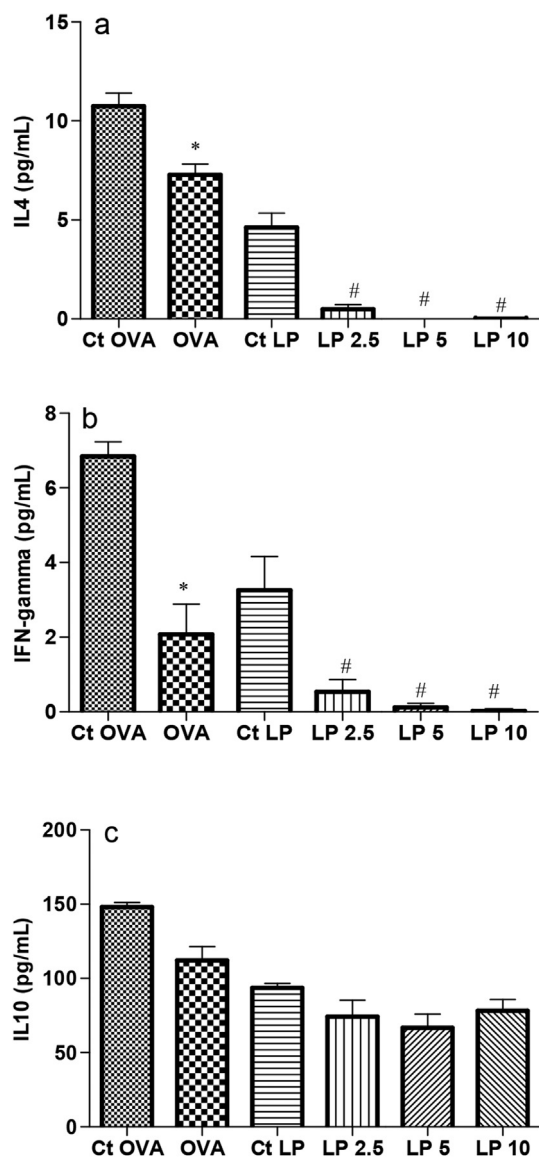


Fig. 6. Cytokine levels in spleen homogenate of treated mice in control OVA, OVA, control LP and LP (2.5, 5 and 10 mg/kg/bw) groups. a. IL-4, b. IFN- γ and c. IL-10. The data are expressed as the mean $\pm$ SEM. ($p < 0.05$) ANOVA – Bonferroni's test: Comparisons between Ct OVA and OVA (*) and comparisons between Ct LP and LP (2.5–10) (#).

this endogenous activity has a negative effect when LP is administered intraperitoneally to animals. The proteolytic activity triggers tissue damage in locci and initiates an inflammatory response that facilitates the uptake of LP content into bloodstream. This stepwise process would explain the ruddiness observed in the peritoneal cavity of the animals, the IgE response and the increment of pro-inflammatory mediators observed in previous studies when LP is given by i.p. [15]. On the other hand, animals given LP orally at very high concentration performed healthily. This result suggests that acute/lethal toxicity of the latex reported in preceding studies is likely to be present in rubber and organic fractions [36,39,40]. Indeed, taken orally, the proteolytic activity of LP seems to contribute to the digestibility of dietary proteins [41]. The implications of the proteolytic activity via intravenous administration were not considered here. However, it is reasonable to propose that the direct injection of proteolytic enzymes into the bloodstream should produce unpredictable results. The proteolytic activity of LP

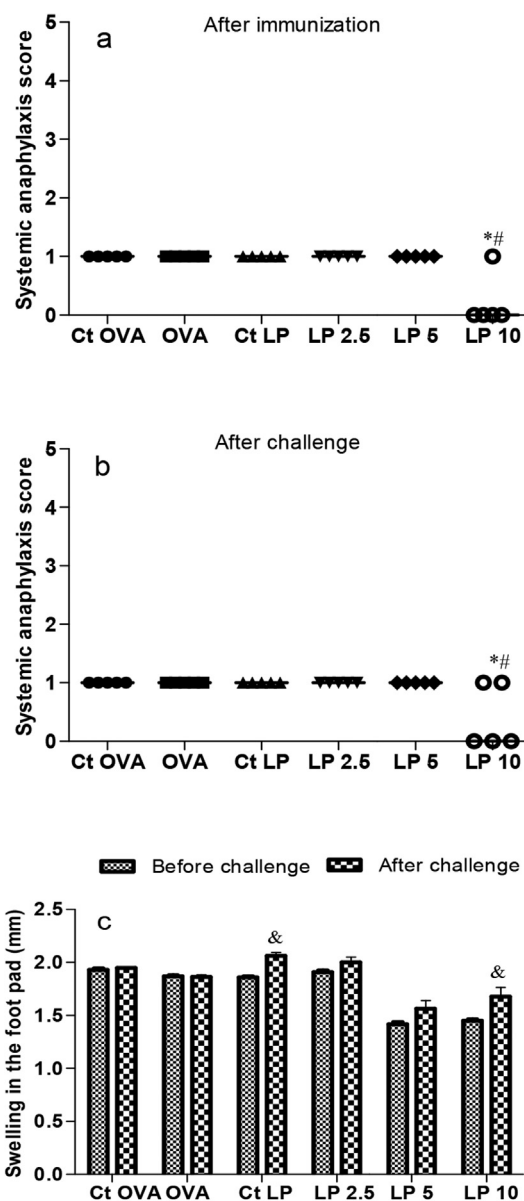


Fig. 7. Hypersensitivity reactions: a. anaphylaxis score after immunization (28 days); b. anaphylaxis score after challenge (42 days); and c. delayed-type hypersensitivity (49 days) in control OVA, OVA, control LP, LP (2.5, 5 and 10 mg/kg/bw) groups. The data are expressed as the mean $\pm$ SEM. ($p < 0.05$) ANOVA – Bonferroni's test: Comparisons between Ct OVA and OVA (*) and comparisons between Ct LP and LP (2.5–10) (#) and 24 h after challenge (&).

was demonstrated to display fibrinogenolytic and fibrinolytic effects on *in vivo* assays [14].

The histological evaluation of tissues of organs recorded of animals given LP (75 mg/kg/bw) (i.p.) did not reveal any adverse effect. LP-IAA (75 mg/kg/bw) displayed discrete, reversible and transitory toxic effects in the kidney. LP-PI but not LP-P-II IAA, at similar concentration, induced discrete degeneration in the liver. These data suggested that the marginal signs of toxicity revealed by histology were probably associated to the proteins present in the fraction LP-PI in addition to the proteases when they are active in LP or LP-P-II. LP-PI fraction was recently described in terms of protein and activity. LP-PI is a complex mix of chitinase isoforms [21]. Earlier, LP-PI was shown to be selectively cytotoxic to different cancer cell lines with IC50 values below 3 μ g/ml [42]. However the

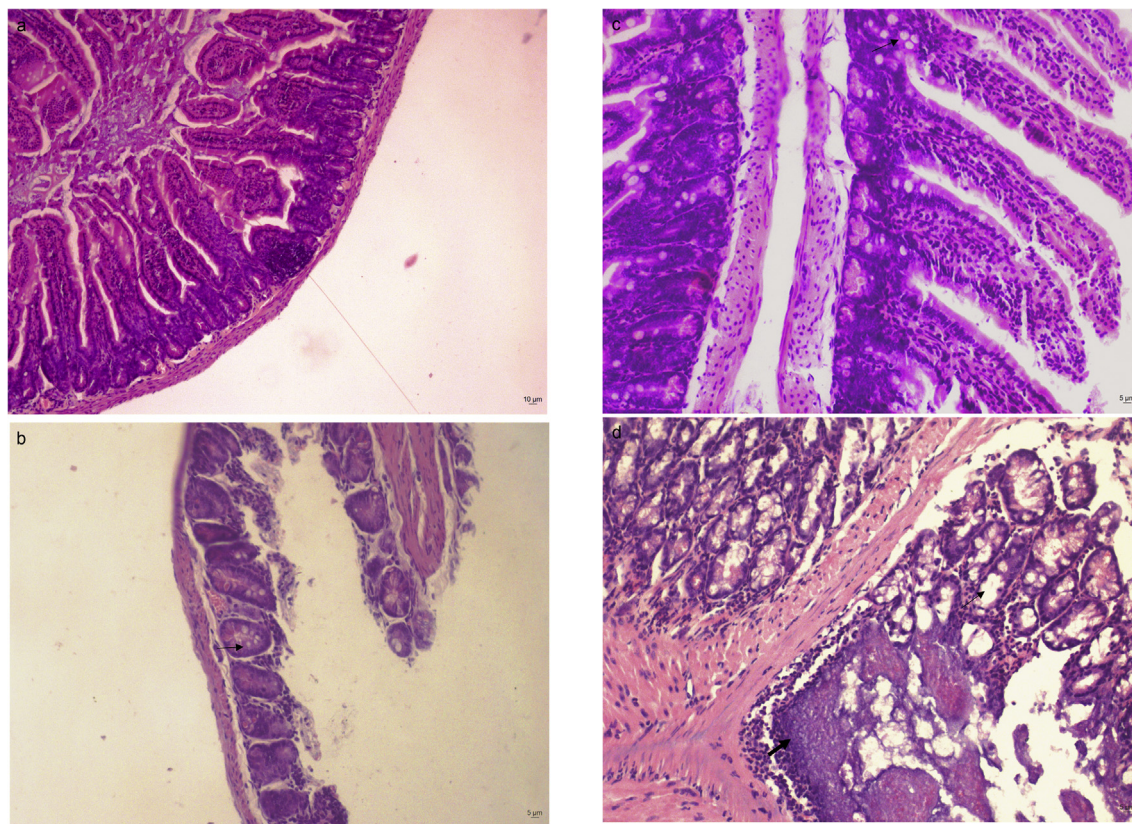


Fig. 8. Effect of LP (2.5, 5 and 10 mg/kg/bw) groups on the microstructures of small intestine (jejunum). (H&E stain, 10 and 20 $\times$) (Bar = 10 and 5 μ m). a. control group: no changes; b. LP 2.5: presence of the mucous cells in normal amounts (black arrow); c. LP 5: mild increase of the mucous cells (black arrow), d. LP 10: moderate increase of the mucous cells (thin arrow) and increased mucus production (thick arrow).

authors observed negligible toxicity of LP over peripheral blood mononuclear cells and did not correlate this effect with LP-PI. Further, *in vitro* studies of LP-PI over macrophages indicated LP-PI did not affect cell viability at 100 μ g/ml [43]. Therefore toxicology associated to proteins found in LP-PI deserves further focused studies.

The literature reports numerous studies of dose-response activities of LP on pharmacological models of clinical relevance. These studies have established that LP is promisingly effective at doses as low as 5 mg/kg. Consequently, the toxicological evaluation performed here suggests LP could be safely studied at this concentration. However, much more data must be monitored in order to progress to advance towards its safe use in humans. It is now established however, that depending of the concentration of LP tested by *i.p.*, the endogenous proteolytic activity becomes adverse, probably masking the expected pharmacological effect of LP.

Another barrier to be overcome before proteins can be proposed as biotherapeutics is allergenicity. Our first approach evaluating LP from this perspective showed that LP triggered IgE response and consistent anaphylaxis response [26]. By previously exposing animals to an oral protocol of LP administration we expected to induce tolerance against LP. The experimental design performed was in accordance with the current literature [27,28]. The lowest response to IgE, after oral administration of LP (10 consecutive days), followed by immunization by *i.p.*, suggested that immunological tolerance was achieved [44]. The reduced levels of IL-4 and IFN- γ supported this belief. However, the level of IL-10 was unaltered instead of being increased as expected [45–47]. IL-10 is an anti-inflammatory cytokine involved in the downregulation of cellular response [48]. However IL-10 release is stimulated in the later

phase of inflammation. Whether acute inflammation does not take place, increment of IL-10 does not occur or it is attenuated. Since release of pro-inflammatory cytokines was inhibited upon LP treatment, this observation could explain the lack of IL-10 increment. However, these prospecting arguments are too preliminary and of limited interpretation. The mechanisms underlying physiological response to LP administration is poorly understood at presently. Since LP displays opposite effects when administered *i.v* (inhibiting inflammation) or *i.p.* (inducing local inflammation and inhibiting far localized inflammation), much more efforts will be necessary to map the cascade of events each situation involves. Although we have accumulated information in both senses, in terms of cellular profile and measurement of key inflammatory/anti-inflammatory markers, we are far from to reach a clear picture of LP actions upon healthy or experimental animals. In this study, however, we aimed at emphasizing some new aspects of the immunological response mediated by immunoglobulins upon LP exposure. The comparative IgE profile observed among the groups investigated here was indicative that oral immunological tolerance was reached. It should be also recorded that oral administration of LP does not induce IgE response [27]. Therefore, the consumption of LP orally would be considered safe in this context. Animals experimentally reaching oral immunological tolerance, with diminished IgE response would be nicely useful to study further effect of LP (or its sub fractions) in the complex pathways that LP triggers inflammation or anti-inflammation. Therefore, this study gives new insights to be prospected soon.

The performance of the animals in response to the DTH test revealed that the hypersensitivity response diminished in animals treated with 2.5 or 5.0 mg/kg LP but not in those treated with

10 mg/kg. This may be due to the level of IL-10, which did not increase and therefore was not enough to suppress DTH response (type IV) at this concentration of LP. On the other hand, the anaphylaxis symptoms (type I) were reduced in animals immunized and challenged when LP was given orally at 10 mg/kg. The studies on oral immunological tolerance suggested that LP could be exploited in terms of pharmacological potentialities by oral route. On the whole, although LP is a promising source of biotherapeutics proteins, identification of individual molecules involved in the desired effects remains the best strategy to be followed before putting forward strategies for overcoming limiting points of the sample. The safe use of latex (the plant) in the herbal context should be urgently addressed.

Conflict of interest

The authors hereby declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.cbi.2017.07.007>.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.cbi.2017.07.007>.

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Table S1 Effects of LP (p.o) on behavioral parameters in mice during evaluation of acute toxicity

Groups (mg/kg)	Symptoms of toxicity				Animals (D/T)
	Central Nervous System	Autonomic Nervous System	Psychomotor	General Activity/ Sensory System	
Control	None	None	None	None	0/5
LP 2000	None	None	-Contortion, 2 h [*]	None	0/5
LP 5000	None	-Hypothermia, 1h [*]	None	- Shaking tail, 6d [*]	0/5

The values are presented as means $\pm$ SEM (5 mice/group). (* $p < 0.05$, ANOVA - Bonferroni's test compared with the control). Central Nervous System: Tremors, convulsions, straubé tail, hypnosis and anesthesia; Autonomic Nervous System: Ptosis, lacrimation, urination, defecation, piloerection, hypothermia, respiration and cyanosis; Psychomotor: Contortion, posterior train, reflection straightening, body weight tone, strength gripping and ataxia; General Activity/ Sensory System: General activity, vocal fremitus, irritability, auricular reflex, corneal reflex, shaking tail, touch response. None: no statistical difference compared to control; D: dead, and T: total treated mice.

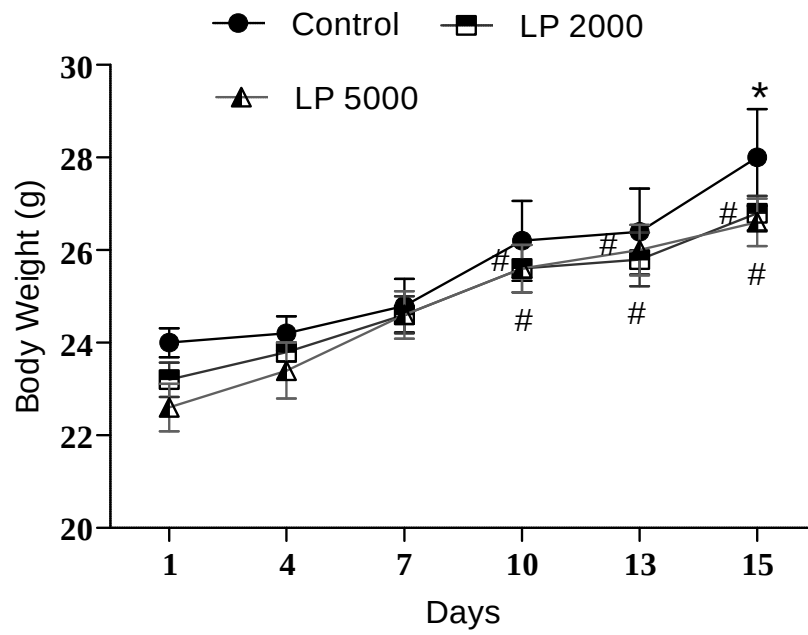


Fig. S1 Body weights in mice treated orally with LP (2000 and 5000 mg/kg/bw) during evaluation of acute toxicity. The values are presented as means $\pm$ SEM (5 mice/group). ($p < 0.05$, ANOVA - Bonferroni's test. Control (*) and LP 2000/5000 (#) in relation to the 1st day).

Table S2 Physiological, hematological and biochemical parameters in mice treated orally with LP 2000 and 5000

Parameters	Groups		
	Control	LP 2000	LP 5000
Physiological[#]			
Excretas (g/day)	1.86 ± 0.09	2.02 ± 0.12	2.05 ± 0.13
Pellets (n°/day)	94.94± 5.21	101.3 ± 5.33	103.4 ± 5.39
Urine (n°/day)	3.53 ± 0.16	3.31 ± 0.27	3.30 ± 0.30
Water (ml/day)	8.33 ± 0.27	8.86 ± 0.27	8.11 ± 0.19
Food (g/day)	4.26± 0.23	4.70 ± 0.28	4.51 ± 0.23
Hematology			
Leukocytes (10 ³ /μL)	1.80 ± 0.15	2.82 ± 0.54	1.84 ± 0.10
Lymphocytes (%)	76.40 ± 1.43	75.80 ± 2.27	78.00 ± 2.96
Neutrophils (%)	19.40 ± 1.21	14.40 ± 3.08	14.40 ± 2.84
Monocytes (%)	4.60 ± 0.51	9.80 ± 0.97*	11.40 ± 0.25*
Eosinophils (%)	0	0	0
Basophils (%)	0	0	0
Biochemistry			
ALT (U/L)	62.93 ± 3.49	60.17 ± 2.21	56.10 ± 4.96
AST (U/L)	120.30 ± 3.88	117.60 ± 6.41	111.70 ± 5.05
Creatinine (mg/dL)	0.40 ± 0.04	0.50 ± 0.03	0.49 ± 0.02
Urea (mg/dL)	55.41 ± 5.28	68.95 ± 3.45	53.71 ± 6.95
Lipid. peroxidation (nmol/g)	195.80 ± 28.63	118.90 ± 14.37	110.10 ± 20.99

Physiological parameters: production of excretas, pellets and n° humid wells of urine and consumption of water and food. The values are presented as means ± SEM (5 mice/group). (* $p < 0.05$, Bonferroni's test compared with the control).

Table S3 Relative organ weights of mice treated orally with LP in evaluation of acute toxicity determined at day 15

Relative Weight	Groups (mg/kg/bw)		
	Control	LP 2000	LP 5000
Liver	4.71 ± 0.21	4.38 ± 0.11	4.15 ± 0.15
Kidney	0.67 ± 0.03	0.66 ± 0.03	0.64 ± 0.01
Spleen	0.24 ± 0.02	0.24 ± 0.01	0.22 ± 0.01
Total Body Weight (g)	28.00± 1.05	24.97± 0.55	24.80 ± 0.64

The values are presented as means ± SEM (5 mice/group). (* $p < 0.05$, Bonferroni's test). No statistical difference compared with the control. Relative weight: (organ weight/total body weight) x 100.

Table S4 Effects of LP (i.p) on behavioral parameters in mice during evaluation of acute toxicity

Groups (mg/kg)	Symptoms of toxicity				Animals (D/T)
	Central Nervous System	Autonomic Nervous System	Psychomotor	General Activity/ Sensory System	
Control	None	None	None	None	0/5
LP 75	None	-Piloerection, 1°day;* -Hypothermia, 30 min. and 8h;* -Respiration, 8h, 1°, 2°, 3° and 4°days;* -Pellets, 1° and 2°days.*	-Contortion, 30 min.*; -Lifting force, 8 h and 1° day.*	-General activity,1, 2, 4, 8h and 1°day;* -Vocal fremitus ,4 and 8 h;* -Irritability, 1 h;* -Shaking tail , 4 and 8h;* -Touch response, 4 and 8h.*	1/5
LP 150	- Tremors, 1h*	- Hypothermia, 30 min.*; -Piloerection, 30 min.*;	-Contortion, 30min.*; -Posterior train, 1h;* -Reflection straightening, 1h.*	-General activity, 30min.*; -Shaking tail, 1h;* -Touch response, 1h;*	5/5
LP 300	- Tremors, 30 min.*; -Convulsions, 30 min.*;	- Hypothermia, 30 min.*; -Piloerection, 30 min.*;	- Contortion, 30min.*; -Posterior train, 30min.*; -Reflection straightening, 30min*	-General activity, 30min.*; -Shaking tail, 30min.*; -Touch response, 30min.*;	5/5
Control	None	None	None	None	0/5
LP 15	None	None	None	-General activity, 30 min., 1, 2, 4, 8 h .	0/5
LP 35	None	None	None	-General activity, 30 min., 1 and 2h*; -Irritability, 2 and 4 h .	0/5
LP 75 IAA	None	None	-Contortion, 4 and 8h* .	None	0/5
Control	None	None	None	None	0/5
LP-PI 75	None	-Piloerection, 30 min., 1, 2 and	None	None	0/5

		4h [*] ; -Respiration, 30min., 1 and 2h [*] .			
LP-P11 75 IAA	None	-Respiration, 30min.,1 and 2h [*] .	None	-Irritability, 1h [*] .	0/5

The values are presented as means $\pm$ SEM (5 mice/group). (* $p < 0.05$, ANOVA - Bonferroni's test compared with the control). Central Nervous System (CNS): Tremors, convulsions, straubé tail, hypnosis and anesthesia; Autonomic Nervous System (ANS): Ptosis, lacrimation, urination, defecation, piloerection, hypothermia, respiration and cyanosis; Psychomotor: Contortion, posterior train, reflection straightening, body weight tone, lifting force and ataxia; General Activity/ Sensory System (G. A/ S.S): General activity, vocal fremitus, irritability, auricular reflex, corneal reflex, shaking tail, touch response. None: no statistical difference compared to control; D: dead, and T: total treated mice.

Table S5 Hematological and biochemical parameters in mice treated intraperitoneally with LP and fractions inhibited with iodoacetamide (LP 75, LP 15-75 IAA, LP-PI and LP-P II 75 IAA) determined at day 15.

Parameters	Groups			
	Control	LP 75	--	--
Hematology				
Leukocytes ($10^3/\mu\text{L}$)	2.89 ± 1.16	2.60 ± 1.21	--	--
Lymphocytes (%)	86.00 ± 3.08	82.00 ± 6.36	--	--
Neutrophils (%)	9.00 ± 2.00	12.75 ± 5.09	--	--
Monocytes (%)	3.60 ± 1.54	4.75 ± 1.60	--	--
Eosinophils (%)	0.40 ± 0.25	0	--	--
Basophils(%)	1.00 ± 0.32	0.50 ± 0.29	--	--
Biochemistry				
ALT (U/L)	71.05 ± 12.01	84.95 ± 20.28	--	--
AST (U/L)	78.67 ± 22.98	95.84 ± 18.52	--	--
Creatinine (mg/dL)	0.31 ± 0.07	0.38 ± 0.06	--	--
Urea (mg/dL)	70.78 ± 10.59	72.81 ± 15.07	--	--
L. peroxidation (nmol/g)	34.95 ± 12.51	51.71 ± 11.79	--	--
	Control	LP 15	LP 35	LP 75IAA
Hematology				
Leukocytes ($10^3/\mu\text{L}$)	3.31 ± 0.91	2.16 ± 1.10	3.36 ± 1.23	2.94 ± 0.81
Lymphocytes (%)	68.80 ± 7.43	79.40 ± 4.41	82.80 ± 3.07	87.80 ± 2.35
Neutrophils (%)	18.00 ± 7.35	13.00 ± 4.18	11.60 ± 3.14	8.40 ± 1.96
Monocytes (%)	7.50 ± 1.04	$1.50 \pm 0.29^*$	$2.40 \pm 0.40^*$	$1.80 \pm 0.49^*$
Eosinophils (%)	2.20 ± 0.58	1.80 ± 0.58	2.00 ± 0.71	0.40 ± 0.40
Basophils(%)	4.80 ± 0.73	2.60 ± 0.75	1.20 ± 0.49	1.60 ± 1.36
Biochemistry				
ALT (U/L)	98.53 ± 34.66	89.68 ± 19.77	95.26 ± 15.34	121.90 ± 26.38
AST (U/L)	130.10 ± 36.06	107.80 ± 14.66	107.90 ± 28.54	129.60 ± 22.87
Creatinine (mg/dL)	2.015 ± 1.32	2.55 ± 0.77	3.30 ± 0.78	3.08 ± 1.49
Urea (mg/dL)	78.86 ± 19.78	62.67 ± 12.89	88.73 ± 25.88	87.59 ± 11.84
Lipid peroxidation (nmol/g)	105.40 ± 39.69	94.25 ± 42.26	89.23 ± 35.93	47.21 ± 24.74
	Control	LP-PI 75	LP-P II 75 IAA	--
Hematology				
Leukocytes ($10^3/\mu\text{L}$)	4.39 ± 0.80	5.04 ± 2.55	4.17 ± 2.18	--
Lymphocytes (%)	94.00 ± 0.45	92.60 ± 1.47	92.40 ± 1.40	--
Neutrophils (%)	3.40 ± 0.51	4.00 ± 1.05	5.20 ± 0.97	--
Monocytes (%)	1.40 ± 0.25	1.60 ± 0.75	1.20 ± 0.20	--
Eosinophils (%)	0.40 ± 0.25	1.00 ± 0.55	0.40 ± 0.25	--
Basophils(%)	0.80 ± 0.20	0.80 ± 0.49	0.80 ± 0.20	--
Biochemistry				
ALT (U/L)	49.89 ± 26.57	39.06 ± 17.49	51.27 ± 4.16	--
AST (U/L)	53.72 ± 12.47	67.05 ± 10.78	88.57 ± 42.01	--
Creatinine (mg/dL)	0.57 ± 0.30	0.62 ± 0.18	0.63 ± 0.11	--
Urea (mg/dL)	72.25 ± 14.27	68.06 ± 34.08	70.74 ± 6.89	--
Lipid peroxidation (nmol/g)	91.22 ± 19.24	98.06 ± 19.78	103.40 ± 45.22	--

The values are presented as means $\pm$ SEM (5 mice/group). (*p < 0.05 ANOVA - Bonferroni's

test compared with the control.

Table S6 Relative organ weights of mice treated intraperitoneally with LP or fractions in evaluation of acute toxicity

Groups (mg/kg)	Relative Weight					Body Weight (g)
	Liver	Kidney (right)	Spleen	Heart	Lung	
Control	4.82 ± 0.51	0.73 ± 0.10	0.27 ± 0.05	----	----	24.38 ± 0.77
LP 75	4.83 ± 0.25	0.71 ± 0.04	0.28 ± 0.05	----	----	23.42 ± 1.93
Control	4.85 ± 0.39	0.68 ± 0.03	0.23 ± 0.02	0.50 ± 0.04	0.55 ± 0.06	25.43 ± 1.10
LP15	4.12 ± 0.44*	0.65 ± 0.04	0.21 ± 0.06	0.50 ± 0.06	0.47 ± 0.02	26.37 ± 0.97
LP 35	4.31 ± 0.15	0.63 ± 0.06	0.27 ± 0.06	0.47 ± 0.02	0.50 ± 0.05	27.53 ± 1.31
LP 75	4.19 ± 0.34	0.63 ± 0.05	0.23 ± 0.04	0.47 ± 0.03	0.53 ± 0.09	28.47 ± 1.17
IAA						
Control	5.14 ± 0.35	0.64 ± 0.08	0.32 ± 0.05	0.59 ± 0.09	0.59 ± 0.07	27.03 ± 0.27
LP-PI	4.91 ± 0.38	0.62 ± 0.08	0.44 ± 0.10	0.50 ± 0.08	0.59 ± 0.04	28.03 ± 0.96
75						
LP-II	5.21 ± 0.46	0.71 ± 0.07	0.39 ± 0.09	0.49 ± 0.05	0.57 ± 0.04	29.33 ± 1.20
75 IAA						

The values are presented as means ± SEM (5 mice/group). (* $p < 0.05$, ANOVA - Bonferroni's test compared with the control). Relative weight: (organ weight/ total body weight) x 100.

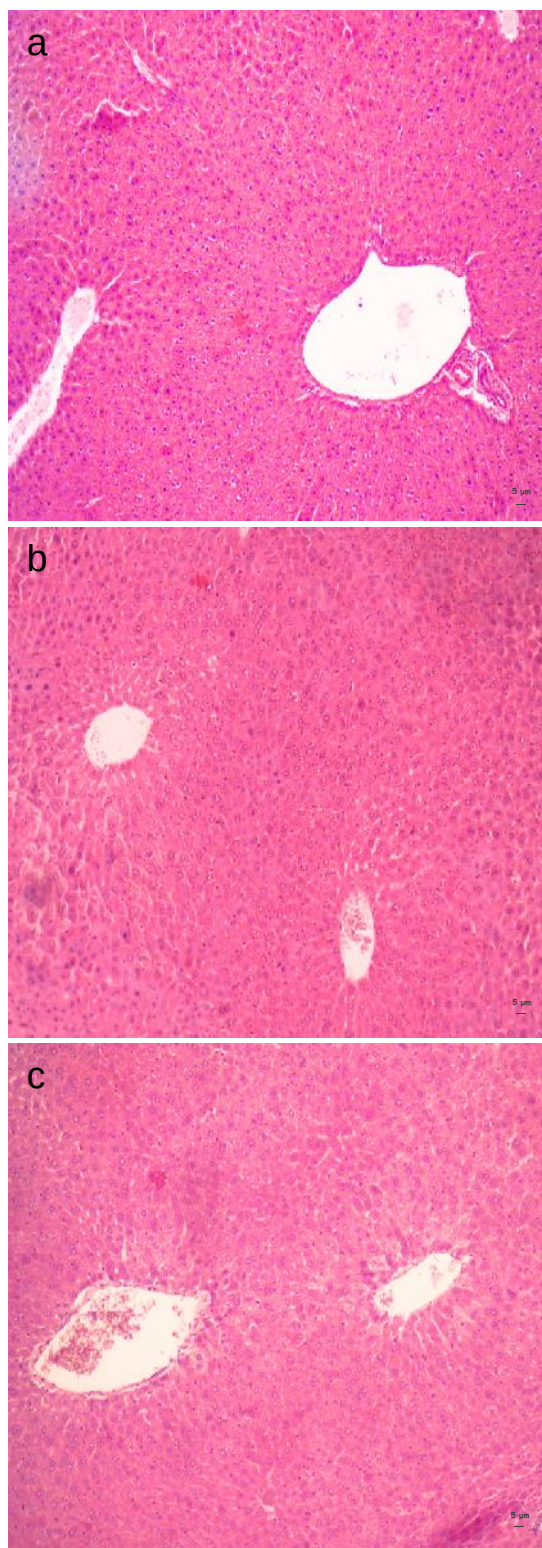


Fig. S2 Effect of LP (75 and 75 IAA, i.p) on the microstructures of liver of mice in evaluation of acute toxicity (H&E stain, 10 x) (Bar = 5 μ m). a. control group; b. LP75 and c. LP75 IAA: no changes.

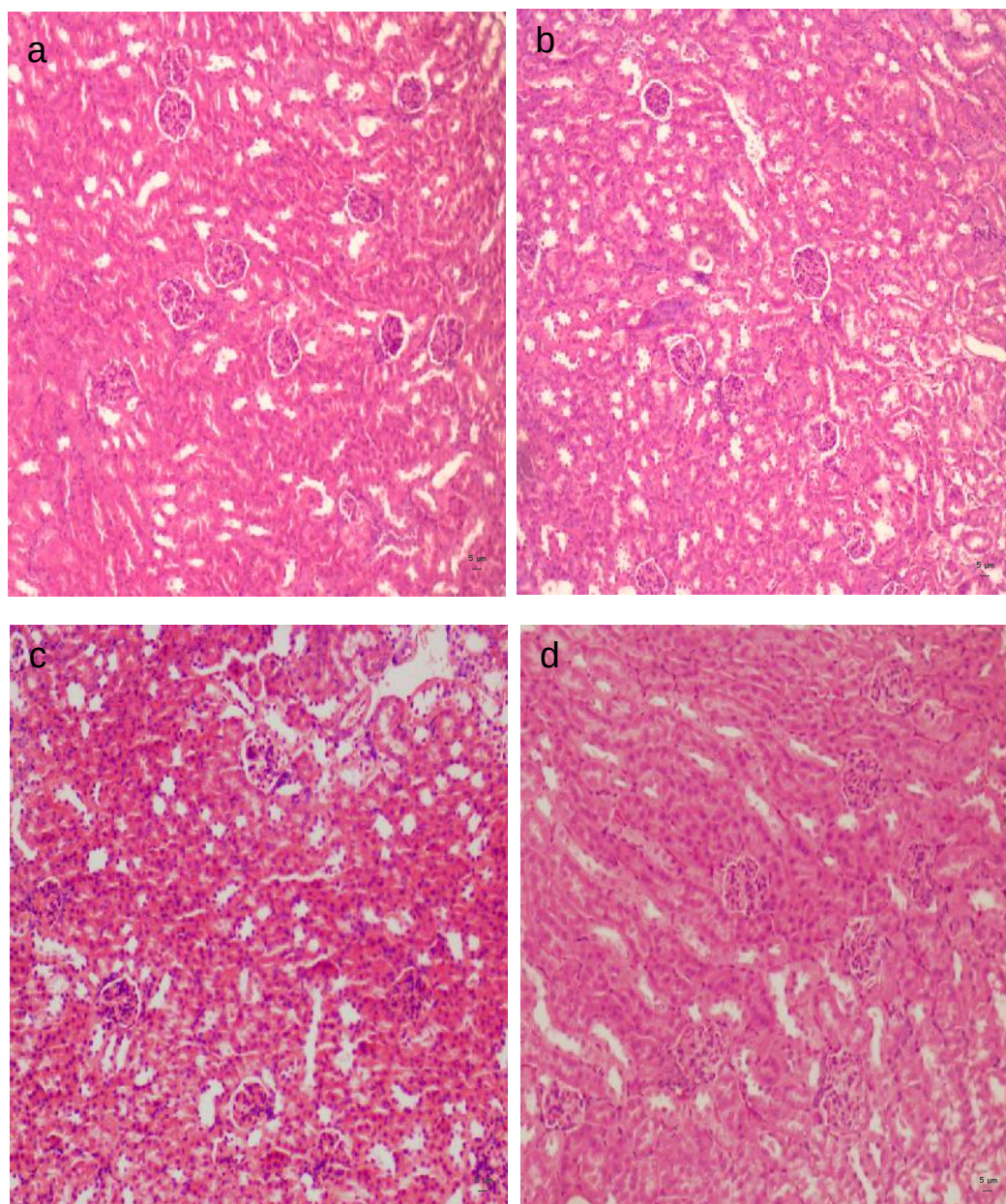


Fig S3. Effect of LP (75, PI and PII 75 IAA, i.p) on the microstructures of kidney of mice in evaluation of acute toxicity (H&E stain, 10 x) (Bar = 5 μ m). a. control group; b. LP75; c. LP-PI 75; d. LP-P11 75 IAA: no changes.

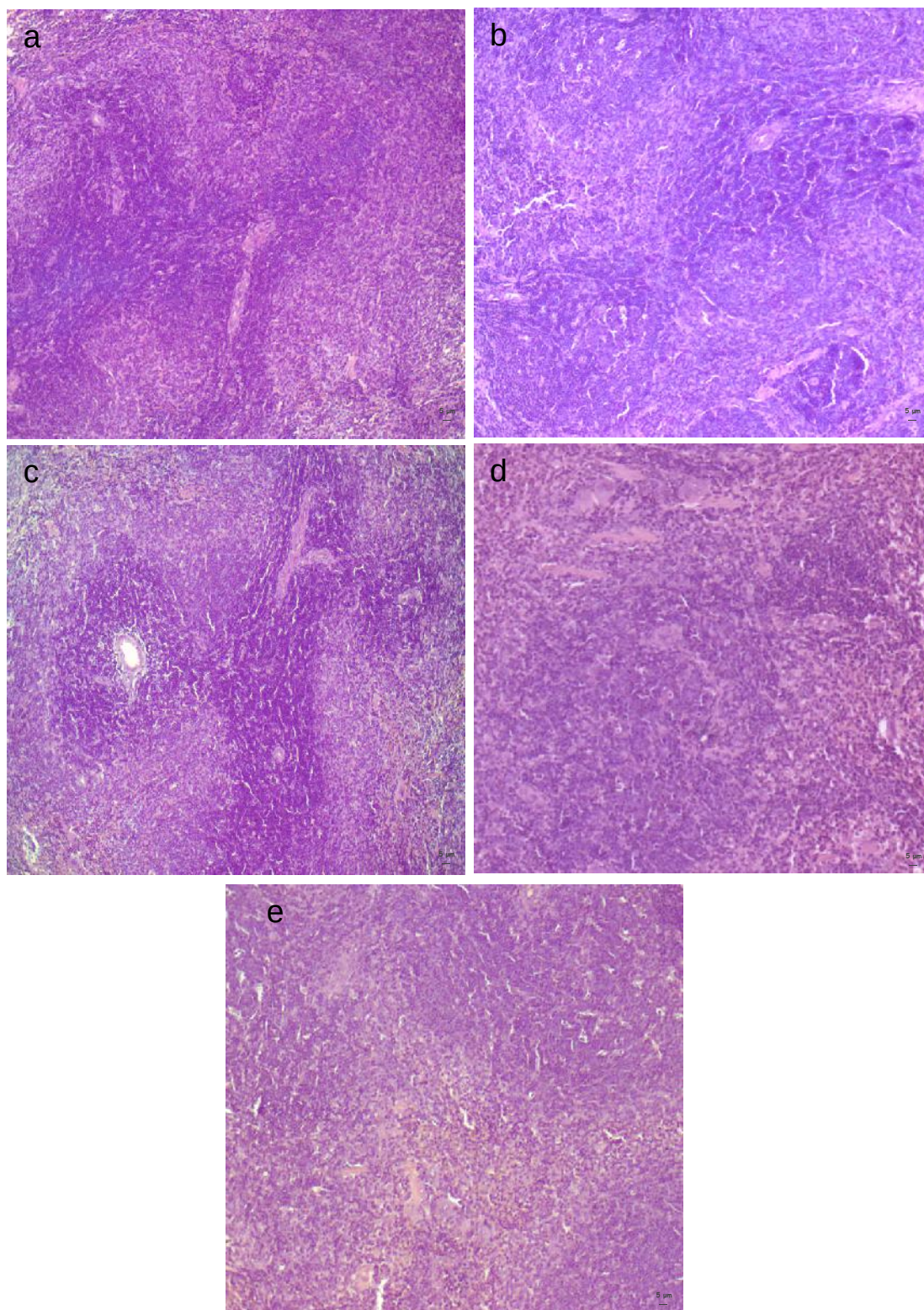


Fig S4. Effect of LP (75, 75 IAA, PI and PII 75 IAA, i.p) on the microstructures of spleen of mice in evaluation of acute toxicity (H&E stain, 10 x) (Bar = 5 μ m). a. control group; b. LP 75; c. LP 75 IAA; d. LP-PI 75; e. LP-PII 75 IAA: no changes.

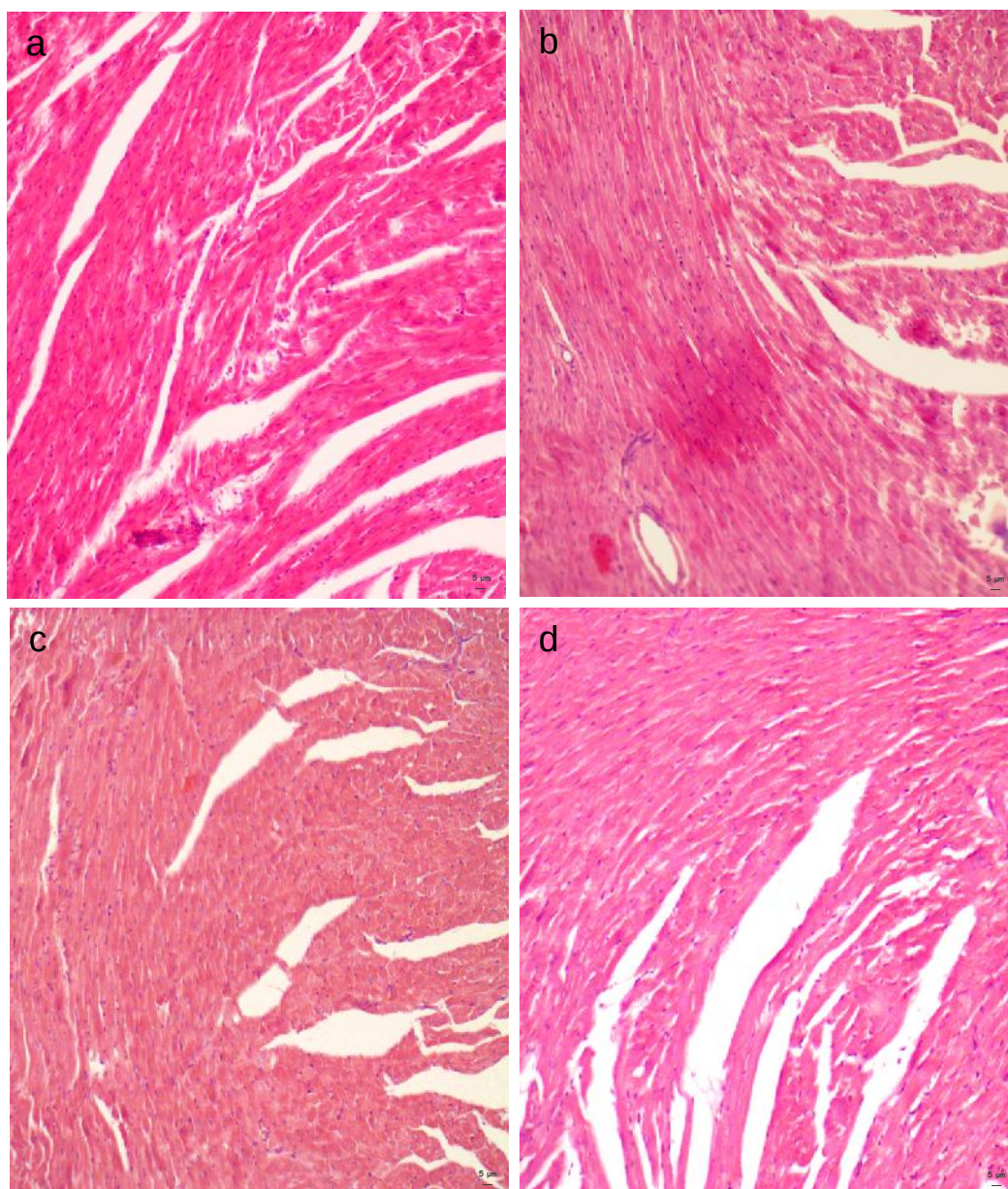


Fig S5. Effect of LP (75 IAA, PI and PII 75 IAA, i.p) on the microstructures of heart of mice in evaluation of acute toxicity (H&E stain, 10 x) (Bar = 5 μ m). a. control group; b. LP 75 IAA; c. LP-PI 75; d. LP-P II 75 IAA: no changes.

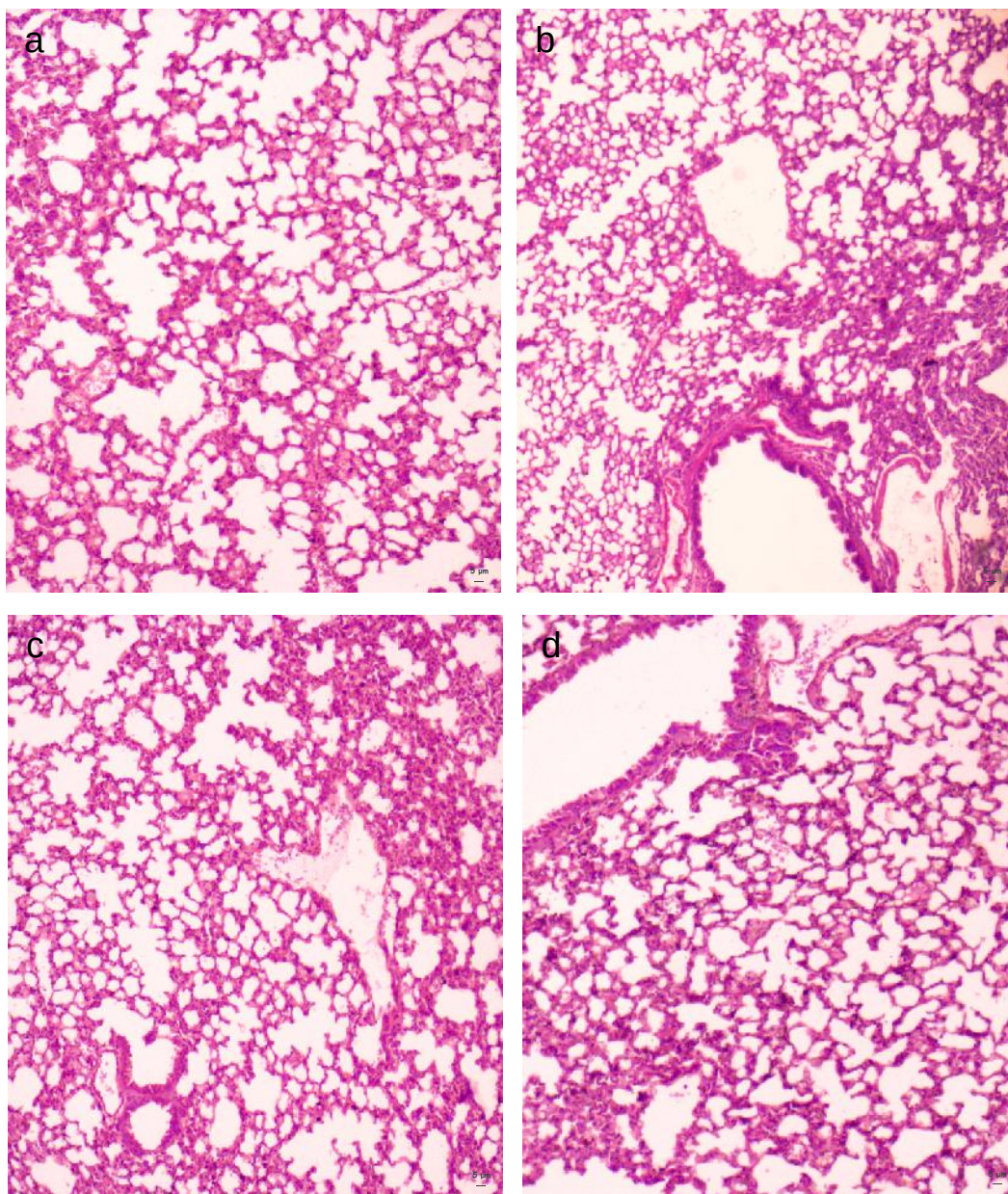


Fig S6. Effect of LP (75 IAA, PI and P11 75 IAA, i.p) on the microstructures of lung of mice in evaluation of acute toxicity (H&E stain, 10 x) (Bar = 5 μ m). a. control group; b. LP 75 IAA; c. LP-PI 75; d. LP-P11 75 IAA: no changes.

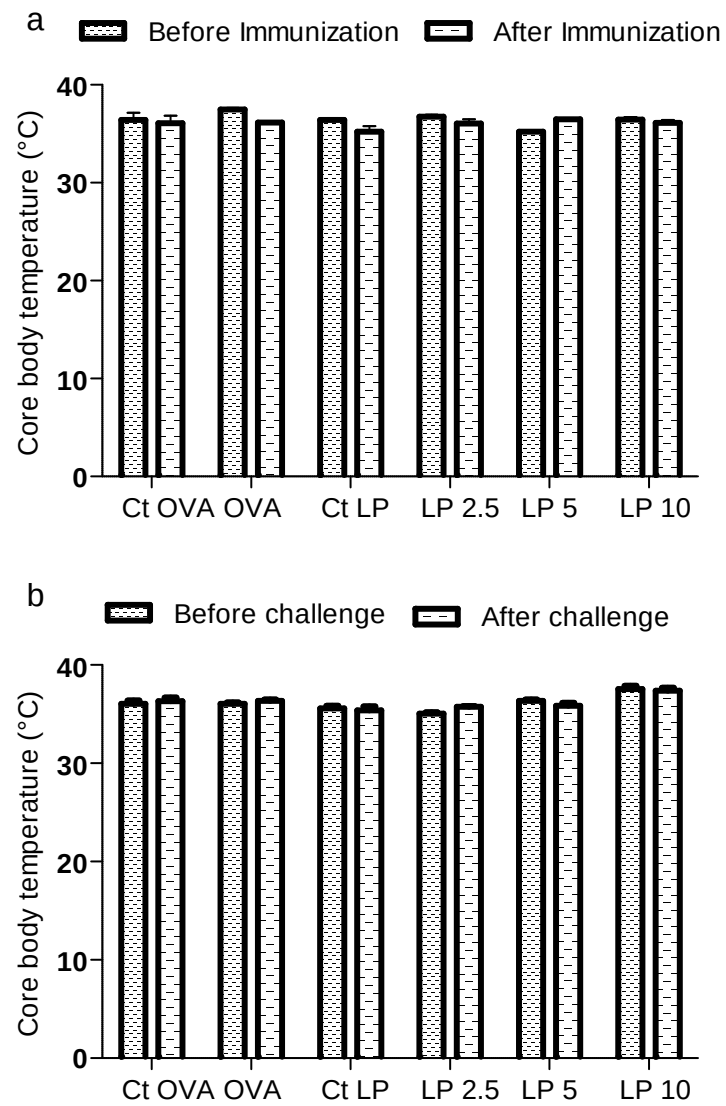


Fig. S7 Core body temperature of mice before and after (a) immunization (Day 28) and (b) challenge (Day 49) with LP or OVA (i.p.). The values are presented as means $\pm$ SEM (5 mice/group). (* $p < 0.05$, ANOVA - Bonferroni's test compared with before of the immunization or challenge).

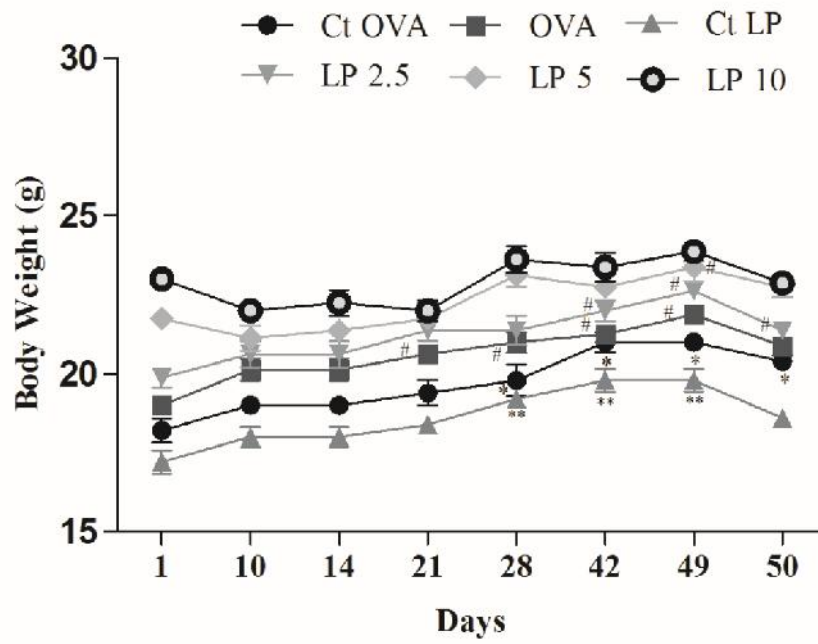


Fig. S8 Body weights evolution of mice treated with different LP fractions or OVA according to the protocol shown in Fig. 4. Animals were first given samples per oral (every day until day 10; immunized by i.p. at day 28; challenged by i.p. at day 42). The values are presented as means $\pm$ SEM (5 mice/group). ($p < 0.05$, ANOVA - Bonferroni's test. Ct. OVA (*), Ct LP (**)) and OVA, LP 2.5, LP 5, LP 10 (#) in relation to the 1st day).

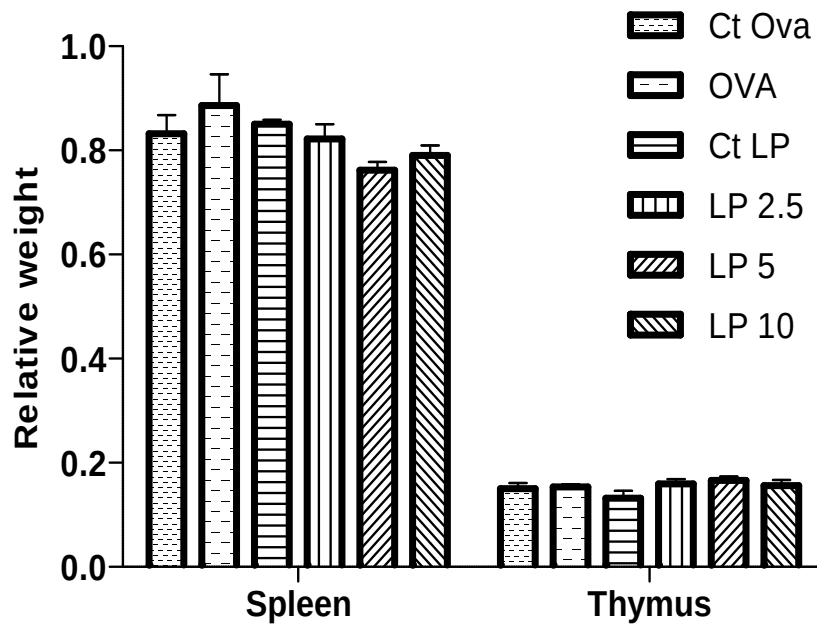


Fig. S9 Relative organ weights (spleen and thymus) of mice in oral tolerance treatment with LP or OVA. The values are presented as means $\pm$ SEM (5 mice/group). (* $p < 0.05$, ANOVA - Bonferroni's test compared with Ct OVA (*) and Ct LP (#)). Relative weight: (organ weight/ total body weight) $\times$ 100.

Table S7 Hematological parameters in treated mice in the control OVA, OVA, control LP, LP (2.5, 5 and 10mg/kg) groups

Parameters	Groups – zero day					
	Ct OVA	OVA	Ct LP	LP 2.5	LP 5	LP 10
Leukocytes (10 ³ /μl)	4.26 ± 0.39	5.73 ± 0.30	4.52 ± 0.36	4.77 ± 0.35	5.01 ± 0.32	5.16 ± 0.36
Lymphocytes (%)	87.80 ± 2.78	84.20 ± 0.86	86.80 ± 0.80	85.60 ± 1.03	89.60 ± 0.51	84.20 ± 1.07
Neutrophil (%)	10.00 ± 2.05	11.80 ± 0.49	12.00 ± 0.63	10.60 ± 0.93	9.60 ± 0.40	11.60 ± 0.40
Monocyte (%)	1.00 ± 0.55	0.40 ± 0.25	0.80 ± 0.20	0.40 ± 0.25	0.80 ± 0.20	1.20 ± 0.20
Eosinophils (%)	0	0.40 ± 0.25	0	0	0.20 ± 0.20	0.20 ± 0.20
Basophils (%)	1.20 ± 0.37	1.20 ± 0.20	0.60 ± 0.25	0.60 ± 0.40	0	0.60 ± 0.25
Parameters	Groups – 49 days					
	Ct OVA	OVA	Ct LP	LP 2.5	LP 5	LP 10
Leukocytes (10 ³ /μl)	9.66 ± 1.41	6.65 ± 0.21	5.16 ± 0.14*	6.60 ± 0.31	7.45 ± 0.59	7.10 ± 0.56
Lymphocytes (%)	84.00 ± 0.71	81.40 ± 0.60*	83.20 ± 0.20	81.00 ± 0.32*#	82.00 ± 0.32	81.80 ± 0.37
Neutrophil (%)	14.00 ± 0.71	14.80 ± 0.58	14.80 ± 0.20	15.80 ± 0.49	15.00 ± 0.55	15.00 ± 0.55
Monocyte (%)	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0
Eosinophils (%)	0	0	0	0	0	0
Basophils (%)	1.00 ± 0.0	0.80 ± 0.20	1.00 ± 0.0	0.80 ± 0.20	0.40 ± 0.25	0.80 ± 0.20

The data are expressed as the mean ± SEM. ($p < 0.05$, ANOVA - Bonferroni's test compared with Ct

OVA (*) and Ct LP (#)).