



BIOMEDICAL SCIENCES

Protective effect of latex proteins from *Plumeria pudica* against acetaminophen-induced acute liver injury

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Abstract: The protein fraction from *Plumeria pudica* latex (LPPp) was evaluated against APAP-induced liver injury in mice. Key measurements included total leukocyte count, liver weight, AST, ALT, liver levels of MDA, GSH, SOD, MPO, NO₃/NO₂ concentration, cytokines TNF- α , IL- β , IL-6, IL-10, IFN- γ , and CXCL1. Histopathological and immunohistochemical analysis were conducted, and proteins in LPPp were identified by mass spectrometry. Molecular docking analyses were performed with LPPp proteins and APAP, NAPQI, TNF- α , and IFN- α . LPPp reduced liver weight, ALT, and AST levels, and prevented the decrease in total leukocyte count, indicating protection against liver injury. It also reduced MDA levels and preserved GSH and SOD levels, showing its antioxidant properties. LPPp significantly inhibited MPO and NO₃/NO₂ levels, reducing inflammation. LPPp decreased levels of TNF- α , IL- β , IL-6, IFN- γ , and CXCL1, while IL-10 levels remained elevated, suggesting an anti-inflammatory response. Histological analysis showed preserved liver tissue, and anti-Ly6C immunostaining indicated reduced neutrophil accumulation. Molecular docking revealed that latex cysteine proteases had a higher affinity for APAP and NAPQI, while a proteinase inhibitor showed affinity for CYP2E1, TNF- α , and IFN- α . These findings suggest that LPPp protects against APAP hepatotoxicity by interfering with APAP and NAPQI metabolism, reducing oxidative stress and inflammation, thus preventing hepatocyte damage.

Key words: APAP, Cysteine proteinase, N-acetylcysteine, Proteinase inhibitor.

INTRODUCTION

Drug-induced liver injury (DILI) is caused by conventional medications, herbal, and dietary supplements and is expressed as liver damage caused by the drug itself or its metabolites (Liu et al. 2021). Acute DILI could progress into chronic liver injury and cause hepatic failure, which necessitates liver transplantation, or even lead to

death (Hayashi & Bjornsson 2018). Acetaminophen, known as N-acetyl-p-aminophenol (APAP), is the most common cause of DILI (Jaeschke et al. 2021b). At therapeutic doses, it is an effective analgesic and antipyretic; however, an excessive dose causes acute liver failure (ALF) (Jaeschke et al. 2021a). APAP overdose is the leading cause of

ALF in countries, including Australia, the United Kingdom, Canada, and the United States (Reuben et al. 2016, Antoniou et al. 2022).

APAP is metabolized into nontoxic metabolites in the liver through sulfation and glucuronidation metabolic pathways. However, APAP overdose saturates these routes, and its excess is metabolized mainly by cytochrome P450 (CYP) into the highly reactive metabolite N-acetyl-p- benzoquinoneimine (NAPQI) (McGill et al. 2012a). NAPQI is a toxic intermediate that contains powerful oxidizing and affinity properties, triggering the depletion of glutathione (GSH) and the formation of protein adducts in hepatocytes (Ramachandran & Jaeschke 2019). The accumulation of NAPQI in mitochondria provokes continuous oxidative stress or nitrosative stress, followed by gradual liver cell necrosis (Jaeschke et al. 2012, Cai et al. 2022). Necrotic hepatocytes release danger-associated molecular patterns, recruiting and activating innate immune cells, which participate in necrotic tissue clearance and inflammatory response expansion (Jaeschke et al. 2012, Kubes & Mehal 2012, Woolbright & Jaeschke 2017). Eventually, liver cells proliferate and regenerate in the presence of certain signal mediators and nonparenchymal liver cells (Bhushan & Apte 2019).

The oxygen free radical scavenger N-acetylcysteine (NAC) is the only treatment approved by the US Food and Drug Administration (FDA). However, NAC can cause side effects, including anaphylactic reactions, and the standard dose may not be sufficient for patients with very high APAP overdoses (Licata et al. 2022). The late presentation of patients with delayed initiation of NAC treatment is the main reason for ALF and death after APAP overdose. Although NAC is effective, there remains a need to identify novel and more potent therapeutic alternatives (Jaeschke et al. 2020). Accordingly, targeting

oxidative stress and the inflammatory response induced by APAP represents a promising strategy (Subramanya et al. 2018).

Plant latex is a fluid produced in secretory structures called laticifers and is usually released after physical damage (Ramos et al. 2020b). It contains a mixture of proteins and enzymes that have been associated with numerous pharmacological properties (Warowicka et al. 2020). In traditional medicine, the latex of different plant species has been diluted in water and used for to treat inflammation, pain, tumors, gastritis, and others (Devprakash et al. 2012). *Plumeria pudica* (Jacq 1760) popularly known as “bridal bouquet” characterized by its intense latex production (Atheaya & Deodhar 2015, Chamakuri et al. 2020). *Plumeria* species has been used in old medicinal preparations in Asia as well Latin America to treat illness (Devprakash et al. 2012, Choudhary et al. 2014). In northeastern Brazil *P. pudica* has been used as a folk medicine due to analgesic properties (Fernandes et al. 2015). In the Indian traditional system of medicine is used to treat rheumatism, bronchitis, diarrhea, venereal disease, leprosy, psychosis, diuresis, and gynecological diseases (Shukla et al. 2023).

A well-defined protein fraction (LPPP) obtained from its latex has demonstrated the ability to interfere in oxidative stress and inflammatory response in different experimental models (Fernandes et al. 2015, Santana et al. 2018, Oliveira et al. 2019, 2022) without promoting signs of toxicity in animals at therapeutic doses (Souza et al. 2021). Studies have suggested the safety and efficacy of plant-derived compounds to restrict APAP-induced hepatotoxicity (Seif 2016, Subramanya et al. 2018, Singh et al. 2022). Traditionally some ethnic groups, have been used medicinal plants to treat liver injuries due to their antioxidant and anti-inflammatory properties (Seif 2016). Thus, natural products

have been highlighted as promising protective agents against paracetamol toxicity by reducing oxidative stress, inflammation, and cellular damage (Seif 2016).

Plant latex has been related to benefits for human health through medicinal use in many different cultures around the world, and scientific investigation has supported this use (Gracz-Bernaciak et al. 2021). Taking into consideration that LPPp has antioxidative and anti-inflammatory properties, key events related to APAP hepatic intoxication were evaluated for its hepatoprotective effect using an experimental model with mice.

MATERIALS AND METHOD

Drugs and reagents

APAP was obtained from Sigma-Aldrich (Saint Louis, Missouri, USA). Reagents for electrophoresis were obtained from GE Healthcare Brasil. Antibody kits for the cytokines (IL-6, IL-10, IL-1 β , TNF- α and IFN- β) and the chemokine CXCL1 (R&D Systems, United States) were used. Histological reagents were obtained from Dinamica®, Diadema. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Latex and extraction of laticifer proteins (LPPp)

The species was identified, and its exsiccated specimen was deposited in the Delta do Parnaíba Herbarium of the Federal University of Delta do Parnaíba under deposit number 2432. The latex was collected in the city of Parnaíba, Piauí State (02° 54' 17" S and 41° 46' 36" W). Latex proteins from *Plumeria pudica* (LPPp) were obtained as previously described (Fernandes et al. 2015). Briefly, the latex was collected after a small break on the shoot apex of healthy plants in Falcon tubes over a volume of distilled water 1:1 (v:v). The samples were then centrifuged, the

rubber-rich precipitate was removed, and the supernatant was collected. After, it was subjected to dialysis against distilled water for 48 hours using membranes with a molecular retention capacity above 8 kDa. The material retained inside the membranes was centrifuged again using the same conditions described above and the supernatant rich in proteins and absent in rubber was lyophilized and named LPPp (Latex proteins from *Plumeria pudica*).

Protein Identification

Ion exchange chromatography

The *P. pudica* latex protein fraction (LPPp) was subjected to ion exchange chromatography in a HiTrap DEAE FF column (1 ml) coupled to a peristaltic pump system. LPPp (5 mg) were dissolved in 1 ml of 50 mM sodium acetate buffer, pH 5.0. The sample was applied into the column and chromatography was performed at a flow rate of 1 ml/min where 1 ml fractions were collected. Retained peaks were eluted after applying a linear gradient of NaCl (0 to 0.4 M). This experimental strategy resulted in 4 chromatographic subfractions, one representing the peak not retained in the column and 3 eluted after application of NaCl.

Polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was performed according to the methodology described by (Laemmli 1970) through 12.5% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS). Chromatographic subfractions of LPPp were prepared at a concentration of 5 μ g/mL and dissolved in 0.0625 M Tris buffer (pH 6.8) containing 2% SDS and 5% 2-mercaptoethanol. The electrophoretic run took place at a constant voltage of 150 V and current of 25 mA, using 0.025 M Tris buffer (pH 8.3) containing 0.192 M glycine and 0.1% SDS. To observe the protein bands after

the run, the gel was stained with a solution of brilliant blue Coomassie R-350 0.1% in water: acetic acid: methanol (8:1:3.5 v/v/v) for 4 hours. After this period, the dye that did not bind to the proteins was removed with this same solution in the absence of the dye.

Mass spectrometry

Protein bands obtained from SDS-PAGE were removed and digested with trypsin. The identification was evaluated from the mass spectrum obtained in an electrospray ionization mass spectrometer. Identification attempts were performed by MS/MS spectra analysis. In each MS map generated, the five most intense ions from the tryptic sample were selected for fragmentation. From the spectra generated after fragmentation, an individual search was performed in databases for the possible identification of the protein studied using the search program “Mascot search engine” (Matrix Science, London-UK) and the NCBI Data Bank as database.

Animals

Animals weighing 20-30 g (*Mus musculus*, variety BALB c), female, were supplied by Federal University of Delta do Parnaíba. The animals were kept in plastic cages, in an acclimatized environment ($24 \pm 1^\circ\text{C}$), obeying the light/dark cycle (12 h/12 h) and receiving food and water *ad libitum*. All experimental procedures were approved and performed in accordance with the guidelines of Institutional Animal Ethics Committee (Protocol N. 028/19).

Evaluation of hepatoprotective efficacy of latex proteins from *P. pudica* in acetaminophen induced injury

The APAP hepatotoxicity treatment model in mice was chosen in accordance with previously published by (Ishida et al. 2002) with some adaptations. All mice were blinded to randomly

divide into groups in each consists of six animals. Animals were fasted three hours before the administration without restricting of water. The untreated control group received only phosphate buffered saline (PBS). APAP solution was prepared by dissolving in warm PBS (pH 7.2). Mice were intraperitoneally injected with APAP at the concentration of 750 mg/kg body weight. The animals were treated with a solution containing LPPp at 40 mg/kg, i.p. one hour before, one hour after and 5 hours after induction of liver injury in mice, totaling 3 treatments. The LPPp dose (40 mg/kg) and route of administration were selected based on prior studies by our group demonstrating its efficacy and safety in inflammatory models (Fernandes et al. 2015, Santana et al. 2018, Oliveira et al. 2019, 2022). In these studies, the 40 mg/kg dose consistently promoted antioxidant and anti-inflammatory responses without inducing toxicity. Moreover, Souza et al. (2021) reported no signs of acute or subchronic toxicity at doses up to 80 mg/kg in mice. Although a formal dose-response curve was not conducted in the present study, preliminary unpublished data from our laboratory have shown that lower doses (10 and 20 mg/kg) elicited weaker protective responses, while doses above 40 mg/kg did not significantly enhance hepatoprotective effects. Thus, 40 mg/kg was chosen as the optimal effective dose for this model. NAC at a dose of 318 mg/kg i.p., was used as standard drug for the treatment of paracetamol-induced liver injury.

Sample obtention

Ten hours after APAP administration the animals were euthanized by administration of Ketamine and Xylazine, 300mg/kg and 30mg/kg. The liver of the animals were excised and weighed, the following formula was used to calculate the relative weight: $\text{Body weight/body weight after treatment} \times 100$. Also, small samples of liver

were fixed in formaldehyde or saved under -80°C for further analysis. Blood samples were collected from each mouse by cardiac puncture for total leucocyte count and serum biochemical analysis.

Serum transaminase activity assay

As the biomarkers of hepatocellular damage, the ALT (Alanine aminotransferase) and AST (Aspartate aminotransferase) activities in serum were detected by using kits according to the manufacturer's protocol (Labtest®).

Antioxidant capacity assay

Liver homogenates (10%, w/v) were prepared for evaluation of the levels of glutathione (GSH), superoxide dismutase (SOD), and malondialdehyde (MDA). All measurements were performed according to methods previously described. GSH was used by (Sedlak & Lindsay 1968). SOD activity was measured by the protocol describe by (Das et al. 2000). The level of MDA was measured previously described (Uchiyama & Mihara 1978).

Determination of myeloperoxidase activity (MPO) and NO_3/NO_2 levels

The level of MPO, a marker for neutrophil infiltration, was estimated using the method described by (Bradley et al. 1982). The Griess method was used to measure the concentrations of nitrate and nitrite (Green et al. 1982).

Inflammatory cytokines and chemokine determination

The concentrations of murine Tumor Necrosis Factor- α (TNF- α , Cat. No. DY410), Interleukin- 1β (IL- 1β , Cat. No. AF-401-NA), Interleukin-6 (IL-6, Cat. No. MAB406), Interleukin-10 (IL-10, Cat. No. AF519), Interferon- γ (IFN- γ , Cat. No. 485-MI), and Chemokine CXCL1 (Cat. No. DY453-05) in liver tissue homogenates were determined using commercially available DuoSet ELISA

Development Kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Histological assessment

At first samples of hepatic tissue were removed and fixed in 10% buffered formaldehyde, after this process were dehydrated in increasing concentrations of ethyl alcohol and immersed in xylene. Thus, were embedded in paraffin. After fixation, the tissue sections were cut into 5 μm slices and then stained with Hematoxylin and Eosin (H&E) reagent to evaluate injury and inflammation parameters. Photomicrographs were taken with a light microscope equipped with a microscope camera (Nova®, Piracicaba). Histological assessment of the liver was scored as described by (Vasconcelos et al. 2019). Stestosis score was categorized according to the percentage of fatty hepatocytes distributed in the liver, as: 0, 0 < 5%; 1, 6 < 25%; 2, 26 < 50%; 3, 51 < 75%; and 4, > 76%. Inflammation and necrosis score were graded as: one focus per field, 1 and two or more focus per field. The foamy vacuolated cytoplasm and nuclear fragmentation were performed using a 100- point mesh in an area of 1,170 μm^2 on the 1,000 $\times$ magnification.

Immunohistochemistry Analysis of inflammatory cells infiltration in liver tissue

For immunostaining of neutrophils in the liver tissue of animals treated or not with LPPp in the face of injury induced by APAP overdose, blocks from samples used for histopathological evaluation were cut and prepared on marked slides, specific for immunohistochemistry. Slides were incubated overnight with anti-ly6G antibody (1:2000; EPR22909-135; ab238132, abcam) followed by a biotinylated secondary antibody and color was revealed using DAB (3, 3'-diaminobenzidine) (Sigma-Aldrich, USA). The sections were counterstained with hematoxylin,

mounted and evaluated using Motic microscope (Carl Zeiss, Gottingen, Germany) adapted to a digital camera (PowerShot A620, Canon, Tokyo, Japan).

Statistical Analysis

The means $\pm$ SEM of the number of events ($n = 6$) in each group were calculated. The Shapiro-Wilk test was used to verify the distribution of the data (parametric or nonparametric data). Statistical significance of differences between the groups was determined by one-way analysis of variance (ANOVA) followed by the student-Newman-Keuls test or Tukey Test. Histological scores were assessed by the nonparametric Kruskal-Wallis test followed by Dunn's multiple-comparison test. For all tests, values of $p < 0.05$ were considered significant. Statistical tests were performed with Graphpad Prism (version 7.0).

In silico Molecular Docking

The 3D structure of possible proteins from *Plumeria pudica* were obtained from the Protein Data Bank (PDB) with the code PDB ID: 3CQL (Chitinase), 4L2J (Osmotin), 9PAP (Cysteine proteinase) and 3S8K (Serine proteinase inhibitor). All docking procedures utilized the Autodock 4.2 package. Protein and ligands were prepared for docking simulations with AutoDock Tools (ADT) version 1.5.6. The binding energy was evaluated by Lamarckian genetic algorithm method. Also, blind molecular docking assays were carried involved proteins 3CQL, 4L2J, 9PAP and 3S8K ligands with CYP 2E1 (3E6I), tumor necrosis factor (PDB ID: 2TNF) and interferon Alpha2 (PDB ID: 7E0E). All molecular docking experiments were performed using the ClusPro 2.0 server (<https://cluspro.org>), which is the best performing server currently available for the CAPRI challenge.

RESULTS

Protein profile of *Plumeria pudica* latex

The LLPp was fractionated by ion-exchange chromatography and four protein peaks were obtained (Supplementary Material - Figure S1). The first (P1) was obtained by washing the column with the equilibrium buffer (50 mM sodium acetate, pH 5.0), while the second and third (P2 and P3) were eluted from the column after gradient elution of NaCl (0 - 0.4M). The fourth peak (P4) was collected after washing the column with a 1M NaCl solution. Analysis of LLPp fraction by 12.5% SDS-PAGE confirmed the presence of many proteins (Figure 1). P1 showed protein bands predominantly in the range of 20 kDa to 60 kDa. P2 and P3 showed protein bands with different apparent molecular weights, ranging from 20 to 40 kDa while P4 contains proteins from 15 to 45 kDa. These results were confirmed by the mass spectrum and all protein spots from SDS PAGE were identified (Table I). Among the proteins found in LLPp we observed cysteine proteinases, chitinases, thaumatin (osmotin), peroxidase, pathogen-related protein 4, endo-1-3- β glucanase, chitin-binding proteins.

Biological benefits of LLPp on APAP-exposed mice

As shown in Figure 2A, APAP-exposed mice exhibited a decrease in total leukocyte count in peripheral blood ($2,200 \pm 302 \text{ mm}^3$) when compared to PBS group ($6,117 \pm 713 \text{ mm}^3$). Moreover, the LLPp-treated groups demonstrated improved leukocyte levels (Figure 2B), reduced serum ALT and AST activities (Figures 2C and 2D), indicating hepatoprotective effects. The treatment of animals with LLPp ($6,275 \pm 493 \text{ mm}^3$) was able to inhibit the reduction in leukocyte count. NAC also inhibited the decrease in the number of leukocytes ($5,190 \pm 300 \text{ mm}^3$). There was no significant difference when comparing the LLPp and NAC groups. To liver weights Figure

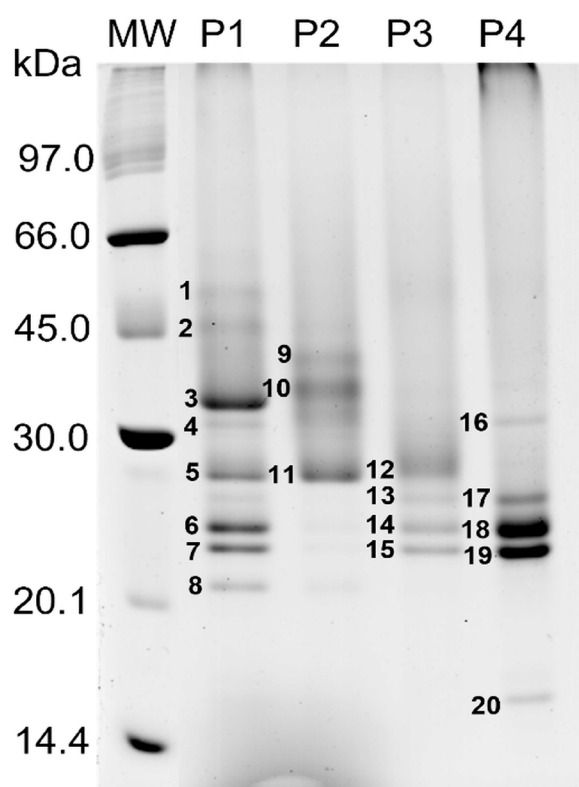


Figure 1. Polyacrylamide gel electrophoresis (SDS-PAGE, 12.5%) of chromatographic peaks of latex proteins from *Plumeria pudica* in DEAE pH 5.0 column. Molecular mass markers used: phosphorylase B (97.0 kDa), bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and alpha-lactalbumin (14.4 kDa). The numbers indicate the protein bands that were selected for further trypsin digestion and identification by mass spectrometry.

2B, APAP-exposed mice resulted in increased liver weight (6.56 ± 0.66 /100g of animal) when compared to these in APAP- free mice (PBS) (3.64 ± 0.12 g/100g of animal). By contrast, this parameter in mice treated with LPPp (4.09 ± 0.11 g/100g of animal) was decreased suggesting its potential hepatoprotective effects. Also, NAC (3.82 ± 0.13 g/100g of animal), used as standard in this study, avoided liver increase weight in mice. There was no significant difference when comparing the LPPp and NAC groups.

Effects of LPPp on biochemical indicators of liver function

To assess the potential protective effect of LPPp against APAP-induced hepatotoxicity, the serum levels of ALT and AST markers of liver injury were determined. As displayed in Figure 2C and D a significant elevation of ALT and AST, levels in mice that received APAP were observed (AST: 141.3 ± 1.9 U/L; ALT: 183.6 ± 10.9 U/L, compared to control PBS (AST: 96.6 ± 7.2 U/L; ALT: 119.3 ± 5.5 U/L). LPPp treatment showed significant inhibition on increasement of these parameters (AST: 63.6 ± 11.0 U/L; ALT: 134.6 ± 2.9 U/L). Meanwhile, the group that received the NAC (AST: 88.6 ± 2.4 U/L; ALT: 140.6 ± 2.3), the increased levels of these markers were inhibited. In relation to ALT, there was significant difference when comparing the LPPp and NAC groups.

LPPp decreased oxidative stress in APAP-exposed mice overdose

As shown in Figure 3, injection of APAP resulted in significant increase of MDA ($1,036 \pm 67.7$ nmol/g of tissue), reduction of SOD (3.66 ± 0.65 μ mol/g of tissue) and GSH levels (82.2 ± 19.3 μ mol/g of tissue) compared to the PBS group (MDA: 316 ± 45.7 nmol/g of tissue; SOD: 7.09 ± 0.57 μ mol/g of tissue; GSH: 304.9 ± 29.8 μ mol/g tissue) indicating APAP-induced oxidative liver stress and decrease of the antioxidant activity. The LPPp treatment significantly prevented all the effects of APAP to MDA (237.3 ± 49.6 nmol/g tissue), SOD (6.24 ± 0.38 μ mol/g of tissue) and GHS (315.3 ± 35.9 μ mol/g of tissue). The group that received NAC it also was observed prevention APAP effects (MDA: 405.3 ± 37.0 nmol/g of tissue; SOD: 7.46 ± 0.46 μ mol/g of tissue; GSH: 213.5 ± 16.6 μ mol/g of tissue). In addition, significant difference was observed to GSH antioxidant enzyme levels between LPPp and NAC groups.

Table I. List of proteins identified in *Plumeria pudica* latex by ESI-QUAD-TOF.

Peak	Band	Acess	Protein and Organism	Molecular Mass (Da)	Protein Score	Peptide Sequence
P1	1	XP_006359049	Peroxidase 12-like <i>Solanum tuberosum</i>	39.395	109	KYYVDLMNRQ RIVSCSDIAALAARD
	2	Q41596	Endochitinase 1 <i>Theobroma cacao</i>	35.794	70	RFDQMLLHRN
	3	KYP77074	Ribonuclease H protein <i>Cajanus cajan</i>	32.247	62	KLNC DGAVADNVRA
	4	XP_011083774	Endo-1,3-beta-glucosidase <i>Sesamum indicum</i>	37.988	72	RIYDPHQPTLQALRG RAIETYIFAMFDENQKNPEYEKH
	5	KHN05804	Vignain <i>Glycine soja</i>	32.359	60	KVGSVPPSVDWRK
	6	AHX59270	Procerain B <i>Calotropis procera</i>	39.060	134	RSGVFSGACGPRV RMIALSEQELLD CERT RVDHAVNIVGYGSEGGVNYW
	7	XP_003550321	Cysteine proteinase <i>Glycine max</i>	53.279	74	KLPDSVDWRK
	8	ALA55569	Thaumatococin-like protein <i>Cryptostegia grandiflora</i>	22.948	218	RTNCFD GAGRG RAPGGC NNPCTVF KT KDDPTSTFTCPGGTNYRVI
P2	9	KHG16133	Endochitinase 1 <i>Gossypium arboreum</i>	35.711	82	RETFNQMLLHRN RVPGYGVITNIINGGLECGKG
	10	XP_006340814	Basic endochitinase-like <i>Solanum tuberosum</i>	29.257	107	KWRPTGADLSAGRV
	11	KHG16133	Endochitinase 1 <i>Gossypium arboreum</i>	35.711	125	RETFNQMLLHRN RVPGYGVITNIINGGLECGKG
P3	12	KDO71433	Chitin-binding type-1 <i>Citrus sinensis</i>	36.082	170	KSFGAFGTTGDTDRK RLPGYGVVTNIINGGIECGKG
	13	KDO71433	Chitin-binding type-1 <i>Citrus sinensis</i>	36.082	144	KSFGAFGTTGDTDRK RLPGYGVVTNIINGGIECGKG
	14	OAY70879	Cysteine proteinase <i>Ananas comosus</i>	37.802	96	KNSWGTTWGENGYMRI
	15	AGI59309	Procerain B <i>Calotropis procera</i>	24.187	74	LPNSVDWRE
P4	16	KDO71433	Chitin-binding type-1 <i>Citrus sinensis</i>	36.082	144	KSFGAFGTTGDTDRK RLPGYGVVTNIINGGIECGKG
	17	OAY70879	Cysteine proteinase <i>Ananas comosus</i>	37.802	83	KNSWGTTWGENGYMRI
	18	AMY15659	Procerain <i>Calotropis procera</i>	38.739	162	RSGVFSGACGPRV RVDHAVNIVGYGSEGGVNYWIVRN
	19	AHX59270	Procerain B <i>Calotropis procera</i>	39.060	251	RTSYGCKGGYYTDAFAYVAKK RVDHAVNIVGYGSEGGVNYWIVRN
	20	AIE39008	Pathogenesis-related protein 4 <i>Vaccinium virgatum</i>	14.914	77	RIVDQCSNGGLDL DAGVFKQ

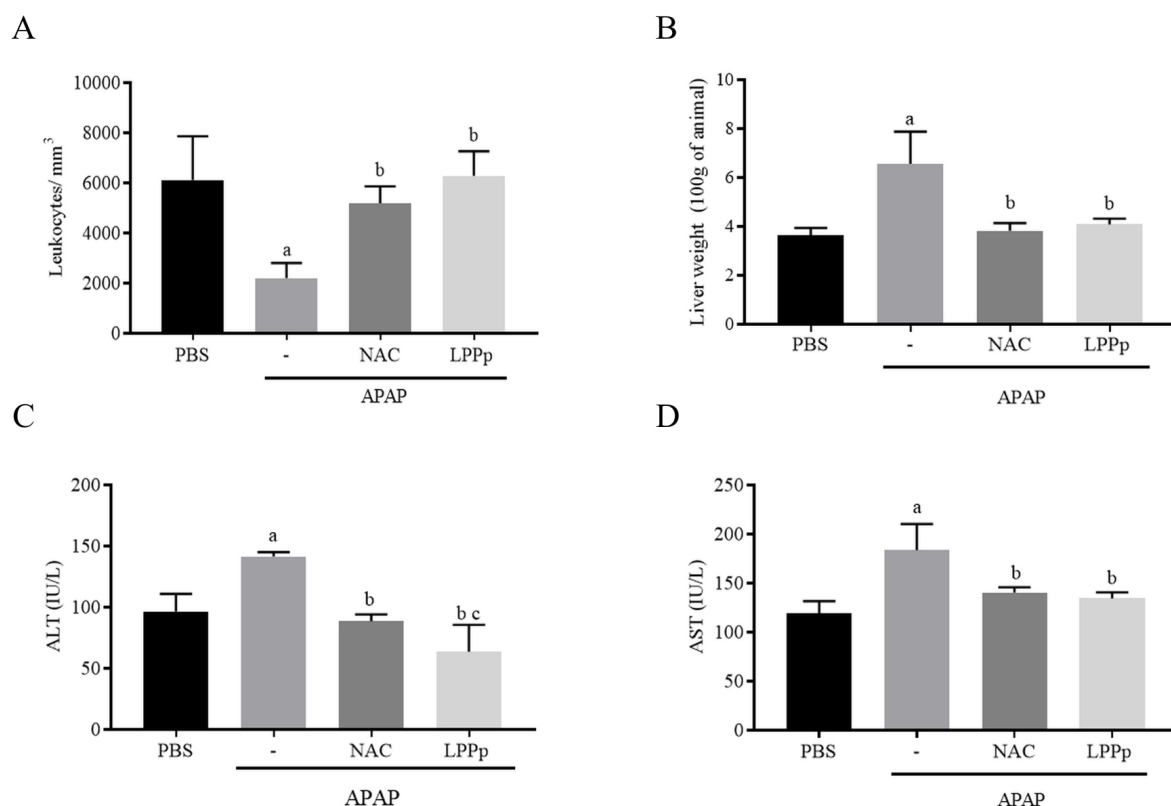


Figure 2. Effects of *Plumeria pudica* latex proteins (LPPp, 40 mg/kg) on paracetamol-induced hepatotoxicity in mice. (A) Total leukocyte count from peripheral blood, (B) Relative weight of mouse liver, (C) Alanina aminotransferase (ALT) and (D) Aspartato transferase (AST). Results are expressed as mean $\pm$ SEM of six animals per group. The letters "a" and "b" indicate statistical difference ($p < 0.05$) in relation to PBS and APAP (750 mg/kg) respectively. ANOVA followed by Tukey's test ($p < 0.05$) for total count. ANOVA followed by Student-Newman-Keuls ($p < 0.05$) test for other parameters.

Effects of LPPp in APAP-induced liver inflammation markers

APAP induced the elevation of NO_3/NO_2 ($0.237 \pm 0.015 \mu\text{M}$) production when compared to PBS group ($0.07 \pm 0.01 \mu\text{M}$) (Figure 4A). Nevertheless, the hepatic concentration NO_3/NO_2 in animals treated with LPPp showed significant reduction ($0.14 \pm 0.01 \mu\text{M}$). A significant inhibition of the hepatic concentration of this marker was observed in the group treated with NAC ($0.09 \pm 0.01 \mu\text{M}$). In addition, there was significant difference between LPPp and NAC groups.

Significant increase on MPO concentration ($6.00 \pm 0.27 \text{ UMPO/mg}$ of tissue) in the liver of mice receiving APAP was seen (Figure 4B) compared to PBS ($0.54 \pm 0.19 \text{ UMPO/mg}$ tissue).

Animals were treated with the LPPp, showed significant inhibition of the increase in this marker of neutrophil migration ($2.72 \pm 0.55 \text{ UMPO/mg}$ tissue). The group that received NAC, presented significant elevation of MPO levels ($0.83 \pm 0.31 \text{ PMU/mg}$ tissue). There was significant difference between LPPp and NAC groups.

LPPp reduced inflammatory cytokines and chemokine in APAP-exposed mice

The systemic and local inflammatory response was evaluated by measuring hepatic levels of $\text{TNF-}\alpha$, IL-6, IL-1 β , IFN- γ , IL-10, and CXCL1. The data, presented in Figure 5, show that LPPp treatment significantly modulated the inflammatory cytokine profile in APAP-exposed mice. The

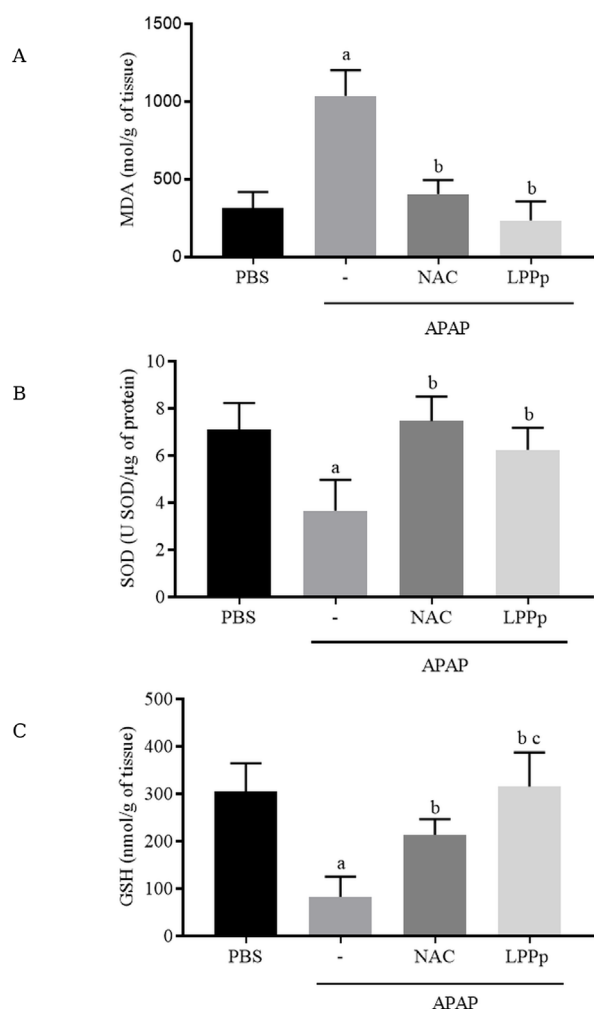


Figure 3. Markers of antioxidant indices and oxidative stress in paracetamol-induced liver injury in mice treated with *Plumeria pudica* latex proteins (LPPp, 40 mg/kg). (A) Maloaldehyde (MDA), (B) Superoxide dismutase (SOD) and (C) Glutathione (GSH). Results are expressed as mean $\pm$ SEM of six animals per group. The letters “a” and “b” indicate statistical difference ($p < 0.05$) in relation to PBS and APAP (750 mg/kg), respectively. Letter “c” indicates statistical difference ($p < 0.05$) in relation to NAC. ANOVA followed by the Student-Newman-Keuls test ($p < 0.05$).

levels of pró-inflammatory cytokines are shown in Figure 5A-D. Mice that received only APAP showed elevated values to TNF- α ($7,434 \pm 875.6$ pg/mL), IL-6 ($4,168 \pm 258.7$ pg/mL), IL-1 β ($4,830 \pm 166.1$ pg/mL) and IFN- γ ($3,843 \pm 318.6$ pg/mL), demonstrating a significant difference when compared to PBS group (TNF- α : 776.6 ± 415.8 ; pg/

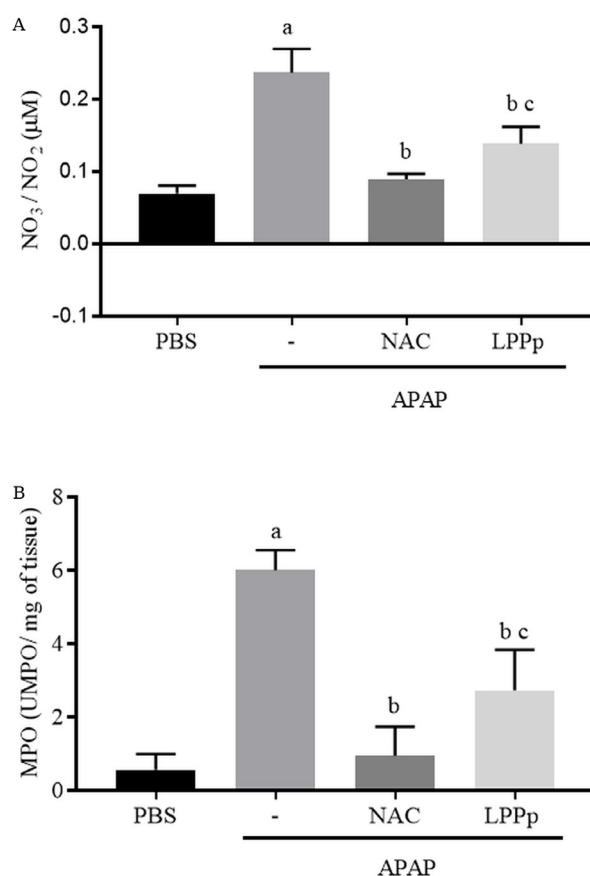
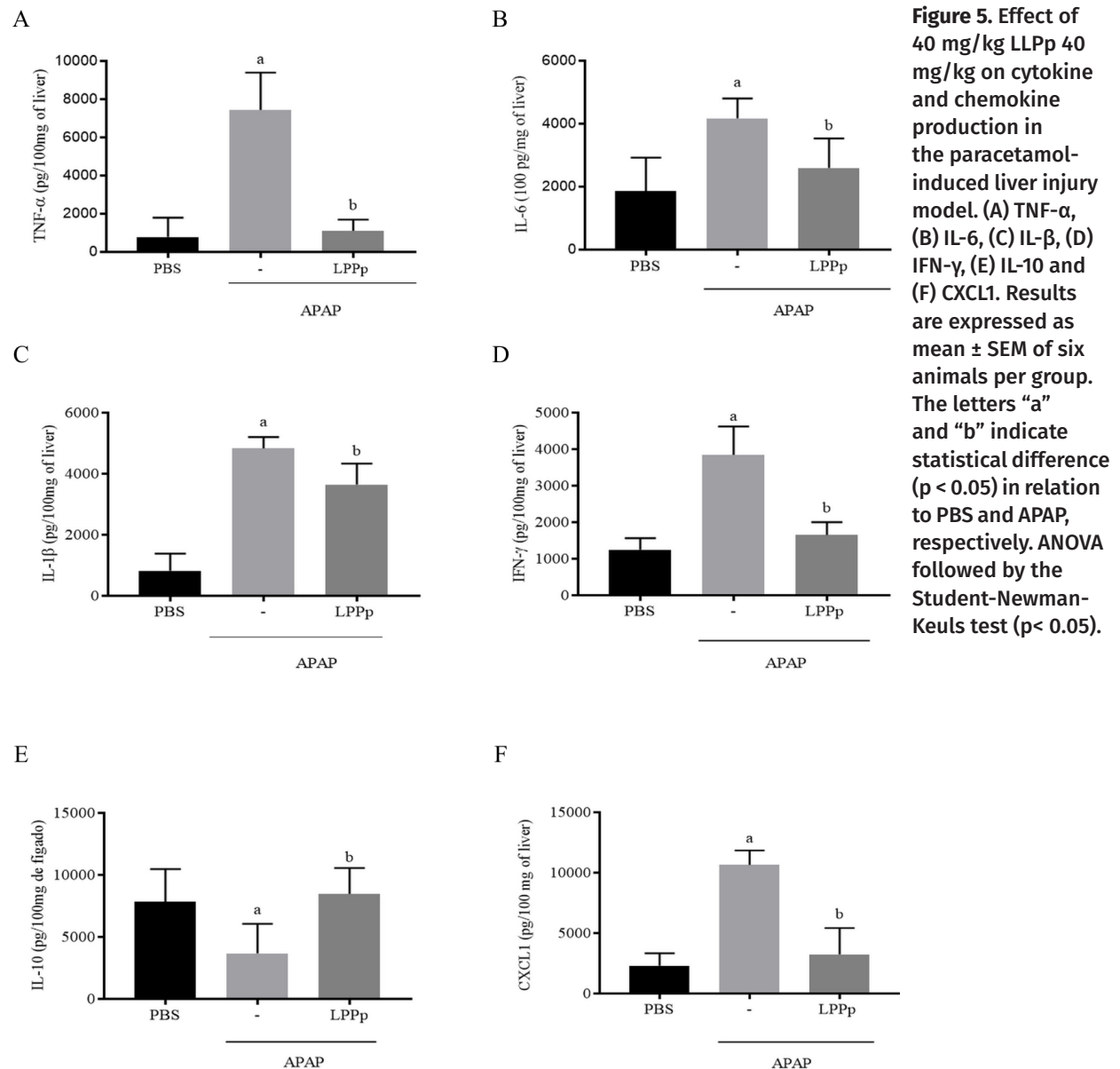


Figure 4. Effects of *Plumeria pudica* latex proteins (LPPp, 40 mg/kg) on inflammatory parameters of paracetamol-induced liver injury. (A) Concentration of nitrite and nitrate (NO₃/NO₂) and (B) Myeloperoxidase (MPO) in the liver of mice. Results are expressed as mean $\pm$ SEM of six animals per group. The letters “a” and “b” indicate statistical difference ($p < 0.05$) in relation to PBS and APAP (750 mg/kg), respectively. Letter “c” indicates statistical difference ($p < 0.05$) in relation to NAC.

mL; IL-6: $1,858 \pm 433.4$ pg/mL; IL-1 β : 818.3 ± 230.1 pg/mL; IFN- γ : $1,248 \pm 128.7$). LPPp treatment significantly inhibited these elevations (TNF- α : $1,107 \pm 241.7$ pg/mL; IL-6: $2,591 \pm 385.3$ pg/mL; IL-1 β : $3,642 \pm 308.7$ pg/mL, IFN- γ : $1,653 \pm 141.9$ pg/mL). For the levels of the anti-inflammatory cytokine IL-10, the mice that received only APAP showed $3,367 \pm 1,069$ pg/mL, demonstrating a significant difference compared to PBS ($7,838 \pm 1,316$ pg/mL). Mice that were treated with LPPp ($8,470 \pm 930.2$ pg/mL) demonstrate significant



increasing compared to the APAP group (Figure 5E). Regarding CXCL1 chemokine (Figure 5F), mice that received only APAP showed increased values ($10,640 \pm 490.2$ pg/mL) demonstrating significant difference to animals that received only PBS ($2,292 \pm 426.3$ pg/mL). For the LLPp group, the value presented was $3,241 \pm 891.2$ pg/mL demonstrating significant inhibition when compared to the APAP group.

LLPp protects to hepatocellular histopathological changes induced by APAP

Samples of liver tissue from animals from different groups evaluated histologically and stained with HE is shown in Figure 6. Histopathological findings in liver tissue in PBS group, indicated normal and undamaged hepatocytes, lobules, and sinusoids (Figure 6A and B). In contrast, microscopic analysis of tissue sections of mice showed that APAP could lead to

alterations such as loss of organization near the central vein, hepatocytes with microvesicular steatosis and nuclear fragmentation mainly in cells close to the central vein. (Figure 6C and D). As expected, the group that received NAC had a more preserved centrilobular region and an area of mild injury (Figures 6E and F). After treatment with LPPp, mice showed more preserved hepatic architecture, smaller lesion area, lower number of hepatocytes with steatosis, in addition to a lower number of cells with morphological characteristics of death (Figures 6G and H). These findings were corroborated by morphometric analysis, as shown in Table II which quantifies histopathological scores reflecting the extent of hepatic tissue injury. It was observed that the PBS group obtained minimum, while APAP group presented maximum injury scores. The treatment of the animals with LPPp significantly contributed to the reduction of the values of scores compared to the APAP group. NAC treatment produced a reduction in scores compared to the APAP control group. In addition, no significant difference was observed between LPPp and NAC treatments.

LPPp inhibited inflammation-related to neutrophils Ly6G accumulation in liver

The result of Ly6G-Hematoxylin immunostaining is presented in Figure 7. Immunohistochemical staining for neutrophils showed extensive accumulation of these leukocytes in centrilobular necrotic areas in the APAP group (56.3 ± 4.7 Ly6G⁺ cells/mm²). The PBS group did not present high values of the presence of these cells (12.5 ± 1.7 Ly6G⁺ cells/mm²). On the other hand, a significant decrease in the number of neutrophils was observed in the liver of the group

treated with LPPp (23.3 ± 2.8 Ly6G⁺ cells/mm²). Immunohistochemistry to Ly6G cells revealed that LPPp- treatment promoted a significant decrease in the number of neutrophils in the liver of mice.

The molecular docking analysis revealed notable binding affinities between APAP or NAPQI and several proteins identified in LPPp, as illustrated in Figure 8. Specific interaction details, such as binding energies and amino acid residues involved in ligand-protein binding, are summarized in Table SI. Among the analyzes performed, the best molecular affinity parameters were obtained from the interaction of APAP with cysteine proteinase (PDB ID 9PAP). Affinity was observed with binding energy equal to -5.79 kcal.mol⁻¹ and an inhibition constant of 56.98 μ M. In the ligands it was possible to observe the interaction with the amino acids Glu50 and Lys17 of the active site via hydrogen bonding (Figure 8A, B and C). Furthermore, the molecular affinity of NAPQI with cysteine proteinase was also considered to be the best, with binding energy equal to -5.59 kcal.mol⁻¹ and an inhibition constant of 80.5 μ M. The interaction occurred through the active site amino acids Lys174 and Lys17 through a hydrogen bond (Figure 8D, E and F).

The results related to the molecular docking between CYP 2E1, TNF- α and IFN- α with proteins identified in the latex of *P. pudica* are presented in Figure S2 and in Table SII. Among the analyzes performed, the lowest binding energy was obtained from the interaction with proteinase inhibitor (3S8K- 3E6I: -1146.0 , 3S8K-7E0E: -927.2 AND 3S8K-2TNF: -922.1).

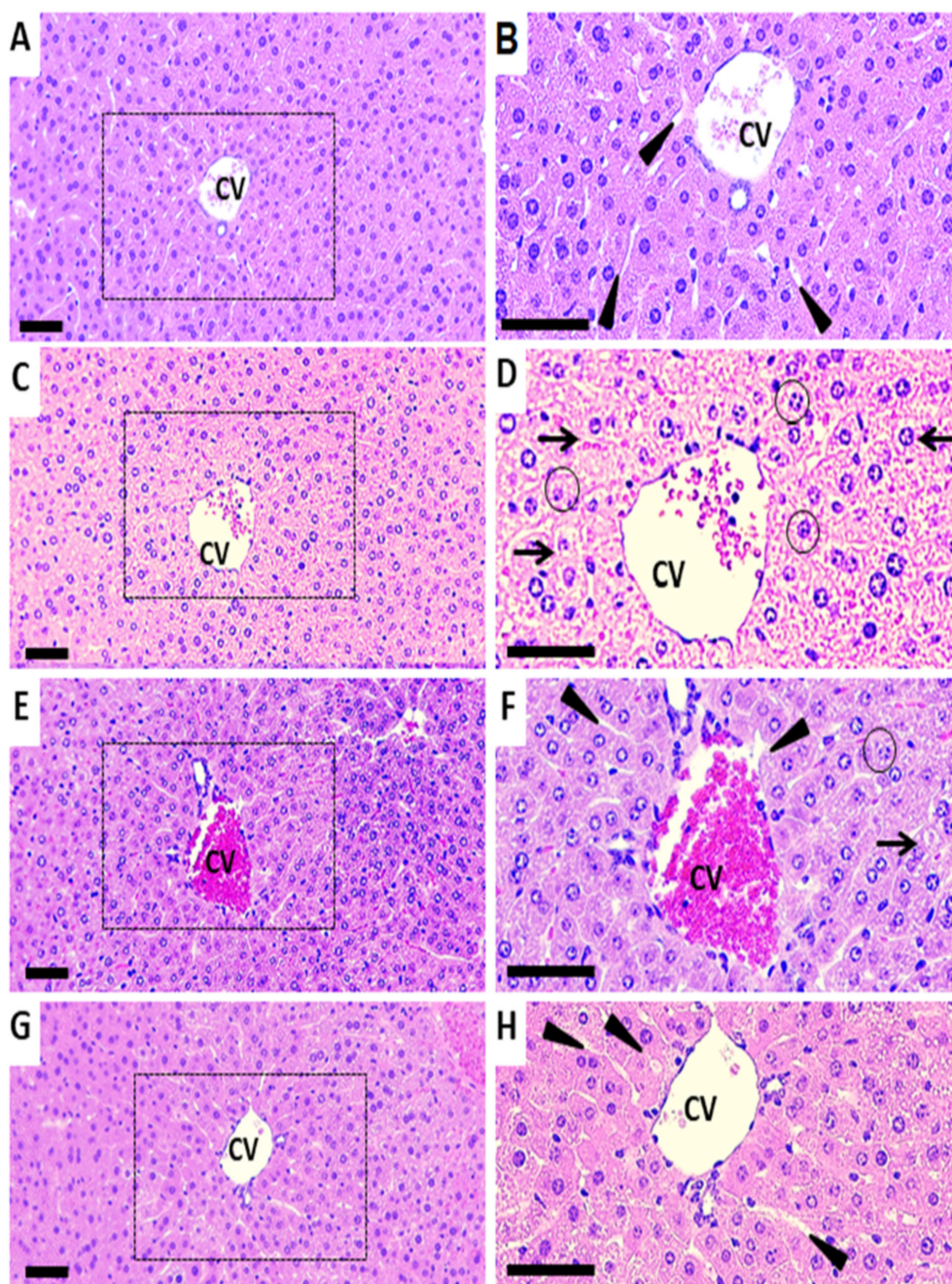


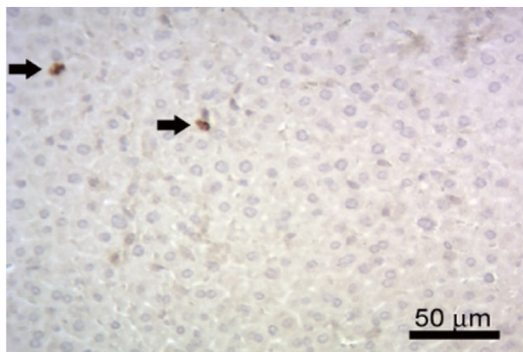
Figure 6. Effects of LPPp treatment (40 mg/kg) on liver tissue of mice submitted to paracetamol- induced liver injury. (A) and (B) Indicate the liver from PBS group (saline) with normal architecture and sinusoid blood vessels (arrowheads) organization near to the central vein (CV) in the middle of the image. (C) and (D) APAP group demonstrate loss of cordon organization near to the central vein (CV), hepatocytes with microvesicular steatosis (arrows) and nuclear fragmentation (circles) of the hepatocytes. (E) and (F) illustrate the liver from NAC group with congestion blood in the central vein (CV) and the hepatocytes demonstrated improve in the cordon organization around the central vein (CV). (G) and (H) Indicate the liver from LPPp group with reduction of microvesicular steatosis compared to the APAP group, the hepatocytes demonstrated improve in the cordon organization near to the central vein (CV), that can be observed by the sinusoid blood vessels (arrowheads) organization treated with LPPp. Hematoxylin and eosin staining in all images; original magnification 150× (short bar) for left column and magnification 600× (long bar) for right column. Pictures are representative samples of tissues from every animal.

Table II. Evaluation score of liver histological changes for each experimental group.

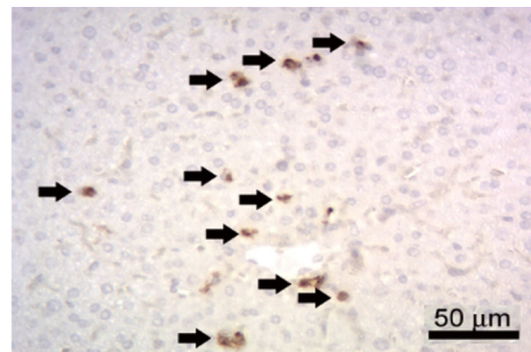
Parameters	PBS	-	PLPp (40 mg/kg)	NAC (300 mg/kg)
APAP (750 mg/kg)				
Steatosis	0 (0-1)	2 (2-3) ^a	0 (1-0) ^b	0 (0-1) ^b
Inflammatory Infiltration cells	0 (0-1)	1 (0-1) ^a	0 (0-1) ^b	0 (0-1) ^b
Necrosis Score	0 (0-1)	2 (2-3) ^a	0 (0-1) ^b	0 (0-1) ^b
Hidropic degeneration	0 (0-1)	3 (2-3) ^a	0 (0-1) ^b	0 (0-1) ^b
Foamy Vacuolated Cytoplasm	0 (0-1)	2 (2-3) ^a	0 (1-0) ^b	0 (0-1) ^b
Nuclear Fragmentation	0 (0-1)	2 (2-3) ^a	1 (1-0) ^b	1 (0-1) ^b

The letters “a” and “b” indicate statistical difference ($p < 0.05$) compared to PBS and APAP (750 mg/kg), respectively.

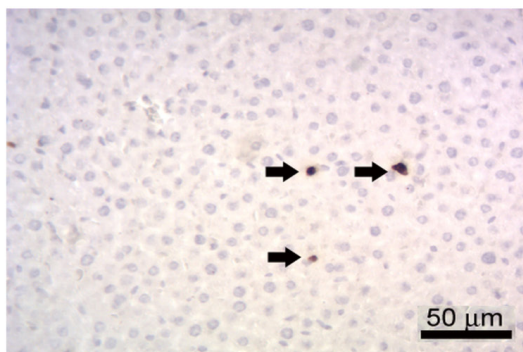
A



B



C



D

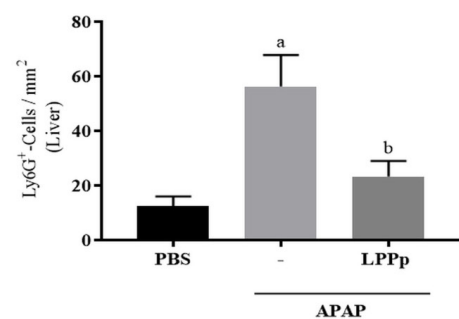


Figure 7. Comparison of neutrophil accumulation in necrosis sites in the liver of mice treated with *Plumeria pudica* Latex Proteins (LPPp 40 mg/kg). Ly6G⁺ neutrophils were detected in peripheral zones of necrosis (black arrow) in groups PBS(A), APAP (B) and LPPp (C). Measurement of Ly6G mm3 cell levels in the mouse liver (D). The letters “a” and “b” indicate statistical difference ($p < 0.05$) in relation to PBS and APAP, respectively. ANOVA followed by the Student-Newman-Keuls test.

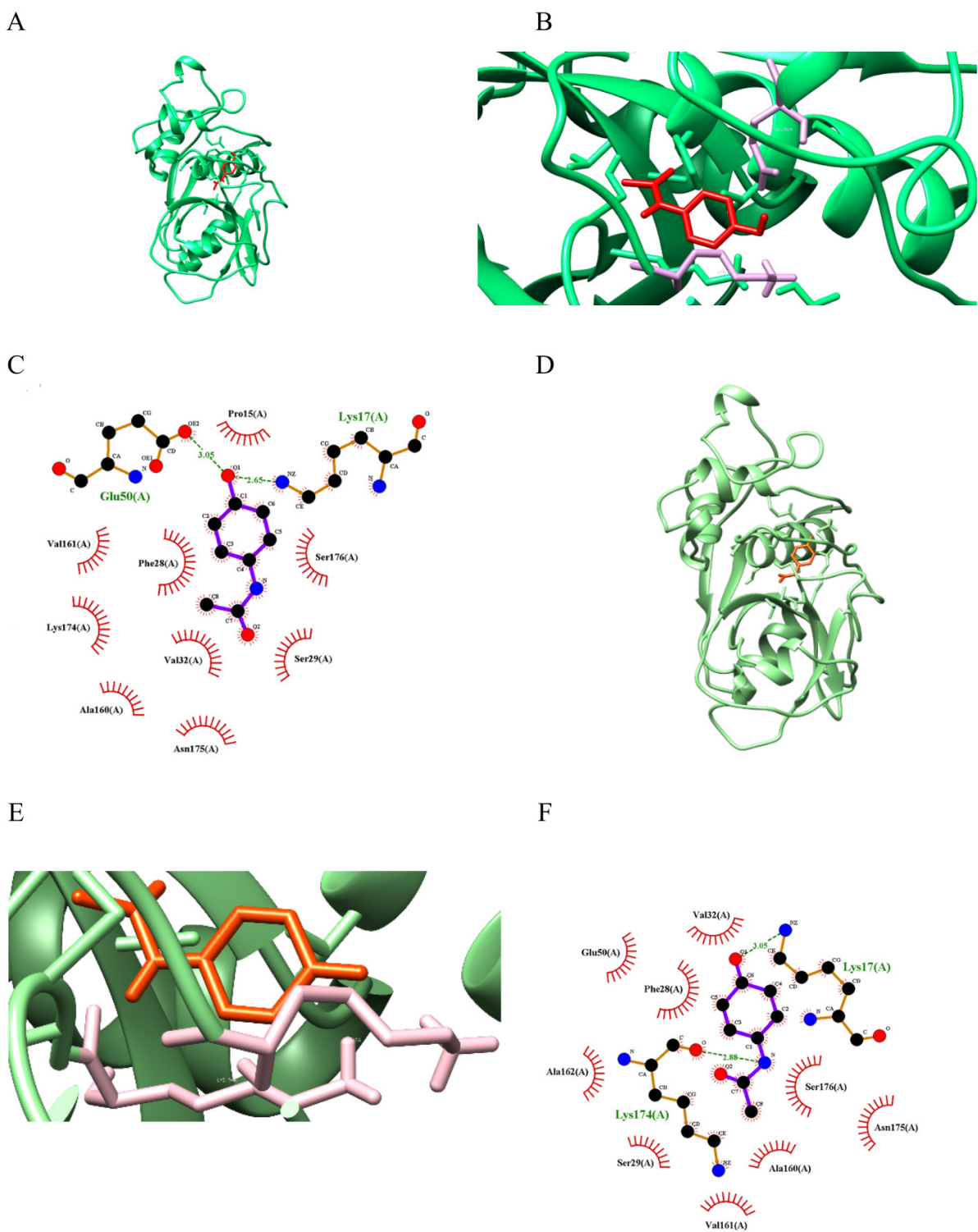


Figure 8. 3D molecular docking of the protein-ligand complex with cysteine proteinase (PDB ID 9PAP) and Acetaminophen ligand (APAP). (A) The active binding site with the respective hydrogen bond interactions (B and C). Red. APAP. Pink. Cysteine proteinase.

DISCUSSION

In the present study, the protective effects of latex proteins from *Plumeria pudica* (LPPp), was investigated against mice APAP-induced liver injury. It is well documented that LPPp have antioxidative and anti-inflammatory effects in some animal experimental models of inflammation and these activities are related to proteins present in this fraction (Fernandes et al. 2015, Santana et al. 2018, Oliveira et al. 2019, 2022). Herein, our results showed that LPPp efficiently protected animals against APAP-induced acute hepatotoxicity, and we suggest that this event involves its protein content, and it happens via inhibition of oxidative stress and inflammatory response that consequently avoided hepatocyte death.

Although several natural products have been shown to counteract APAP-induced hepatotoxicity, LPPp appears to act via a multifactorial mechanism involving both antioxidant and anti-inflammatory pathways. For example, flavonoids from *Ziziphus jujuba*, polyphenols from *Garcinia mangostana*, and triterpenoids from *Sorbus pohuashanensis* have been reported to attenuate oxidative stress and inflammation in similar models (Huang et al. 2017, Ibrahim et al. 2018, Yin et al. 2019). However, unlike many isolated phytochemicals, LPPp contains a complex mixture of proteins with diverse bioactivities, including chitinases, osmotins, and cysteine proteinases, which may exert synergistic effects. This multifaceted composition may offer advantages in terms of breadth of action, which should be explored in future head-to-head comparative studies.

When the liver is injured by APAP many different biochemical, physiological, and cellular events are triggered. AST and ALT are enzymes located within hepatocytes and are released into the bloodstream upon cellular damage.

Thus, elevated serum levels of these enzymes are routinely used as biochemical markers of liver injury and reflect the loss of hepatocellular membrane integrity, typically associated with necrosis (Giannini et al. 2005, Chalasani et al. 2014). Thus, serum levels of these enzymes are often assessed as biochemical markers of liver injury and their increase are suggested loss of functional integrity of hepatocellular cell membranes and it is associated to necrosis (Uchida et al. 2017). Our results demonstrated that LPPp treatment significantly reduced the serum levels of both biochemical markers in APAP-treated mice. Concurrently, APAP metabolism leads to the formation of the toxic reactive metabolite NAPQI, which damages hepatocytes, leading to inflammation and subsequent liver tissue injury, potentially resulting in decreased total leukocyte counts in the blood (Rainsford & Velo 2012). Interestingly, we observed normal total leukocyte count in the blood of mice treated with LPPp. These liver alterations during overdose of APAP are described to be associated to accumulation of collagen and extracellular matrix protein, liver congestion, as well as microsomal induction, resulting in increment in liver weight (Hall et al. 2012, Elsayed et al. 2020). Animals treated with LPPp have the reduction in liver weight associated with APAP overdose significantly inhibited. These results demonstrate the protective effect of LPPp.

Liver oxidative stress, is described as one of the main mechanisms involved in the development of hepatotoxicity, is an important parameter to be evaluated during APAP toxicity (Du et al. 2016). APAP overdose typically leads to reduced activity of antioxidant enzymes, particularly GSH and SOD, promoting ROS accumulation and lipid peroxidation. GSH serves as the first line of defense against oxidative stress, and its depletion enables the accumulation of NAPQI, triggering

mitochondrial dysfunction, lipid peroxidation, and DNA fragmentation, ultimately resulting in hepatocyte death (Hinson et al. 2010, McGill & Jaeschke 2013). GSH is considered as the first defense barrier against oxidative stress. When GSH is deficient it is observed an accumulation of NAPQI that will bind to cellular proteins and induce a series of toxic reactions, including lipid peroxidation, mitochondrial dysfunction, and DNA fragmentation, leading to massive hepatocyte damage or death (McGill et al. 2012a).

Our results demonstrated that LPPp treatment preserved hepatic GSH levels, thereby sustaining antioxidant capacity and reducing NAPQI accumulation. Another important enzyme that participates of the antioxidant defense system is the SOD (Yin et al. 2019). It protects the cell integrity by resisting free radicals in the process of biological oxidation, thus its evaluation can be used to assess the intensity of liver damage (Wang et al. 2021). In this study, LPPp-treatment was able to maintenance SOD activity. In relation to MDA measurement, a sensitive biomarker of oxidative stress and one of the products of lipid peroxidation, that indicates hepatic injury (Ibrahim et al. 2018), LPPp also significantly inhibited it increment. The improvement of all these parameters indicated that LPPp could reduce the APAP-induced liver oxidative stress, significantly inhibited the effects of ROS and lipid peroxidation, thereby exerting the hepatoprotective effects. These observations corroborate with previous studies showing that LPPp acts *in vivo* by preventing the oxidation of biomolecules by improving the antioxidant activity enzymes (Santana et al. 2018, Oliveira et al. 2019).

Oxidative stress during APAP-induced acute liver injury is rapidly followed by an intense inflammatory response. Excess ROS and cell necrosis activate Kupffer cells and macrophages to release pro-inflammatory

mediators, which in turn recruit neutrophils and monocytes to the liver, exacerbating inflammation (Cover et al. 2006, Jaeschke & Ramachandran 2020). Uncontrolled oxidative stress associated to cell necrosis, stimulate macrophages to generate pro-inflammatory mediators that are responsible for hepatic recruitment of neutrophils and monocytes resulting in inflammation (Antoniades et al. 2012). The hepatic APAP-inflammatory response occurs with the release of pro-inflammatory mediators, such as NO (Huang et al. 2017). NO can react with ROS and produce peroxynitrite, which induces cytotoxic effect on neutrophils, induction of apoptosis and aggravation of lipid peroxidation (Huang et al. 2017, Woolbright & Jaeschke 2017). Our results demonstrated that LPPp significantly inhibited the elevation of NO₂/NO₃, a marker of NO synthesis (James et al. 2003). Moreover, MPO activity, used as indicative of neutrophil migration (Bradley et al. 1982) was significantly inhibited in LPPp-treated animals. These data corroborate with previous observation that LPPp can inhibit NO synthesis and neutrophil migration during inflammatory response (Fernandes et al. 2015, Oliveira et al. 2019, 2022).

Despite the promising results, some limitations should be acknowledged. First, the protein composition of LPPp may vary due to environmental factors, plant maturity, and extraction conditions, which could affect reproducibility between batches. Establishing a standardized protocol and performing proteomic fingerprinting for quality control will be essential for future preclinical or clinical use. Second, although the selected dose was based on prior efficacy data, a comprehensive dose-response study was not conducted in the present work. This remains an important step to identify minimal effective and maximal tolerated

doses and to better understand the therapeutic window of LPPp.

Cytokines and chemokines are key mediators of the inflammatory cascade in APAP-induced liver injury. The observed increase in hepatic levels of TNF- α , IL-6, IL-1 β , IFN- γ , and CXCL1 during APAP overdose is consistent with their known roles in promoting liver inflammation and neutrophil recruitment (Imaeda et al. 2009, Marques et al. 2012, Ishida et al. 2023). On the other hand, IL-10 levels, an anti-inflammatory cytokine, remained at normal levels in the group that received LPPp. All these pro and anti-inflammatory mediators are directly involved with the control of neutrophil migration to inflammatory site during inflammatory response (Krenkel et al. 2014, Jaeschke & Ramachandran 2020).

Liver biochemical markers together oxidative stress and the intense inflammatory response observed during APAP toxicity results in significant cells morphological alterations, such as increasing of hydropic degeneration, microvesicular fat alteration, vacuolization of hepatocytes, number of foci of necrosis and cells with nuclear alterations and increase of infiltrated neutrophils (Hinson et al. 2010, McGill et al. 2012b). We observed that the treatment of animals with LPPp significantly improved all these histopathological parameters of animal's analysis. Histopathological and immunohistochemical assays corroborate with measurement of MPO, pro-inflammatory cytokines and CXCL1 chemokine and reinforce the hypothesis that LPPp also act by preventing liver damage due its anti-inflammatory effect by inhibiting neutrophil migration.

Cysteine proteinases, proteinase inhibitors, chitinases and osmotins are present in LPPp fraction, it is documented that these plant proteins have anti-inflammatory and antioxidant properties (Hiemstra 2002, Arsenescu et al. 2011,

Rathnavelu et al. 2016, Viana et al. 2017, Ramos et al. 2020a), suggesting that these proteins should be related to biological properties observed here. To investigate the involvement of these class of proteins in the protective effect LPPp against APAP-induced hepatotoxicity, we select the 3D structure of a plant chitinase, a cysteine proteinase and an osmotin, taking into consideration that mass spectrometry revealed the presence of them and the 3D structure of one plant proteinase inhibitor considering its identification in LPPp in a previous study developed by (Santana et al. 2018).

Molecular docking analyses were conducted to evaluate the binding affinity between selected LPPp proteins and hepatotoxic agents, including APAP, NAPQI, CYP2E1, and inflammation-related targets such as TNF- α and IFN- α . Computational docking methodologies are widely used to predict ligand-protein interactions and guide mechanistic hypotheses (Daina et al. 2017). Notably, CYP2E1 plays a central role in the bioactivation of APAP to its toxic metabolite NAPQI (Zaher et al. 1998). Our results indicated strong affinity between cysteine proteinase and both APAP and NAPQI, suggesting a potential neutralizing interaction. Changes in the metabolism or bioactivation of APAP are considered therapeutic targets, the activity of cytochrome P450 as CYP 2E1 the main CYP enzyme, acting as an effective catalyst for the formation of NAPQI (Chowdhury et al. 2020), formation of protein-NAPQI adducts and APAP metabolites in the liver or plasma have been suggested as potential targets (Antonίου et al. 2022, Licata et al. 2022), thus any intervention in these aspects would block all subsequent events (Subramanya et al. 2018, Chowdhury et al. 2020). This suggests that cysteine proteinases present in LPPp should participate in the hepatoprotective effect of LPPp on APAP-induced injury by not only through their antioxidant

and anti-inflammatory properties but also by reducing APAP and/or NAPQI toxicity. In relation to CYP 2E1, TNF- α and IFN- α interactions, the best molecular affinity was obtained from the interaction with the proteinase inhibitor.

The molecular docking results support and expand the *in vivo* findings by suggesting specific interactions between LPPp proteins and critical targets involved in APAP hepatotoxicity. For instance, cysteine proteinase demonstrated high binding affinity with both APAP and its toxic metabolite NAPQI, suggesting a potential for direct interference in their bioactivation or toxicity. This aligns with the observed preservation of GSH levels and reduced MDA formation *in vivo*, indicating that such proteins might reduce oxidative damage not only via classical antioxidant pathways but also through molecular sequestration or inhibition. Similarly, the interaction of proteinase inhibitors with CYP2E1, TNF- α , and IFN- α may help explain the anti-inflammatory and hepatoprotective effects observed. These mechanistic insights underscore the potential multifaceted action of LPPp in modulating liver injury pathways.

Taken together, these results suggest that the beneficial effects observed in this study are likely mediated by the combined actions of multiple proteins present in LPPp. Further chromatography-based fractionation of LPPp is required to isolate and identify its protein components and determine their role in this model. This step is now in progress in our lab and the determination of putative protein(s) involved in the investigated activity will give light to knowledge of how LPPp fraction presented its biological effects. These findings would be helpful for developing a potential hepatoprotective agent for alleviating APAP hepatotoxicity or which may be a complementary therapy for NAC.

While the current findings demonstrate promising hepatoprotective effects of LPPp in an animal model, further steps are required to enable translational application. These include establishing pharmaceutical-grade standardization of LPPp, long-term toxicity assessments, and pharmacokinetic profiling. Given the limitations of NAC, particularly in delayed treatment windows or high-overdose cases, LPPp, or bioactive proteins isolated from it, could be explored as adjunct or alternative therapies. Additionally, recombinant expression of key proteins or synthetic peptides could provide a route toward clinical formulations with defined composition and reproducibility.

CONCLUSIONS

The present study demonstrated that LPPp treatment attenuated APAP-induced liver injury. This effect should be related to combined action of different proteins present in LPPp, such as cysteine proteinases and proteinases inhibitors. They act on different targets related to APAP-induced liver injury, interfering in key events of APAP and NAPQI metabolism and CYP 2E1 activity, diminishing development of oxidative stress, reducing inflammation, and consequently preventing hepatocyte damage and death cell.

Acknowledgments

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SUPPLEMENTARY MATERIAL

Table S1, SII.

Figure S1, S2.

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Data Availability

Data will be made available upon reasonable request.

