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Original Article

Antioxidant and Hepatoprotective Activity of Ethanol Extract of *Annona cherimola* Mill. On Paracetamol-Induced Liver Toxicity in Rats

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ABSTRACT

Background: *Annona cherimola* Mill. (*A. cherimola*) is mainly characterized by its antioxidant and cytoprotective properties due to their content of phenolic compounds. **Objective:** To evaluate antioxidant and hepatoprotective activity of ethanol extract of leaves from *A. cherimola* against induced toxicity by paracetamol in rats. **Methods:** Amount of total phenolics compounds of ethanol extract of *A. cherimola* Mill. was determined by the Folin-Ciocalteu method and antioxidant activity was evaluated by DPPH method. Three doses of the ethanol extract of leaves of *A. cherimola* (250, 500 and 750 mg/Kg/day) were administered to rats and it was evaluated biochemical blood parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were measured, liver tissue was removed for histopathological analysis. **Results:** Ethanol extract of leaves from *A. cherimola* had 41.26 mg GAE/g extract and antioxidant DPPH Scavenging Activity had 85.51%. *A. cherimola* reduced blood levels of ALT, AST and ALP, compared to control group Paracetamol, ethanol extract, being more effective at doses of 750 mg/Kg/day. Histopathological evaluation suggested that *A. cherimola* decreased hepatic necrosis and degenerative process induced by paracetamol. **Conclusions:** Hepatoprotective activity of ethanol extract of leaves of *A. cherimola* was demonstrated, being hepatoprotective activity dose dependent and the mechanism may involve antioxidant activity and total polyphenols found in extract of this plant.
Key words: Liver, Antioxidants, DPPH, Paracetamol, Rat.

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INTRODUCTION

Liver diseases are a global health problem, which includes conditions such as acute or chronic hepatitis, steatosis and cirrhosis. Unfortunately, liver disease treatments are controversial because conventional medications for liver disease are insufficient and sometimes cause serious side effects.[1-2]

Conventional and/or synthetic medications such as steroids, vaccines, antivirals, and other medications can cause serious side effects, such as liver damage, especially when used for long periods.[3] Most drug-induced liver injury occurs due to accidental or intentional drug overdose.[4]

Paracetamol or acetaminophen is an antipyretic pain reliever and is available with no prescription. However, excessive use of paracetamol has the potential to precipitate liver damage in both humans and animals.[5-6] In the liver, by action of cytochrome P450, paracetamol is converted into a highly toxic metabolite called N-acetyl-p-benzoquinamine (NAPQI), which under normal conditions, is easily detoxified by conjugation with the enzyme glutathione antioxidant, but an excess of NAPQI causes an imbalance in reactive oxygen species (ROS) and antioxidant enzymes, causing severe liver damage.[5,7,8]

Given that, natural products are attracting the interest of many researchers to determine their potential usefulness for the treatment of liver diseases.[4] Some plants have been studied for their hepatoprotective activity, these plants include *Silybum marianum*, *Glycyrrhiza glabra*, *Phyllanthus* species (*P. amarus, P. niruri, P. emblica*) and *Rosmarinus officinalis, Psidium guajava, Picrorhiza kurroa*.[1,9]

Annona cherimola Mill. (*A. cherimola*) known as "anona" or "cherimoya", belongs to the Annonaceae family and is an exotic fruit found in different subtropical areas around the world.[10-11] *A. cherimola* has not been well studied, but it is known that its fruits have antioxidant and cytoprotective properties due to their content of phenolic compounds. The decoction of its leaves is traditionally used as anti-inflammatory, lipid-lowering, antimicrobial, digestive and hepatoprotective.[12-15] The chemical compounds reported in *A. cherimola* are the compounds Cherimolacyclopeptide D, cherimolacyclopeptide E, rutina, acetogeninas como Aromin-A, cherianoine, cherimoline, cheritamine, annomolin, annocherimolin, anonaine andasimilobine, lanuginosina, liriodenina, lisicamina, pronuciferina y esteparina.[16-21]

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Considering these antecedents, the present study evaluated the hepatoprotective activity of ethanol extract of *A. cherimola* on hepatic toxicity induced by paracetamol in rats.

MATERIALS AND METHODS

Vegetal material

The fresh leaves of *A. cherimola* were collected in Chocope town (La Libertad, Peru). The specie was identified for José Mostacero (Botanist and Taxonomist of *Herbarium Truxillense* of the Universidad Nacional de Trujillo - HUT), getting HUT code 59574.

Preparation of ethanol extract

The leaves were cleaned and washed with water, dried in the shade and then pulverized with an electric mill, to be later stored in amber glass containers. Ethanol extract was prepared by maceration for 72 hours, then filtered under sterile conditions in the biological safety cabinet and stored in amber containers under refrigeration until use.

Determination of total phenolics compounds (TP)

The TP was determined with the Folin – Ciocalteu reagent. A calibration curve was obtained by using gallic acid as standard. The TP was calculated from the standard curve prepared by the addition of two milligrams of gallic acid with 10 ml of methanol. Concentrations of 100, 50, 25 and 12.5 µg/ml were prepared from the stock solution. Both 0.5 mL of standards and extract were taken and mixed with 2.5 mL of Folin – Ciocalteu 50% and 2.5 mL of distilled water, after incubated for 5 min at 40 °C. Finally, 2 mL of Na_2CO_3 solution (7.5%, w/v) was added. The final mixture was shaken and then incubated for 15 min at 40 °C. The absorbance of all standards and samples were measured at 765 nm using UV – Vis spectrophotometer. The results were expressed as mg of gallic acid equivalents (GAE)/g extract.[22-23]

Determination of antioxidant activity

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay was used to evaluate the antioxidant activity of ethanol extract of *A. cherimola*. Ascorbic acid was used as standard. The test was conducted in a 96-well plate, 20 µL stock solution of extract and 180 µL of DPPH solution 0.147 mM were added to each well. After 30 min incubation at room temperature in a dark room, absorbance was read at 517 nm using a microplate reader and methanol was used as blank.[22] All tests were performed in triplicate and scavenging ability (%) was calculated as follows:

$$\%\ Inhibition = \frac{Absorbance\ of\ standard - Absorbance\ of\ extract}{Absorbance\ of\ standard}\ x\ 100$$

Experimental animals

Albino Holtzman rats of both sexes, weighing 220-250 g and 18 weeks old, were used for this research. The animals were obtained from the National Institute of Health (INS, Perú). All rats were kept in plastic cages and Wood shavings were used as bedding. The animals were kept in the bioterium of the School of Pharmacy and Biochemistry of the National University of Trujillo, under standard environmental conditions of photoperiod (12:12 h dark: light cycle) and controlled temperature [(25 ± 2) °C]. Rats were feeded with balanced food (Food purchased from INS) and water administered *ad libitum*. This study was approved by the Ethics Committee of the School of Pharmacy and Biochemistry of the National University of Trujillo with the document COD. N°: 005 - 2017/C.Fac.Farm.

Hepatoprotective assessment

The rats were divided into 6 groups with 6 rats in each group. Group I (control) received only the vehicle (sterile water 10 mL/kg body weight p.o.) once a day for 7 days. Group II (paracetamol control) received paracetamol (2 g/kg) for seven days. Group III (standard) was treated with the standard drug silymarin (200 mg/kg) once a day for 7 days together with Paracetamol (2 g/kg). Groups IV (*A. cherimola*-250), V (*A. cherimola*-500) and VI (*A. cherimola*-750) were treated with *A. cherimola* at a dose level of 250 mg/kg, 500 mg/kg and 750 mg/kg body weight p.o. per day, respectively, for 7 days together with Paracetamol (2 g/kg). 24 h after the last treatment dose, blood was drawn and euthanized with sodium pentobarbital 60 mg/kg v.ip.[24]

Measurement of biochemical parameters

Blood serum was obtained, which was used to determine alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) levels.[25]

Histopathological study

Liver tissue samples were obtained that were fixed in 10% formaldehyde for 8 days before being analyzed. After embedding in paraffin wax, 4 µm thick thin sections were cut and stained with Hematoxylin-Eosin (H&E). Thin sections of the tissue were placed on permanent slides and submitted for histopathological analysis by a veterinary pathologist.[25]

Statistic analysis

The data obtained from the experiment were expressed as mean ± standard deviation (SD). Plots were prepared using SigmaPlot 12.0 and data was subjected to analysis of variance (ANOVA) and post Hoc Tukey test. Values were considered statistically significant at $p<0.05$.

RESULTS

Determination of total phenolics compounds (TP) and antioxidant activity

The amount of TP of ethanol extract of *A. cherimola* Mill. was determined by the Folin-Ciocalteu method reported as gallic acid equivalents (Figure 1). The antioxidant activity was evaluated by DPPH method (Table 1).

Biochemical analysis

Comparison of the biochemical parameters are shown in Figure 2. The extracts of A. *cherimola*-250, *A. cherimola*-500 and *A. cherimola*-750 showed a significant decrease in the AST, ALT and ALP parameters compared to the paracetamol control group ($p<0.05$), however, the decrease in the *A. cherimola*-750 extract was greater.

Histopathological changes

Liver lesions decreased mainly with the administration of the ethanol extract of *A. cherimola*, showing a hepatoprotective effect by reducing degeneration and liver necrosis. The protective effect was also observed in the group treated with silymarin. The paracetamol control group revealed liver lesions characterized by degenerative hepatocytes, necrosis and dilated sinusoids containing red blood cells. On the other hand, in the normal control group, the histological sections of the liver showed normal cells (Figure 3). That agrees with results obtained from biochemical parameters.

Table 1: Total Phenolic Content and Antioxidant DPPH Scavenging Activity of Ethanol Extract of *A. cherimola* Mill.

Sample	Folin-Ciocalteau (mg GAE/g extract)	DPPH assay (% inhibition)
Ethanol Extract	41.26 ± 1.75	85. 51 ± 2.02

Data are mean ± SEM for triplicate measurements

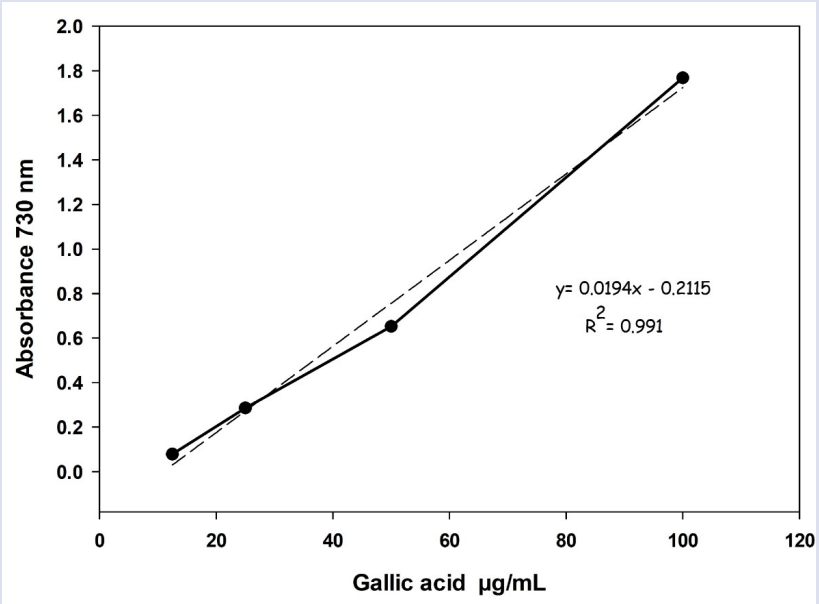

Figure 1: Gallic Acid calibration curve.

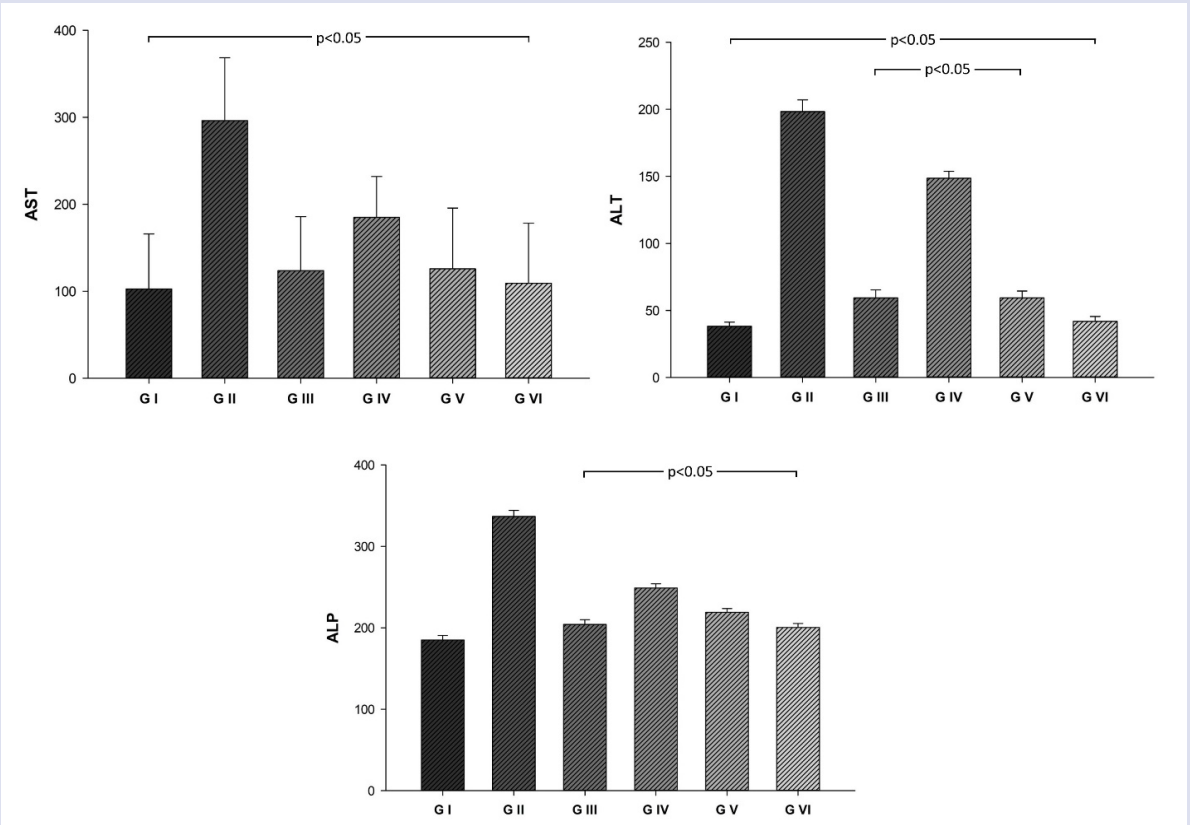

Figure 2: Effect of the ethanol extract of *A. cherimola* on biochemical parameters in hepatic damage induced by paracetamol in rats. Group I (G I): Control. Group II (G II): Paracetamol Control. Group III (G III): Silymarin 200 mg/kg + Paracetamol. Group IV (G IV): *A. cherimola*-250 mg/kg + Paracetamol. Group V (G V): *A. cherimola*-500 mg/kg + Paracetamol. Group VI (G VI): *A. cherimola*-750 mg/kg + Paracetamol. Results expressed as mean ± SD (n=6), p <0.05.

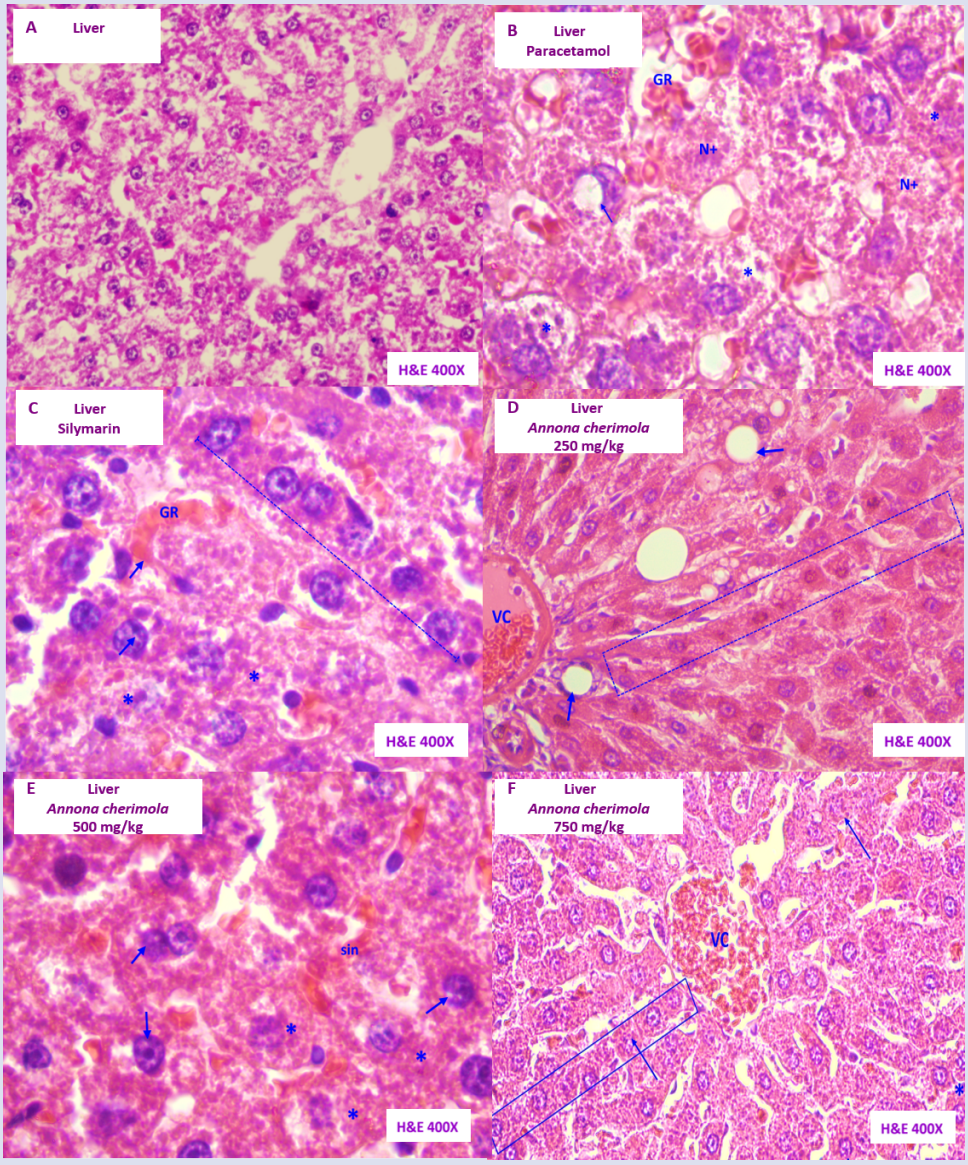

Figure 3: Histopathological sections of the liver. (A) Group I (Normal Control), normal architecture of liver tissue. (B) Group II (Paracetamol Control). The presence of several hepatocytes in a degenerative process (*) and in necrosis or cell death (N +), dilated sinusoids containing red blood cells (GR) is observed. (C) Group III (Silymarin). Most hepatocytes with nuclei and nucleoli, as well as normal-appearing cytoplasm (line and arrows), few cells show a reversible cloudy appearance (*). (D) Group IV (*A. cherimola*-250 mg/kg). Dilated sinusoids containing red blood cells (RBC), but most hepatocytes retain their architecture and cord arrangement (inset) around the central vein (VC), several hepatocytes (arrows) contain fat in the cytoplasm. (E) Group V (*A. cherimola*-500 mg/kg). Several hepatocytes are observed (arrows) that show the nucleus and nucleolus well stained, indicative of a normal state, in some hepatocytes the cytoplasm of cloudy appearance (*) due to cell swelling in response to the effect of paracetamol, slightly dilated capillaries (without). (F) Group VI (*A. cherimola*-750 mg/kg). Cords of hepatocytes (inset) are irradiated from the central vein (CV), showing stained nuclei and nucleolus (*), some hepatocytes with mild reversible degeneration (arrows), slightly dilated capillaries (c).

DISCUSSION

Excessive use of paracetamol allows the accumulation of NAPQI that binds covalently to cellular macromolecules (proteins and DNA) to produce a protein adduct could produce acute liver necrosis. Oxidative stress is generally caused by a very high level of NAPQI and can contribute to the hepatotoxicity of paracetamol through lipid peroxidation and mitochondrial damage.[4-5]

Measurement of serum markers such as AST, ALT and ALP is of great value in evaluating clinical and experimental liver damage.[7] The ALT enzyme is more specific to the liver and is a better parameter to analyze liver injury, whereas, high levels of AST indicate cell loss, as well as loss of the functional capacity of the cell membrane in the liver and serum ALP is also related to liver cell damage.[2] In hepatotoxicity, the transport function of liver cells is altered, causing an alteration of the plasma membrane, which results in an outflow of these liver enzymes that leads to an increase in their serum level.[26] Paracetamol administered to rats increased AST, ALT, and ALP levels, indicating liver damage. The increase in these values decreased with the administration of *A. cherimola* extracts in a dependent dose.

Histopathological sections of the liver showed recovery of hepatocytes induced by the administration of *A. cherimola*, which could counteract the effect of paracetamol. The histological architecture of the liver sections from the paracetamol-treated rats showed disorder and degeneration of normal liver cells with intense centrilobular necrosis. The histopathological profile of the rat treated with ethanol extract of *A. cherimola* showed visible changes in the hepatocytes with slight degeneration, slight sinusoidal congestion, less infiltration of inflammatory cells compared to the control group.

The hepatoprotective effect of *A. cherimola* has not been studied previously, but the effect of other *Annona* species such as *A. muricata*[27], *A. squamosa*[28], *A. reticulata*[29], *A. crassiflora*[30], the effect being similar to that presented by *A. cherimola*.

Annona species contain a considerable amount of polyphenolic compounds and these compounds are antioxidants and may help to prevent diseases associated with oxidative stress, such as hepatoxicity.[10] These compounds are very important plant constituents and they have received considerable attention because of their potential antioxidant activities because confer capacity of scavenging free radicals [31]

The hepatoprotective activity can be attributed to the antioxidant properties of *A. cherimola*. The presence of phenolic compounds as antioxidant compounds could reduce lipid peroxidation by controlling the production of reactive oxygen species,[13,32] and reducing the hepatotoxic damage induced by paracetamol, which is characterized by an oxidative stress process that contributes to the initiation and progression of hepatic injury.[33-34] The phenolic compounds stabilizing cell membrane networks and inhibiting the formation and expression of inflammatory cytokines like tumor necrosis factor alpha (TNF-α), Transforming Growth Factor beta (TGF-β) and varieties of interleukins (IL-6, IL-2, IL-8).[35]

In many plant species the relationship between antioxidant activity and its phenolic content has been evaluated; but the antioxidant activity is not only determined by phenolic compounds, but also by other compounds reported as flavonoids, acetogenins and catechins in *A. cherimola* leaves that could also be contributing to its hepatoprotective activity.[36-38]

CONCLUSION

In conclusion, ethanol extract of leaves of *A. cherimola* was effective in preventing liver damage caused by paracetamol in rats, the highest dose being the most effective. However, the mechanisms of hepatoprotection have not yet been determined, although the antioxidant effects of the total polyphenols determined in the plant species may be involved.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHORS 'CONTRIBUTIONS

CSC and VVLT carried out the preparation of the first draft and preparation of the extract. ACP and CAV administered the treatments. JCR and AGS performed liver tissue extraction for histopathological analysis. JRC collected the plant species and entered the herbarium. MGB and JHV performed the blood collection and biochemical analysis. WSG and LGE performed the quantification of total polyphenols and evaluation of the antioxidant activity.

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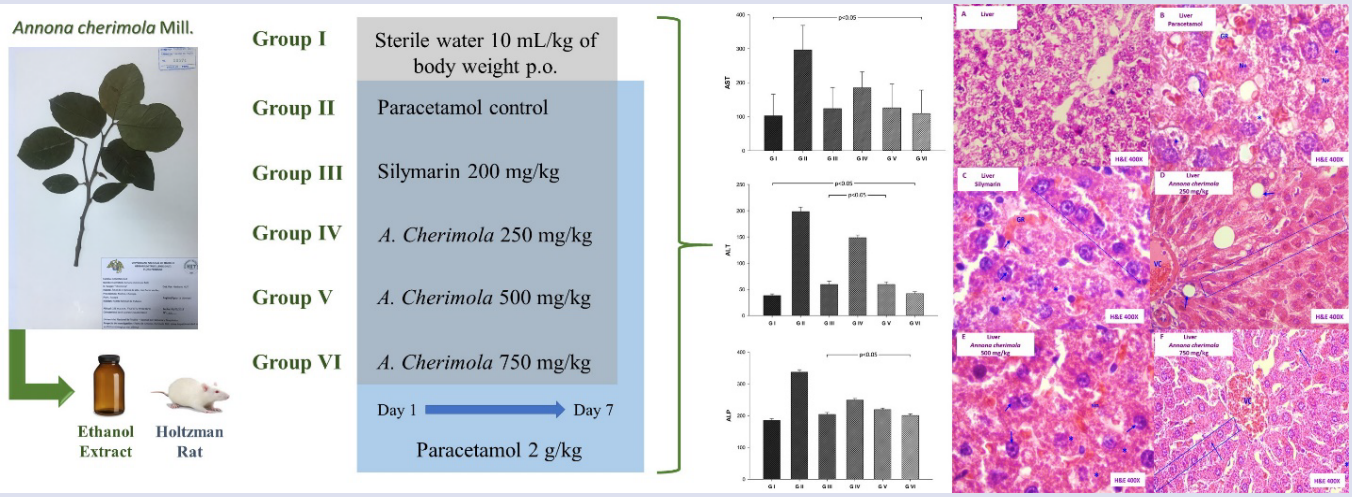
GRAPHICAL ABSTRACT



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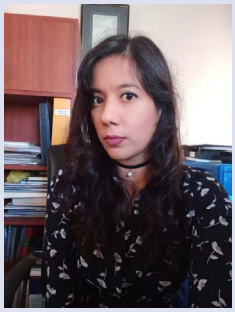

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