HEPATOPROTECTIVE EFFECT OF CALYCES EXTRACT OF *Physalis peruviana* ON HEPATOTOXICITY INDUCED BY CCl$_4$ IN WISTAR RATS

EFECTO HEPATOPROTECTOR DE UN EXTRACTO DE CÁLICES DE *Physalis peruviana* EN HEPATOTOXICIDAD INDUCIDA POR CCl$_4$ EN RATAS WISTAR

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ABSTRACT

Background: *Physalis peruviana* ("uchuva", Solanaceae) is a widespread species of the South American Andes and widely used in traditional medicine. Its fruits are consumed as food and for the treatment of diabetes. The juice of *Physalis peruviana* fruits is topically applied in the eyes for pterigyum treatment. Previous works reported that the fruit extracts has modulating activity of oxidative stress in experimental diabetes models induced by streptozotocin. It has been attributed antipyretic, antimicrobial, analgesic and anti-inflammatory properties to the calyces enveloping the fruit. Reported literature demonstrates *in vivo* and *in vitro* that different calyx's extracts have antioxidant and anti-inflammatory activities. **Objectives:** To evaluate the *in vivo* hepatoprotective effect of the extract of *Physalis peruviana* calyces, involving inflammation and oxidative stress models at hepatic level. **Methods:** Hepatotoxicity was induced by single oral administration of CCl$_4$ (2 mL / Kg in olive oil) in Wistar rats. *Physalis peruviana* extract (250 mg/Kg) and silymarin (200 mg/Kg), used as control drug, were administrated twice a day for five days. At the end of the experiment, animals were euthanized and the liver enzymes alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase were measured as well as some parameters of hepatic antioxidant status like superoxide dismutase and catalase activities, protein oxidation and lipid peroxidation. **Results:** Extract of *Physalis peruviana* calyces inhibited significantly ($p < 0.001$) liver oxidative stress caused by CCl$_4$, maintaining superoxide dismutase and catalase activities close to normal. Studied extract also reduced significantly liver enzymes levels increased by CCl$_4$ administration. **Conclusion:** It was suggested that the extract of *Physalis peruviana* calyces presents a hepatoprotective effect related to its antioxidant activity, especially regarding to lipid peroxidation inhibition.
Keywords: *Physalys peruviana*, hepatotoxicity, oxidative stress, antioxidant.

RESUMEN

Antecedentes: *Physalis peruviana* ("uchuva", Solanaceae) es una especie distribuida en los Andes suramericanos y de amplio uso a nivel etnofarmacológico. Sus frutos, además de ser usados como alimento, también son consumidos para el tratamiento de la diabetes. Por su parte, el zumo del fruto es aplicado localmente para el tratamiento de pterigios. En modelos de diabetes experimental inducidos por estreptozotocina, trabajos previos han reportado que los extractos de los frutos poseen actividad moduladora del estrés oxidativo. A los cálices que envuelven el fruto, se les atribuyen propiedades antipiréticas, anti-

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microbianas, analgésicas y antiinflamatorias, entre otras. Estudios previos han demostrado en modelos *in vivo* e *in vitro* que diferentes extractos de los cálices presentan actividad antioxidante y antiinflamatoria. **Objetivo:** Evaluar el posible efecto hepatoprotector de un extracto de cálices de *Physalis peruviana* en un modelo experimental *in vivo* que involucra procesos de inflamación y de estrés oxidativo a nivel hepático. **Métodos:** Se empleó un modelo de hepatotoxicidad inducida por CCl_4 en ratas Wistar, mediante una única administración oral de CCl_4. Tanto el extracto de *Physalis peruviana* (250 mg/Kg) como la silimarina (200 mg/Kg), empleada como patrón de referencia, fueron administradas dos veces al día durante cinco días. Al final del experimento, los animales fueron sacrificados y se evaluaron los niveles hepáticos de las enzimas alanina-aminotranferasa, aspartato-transaminasa y fosfatasa alcalina, al igual que algunos parámetros del estado antioxidante hepático como actividad de superóxido dismutasa y catalasa, oxidación proteica y peroxidación lipídica. **Resultados:** El extracto evaluado de *Physalis peruviana* inhibió significativamente (p < 0,001) el estrés oxidativo causado a nivel hepático por el CCl_4, manteniendo las actividades de superóxido dismutasa y catalasa con valores cercanos a los normales. El extracto también redujo significativamente los niveles de las enzimas hepáticas que fueron incrementados después de la administración de CCl_4. **Conclusi**ón: Estos resultados sugieren que el extracto de cálices de *Physalis peruviana* posee un efecto hepatoprotector relacionado con su actividad antioxidante, especialmente en lo referente a la inhibición de la peroxidación lipídica.

Palabras clave: *Physalys peruviana*, hepatotoxicidad, estrés oxidativo, antioxidante.

INTRODUCTION

Oxidative stress is employed to indicate the oxidative damage produced to biological molecules such as nucleic acids, proteins, lipids, and carbohydrates (1). This phenomenon occurs when reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH) exceed the cellular antioxidant defense. This imbalance produces adverse effects on the tissues and eventually cardiovascular or liver diseases, inflammatory processes and other chronic diseases such as hepatic fibrosis (2, 3).

The administration of carbon tetrachloride (CCl_4) in rodents is the most studied experimental model of liver inflammation. The CCl_4 is biotransformed in the liver by cytochrome P450 and produces trichloromethyl radical (CCl_3•) and ROS, initiating a lipid peroxidation leading to hepatocyte death and subsequent inflammation of the organ (4). These changes modify the normal performance of liver and alter serum levels of liver alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (AP).

Cape gooseberry (*Physalis peruviana* L., *Solanaceae*) is an abundant specie of the South American Andes that has been widely used and studied for medicinal purposes; this specie has been used for its anti-mycobacterial, antipyretic, analgesic, diuretic, and anti-inflammatory properties (5, 6). Extracts from the calyces of *Physalis peruviana* have demonstrated anti-inflammatory (7), immunomodulatory (8) and anti-nociceptive activities (9). Furthermore, it has been reported antioxidant and hypoglycemic activity in fruit extracts (10), and effectiveness in the treatment of pterygium, due to the inhibition of fibroblasts proliferation (11). Therefore, the aim of the present study was to evaluate the possible hepatoprotective effect of calyces extract of *P. peruviana* in CCl_4 hepatotoxicity model.

METHODS AND MATERIALS

Plant Material and extraction

Physalis peruviana calyces were obtained from a local market of Bogota city (voucher specimen was deposited at The Herbario Nacional Colombiano -COL- 51200, identified by the Botanic C.I. Orozco-). Fresh calyces were selected and dried in air circulating oven at 50°C for 36 h. Dried material was milled and then was extracted by exhaustive percolation with petroleum ether according to other works (7). The extract in petroleum ether was fractionated by a liquid - liquid partition with methanol-water (9:1). The methanol-water fraction was dried in a rotary evaporator and used for this work.

Animals

Male Wistar rats (220-250 g) were bred and housed under standard conditions (12 h light/12 h

dark cycles, temperature 22 ± 2 °C). Food and water were available *ad libitum*. The protocol was approved by the Ethics Committee of Science Faculty of the National University of Colombia. The animals were cared for in accordance with the international ethical guidelines (12).

Treatment

The rats were randomly divided into five groups of 10 animals each one. Table 1 describe the treatments.

Table 1. Groups of treatment in hepatotoxicity induced by CCl$_4$.

Groups	Hepatotoxicity induction	Treatment
VH	Carbon tetra-chloride (2 mL/Kg) in olive oil (1:1), by *oral via*.	Vehicle (glycerin: propylenglicol: distilled water; 1:1:8) twice daily, by *oral via*
SI		Silymarin (200 mg/Kg) twice daily, by *oral via*
PP		*Physalis peruviana* extract (250 mg/Kg) twice daily, by *oral via*
V	Olive oil 2 mL/Kg, by *oral via*	Vehicle (glycerin: propylenglicol: distilled water; 1:1:8) twice daily, by *oral via*
P		*Physalis peruviana* extract (250 mg/Kg) twice daily, by *oral via*

At the beginning of the experiment, animals were fasted for 12 h. In groups V and P hepatotoxicity was not induced.

At the end of the experiment, all animals were anesthetized for cardiac puncture in order to obtain blood for ferric reduction ability of plasma (FRAP) and biochemical determinations (alanine aminotransferase-ALT, alanine aminotransferase-AST, and alkaline phosphatase-AP). Finally, the rats were sacrificed by cervical dislocation and the livers were quickly removed, place in ice cold, rinsed, cut and homogenized (Polytron® PT-2100) in cold Tris–HCl (5 mM, pH 7.4). Homogenates were centrifuged (10000 rpm, 4°C, 10 min), and supernatants were used to evaluate the oxidative stress parameters. The protein concentration was determined using Bradford reagent.

Histopathological studies

Sections of liver samples were washed and fixed with 10% neutralized formalin (pH 7.4) and then stained with hematoxylin and eosin, and were kept under observation for pathological changes.

Ferric reduction ability of plasma (FRAP)

This method measure the antioxidants ability in biological sample by reducing the 2,4,6-tripyridyl-s-triazine (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}) (13). The FRAP was expressed as mg FeSO$_4$/mg protein.

Index of lipid peroxidation

This parameter was evaluated by the measure of thiobarbituric acid reactive substances (TBARs). Briefly, 450 μL of homogenated were added to 50 μL of phosphate buffers (50 mM, pH 7,4) and 1 mL of trichloroacetic acid (10%). The mix was centrifuged (2000 rpm, 10 min, 4°C) and 1 mL of supernatant was added to 1 mL of thiobarbithuric acid (0.67%). This mixture was boiled for 30 min, with the subsequent absorbance measurement at 532 nm. Concentration of the TBARs were expressed as (mmol/mL)/mg protein, using the expression $\varepsilon = A/c*h$, where $\varepsilon = 153000$ M^{-1}cm^{-1}.

Protein carbonyl content (COP)

This parameter was measured using 2,4-dinotrophenylhydrazine (DNPH) according to methods previously described (14). Briefly, 50 μL of homogenate was added to 250 μL of 2,4-dinitrophenylhydrazine (DNPH, 10 mM in HCl 2M) and left to stand at room temperature for 1h stirring every 15 min. Thereafter, 500 μL of cold trichloroacetic acid (TCA, 20%) were added and left to stand for 15 min at 4°C. Then, the mixture was centrifuged at 11000 rpm for 5 min, the supernatant was removed and the pellet was rinsed three times with ethanol: ethyl acetate (1:1), and centrifuged after each rinse for 7 min at 3000 rpm. Finally, the pellet was dissolved in 250 μL of Guanidine 6 M and incubated at 37ºC for 10 min. The absorbance was measured at 360 nm. The carbonyl content was calculated using the expression $\varepsilon = A/c*h$, where $\varepsilon = 22000$ M^{-1}cm^{-1} and expressed as nmol/mg protein.

Superoxide dismutase activity (SOD)

The Cu-SOD and Mn-SOD were determined by the riboflavin-o-dianisidine test (13). 4 μL of homogenate was added to 200 μL of the reaction mixture (0,1 mL of *o*-dianisidine 6 mM; 0,1 mL of riboflavin 0.29 mM and phosphate buffer, 50 mM, pH 7.8). The mixture was incubated at dark for 5 min at 25°C and then exposed for 8 min to fluorescent light; the absorbance was measured at 415

nm before and after the light exposition. The SOD concentration was extrapolated from a calibration curve and expressed as U/mg protein

Catalase activity (CAT)

The method is based on the H_2O_2 decomposition and the rate constant determination k (s^{-1}) (15). Briefly, 250 μL of H_2O_2 (10 mM) were added to 5 μL of homogenate and 45 μL of phosphate buffer (50 mM, pH 7.0). Immediately, the change in the absorbance was measured at 240 nm for 30 sec. The enzyme activity was expressed as k (s mg protein)$^{-1}$.

Statistical Analysis

The results were expressed as means ± SD and were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's test. Statistical differences were considered (*) when p < 0.05.

RESULTS

Significant increases of enzymes AST, ALT and ALP (more than 5 times baseline levels) were found in the control group (VH) (figure 1). These results are similar to those reported by other authors and were consistent to the mode of action of CCl_4, which induces a large lipid peroxidation in the endoplasmic reticulum of hepatocytes (16, 17).

Histopathological studies showed that rats without CCl_4 had a normal hepatic architecture, indicating that the vehicle and the extract of *Physalis peruviana* do not have hepatotoxic effect (figure 2). The administration of CCl_4 induced sub-massive hepatic necrosis and a pronounced hepatic micro-vacuolar steatosis (figure 2).
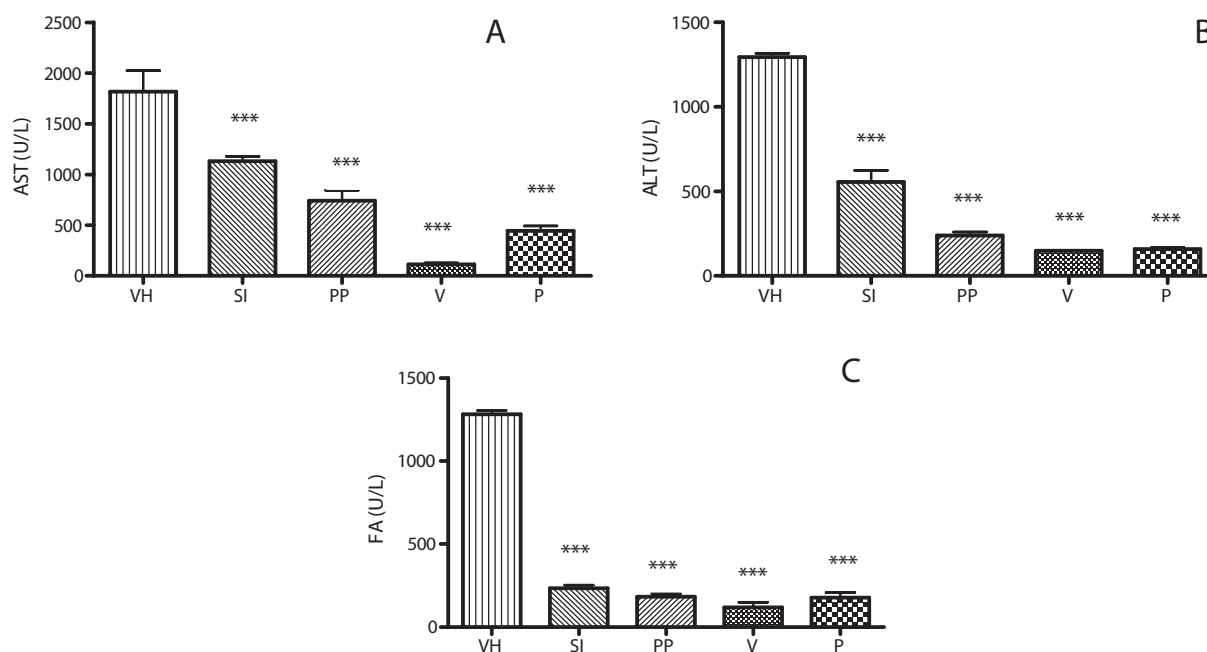

Figure 1. Liver functional parameters assessed in plasma of Wistar rats. A) *Aspartate Amino Transaminase*, B) *Alanine Amino Transaminase*, C) *Alkaline Phosphatase.* **VH:** CCl_4 + Vehicle; **SI:** CCl_4 + silymarin; **PP:** CCl_4 + P. peruviana; **V:** Olive oil + vehicle; **P:** Olive oil + *P. peruviana*. *** p < 0,001 Dunnett's test.
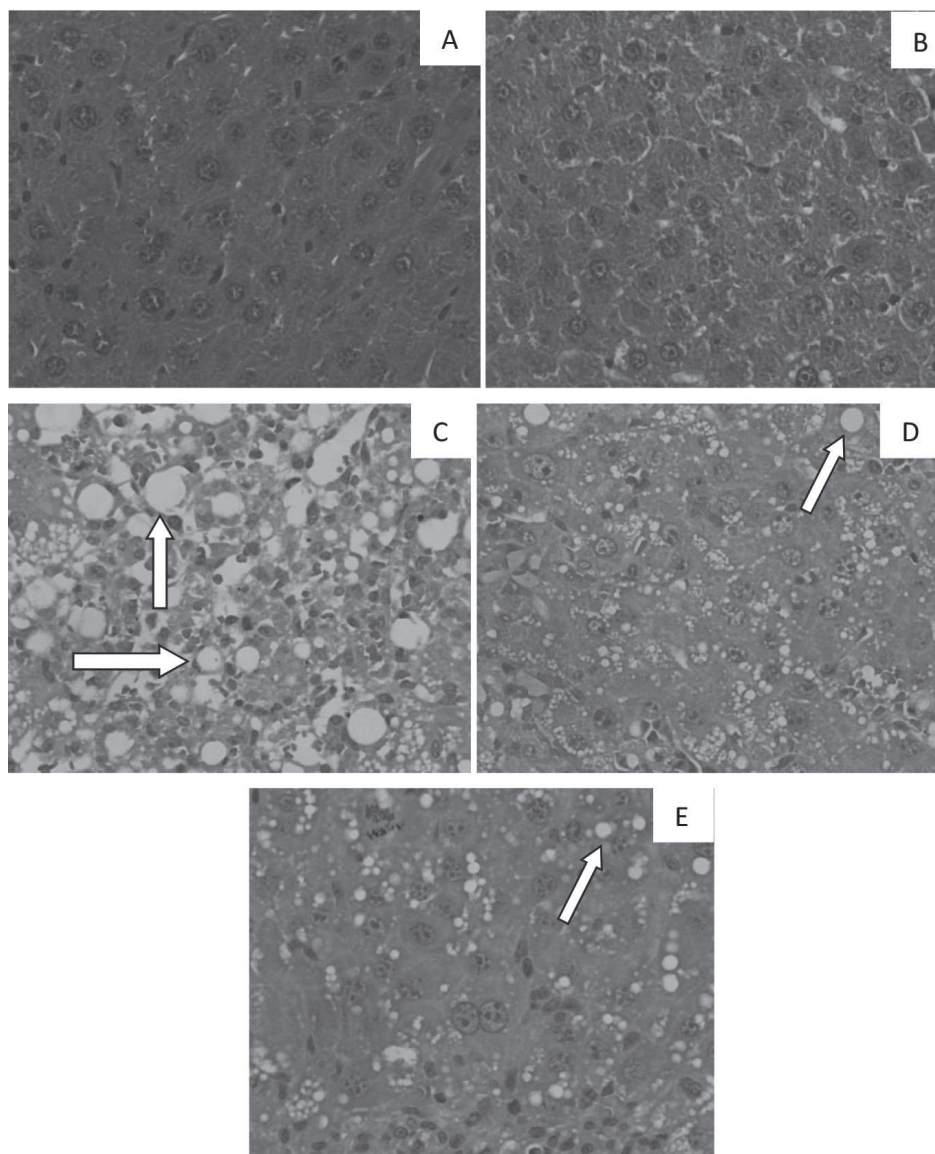
Figure 2. Photomicrographs of liver histopathology in rats. *Hematoxylin and eosin stain. Steatosis is shown with white arrows.* A) *Olive oil (2 mL/kg) + Vehicle (2 mL/kg)*, B) *Olive oil (2 mL/kg) + P. peruviana (250 mg/kg twice daily)*, C) *CCl$_4$ (2mL/kg) + Vehicle (2 mL/kg)*, D) CCl$_4$ *(2mL/kg) + silymarin (200 mg/kg twice daily)*, E) CCl$_4$ *(2mL/kg) + P. peruviana (250 mg/kg twice daily).* Sections were studied under a light microscope at 40 × magnification.

Regarding to oxidative stress parameters, a significant increase (around 50%) in SOD and CAT activities [1.87 ± 0.13 units/ mg protein and 3.16 ± 0.04 k/(s* mg protein), respectively] in the group VH respect to group V was found [0.60± 0.16 units/ mg protein and 1.15 ± 0.17 k/(s* mg protein), respectively], while silymarin and *P. peruviana* maintain enzyme activities near to normal (figures 3A y 3B).

Furthermore, a high level of carbonyl (figure 3C) was found in group VH (5.78 ± 0.94 nmol/mg protein) being three times more than in group V (1.45 ± 0.06 nmol/mg protein). Similar to the other parameters evaluated, silymarin and the extract of *P. peruviana* showed a decrease in the levels of carbonyl groups. Lipid peroxidation, expressed as nmoles of malondialdehyde (MDA), were increased almost twice in the group VH (9.29 ± 0.19 nmol MDA/mg protein) (figure 3D). In this case, silymarin prevented lipid peroxidation produced in the liver by CCl$_4$ in a 50%, while the extract of *P. peruviana* reduced significantly this damage, in a 30% respect to group VH.
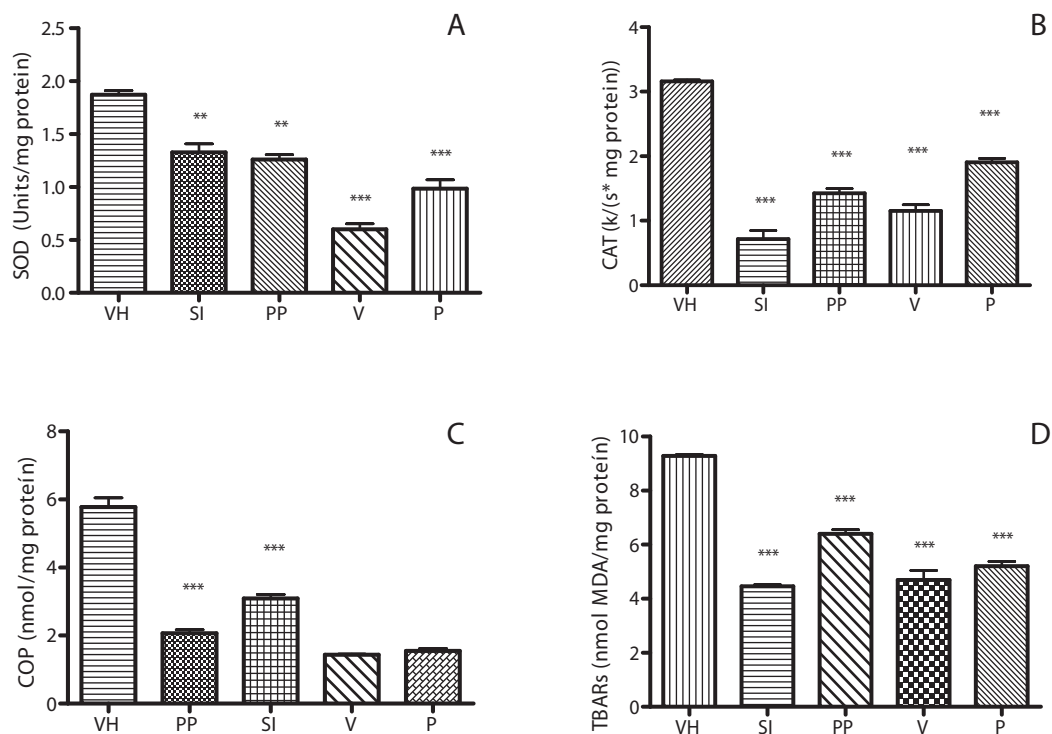
Figure 3. Oxidative stress parameters evaluated in liver homogenates of Wistar rats. A) *Superoxide dismutase enzyme activity*, B) *Catalase enzyme activity*, C) *Oxidation of carbonyl groups*, D) *Lipid peroxidation, MDA (malonildialdehyde)*. **VH:** CCl_4 + Vehicle; **SI:** CCl_4 + silymarin; **PP:** CCl4 + *P. peruviana*; **V:** Olive oil + Vehicle Group; **P:** Olive oil + *P. peruviana*. **COP: *** p < 0,001 Dunnett's test.

DISCUSSION

Serum levels of liver enzymes such as ALT, AST and ALP, and other compounds such as urea, are important markers of liver function and the possible damage to hepatocytes. These enzymes are released from the cytosol and mitochondria, therefore lesions that alter the permeability of hepatocytes elevate the normal levels of ALT, AST and ALP more than three times, allowing the passage of these enzymes to peripheral blood (16, 18).

Treatment with silymarin (SI) and *P. peruviana* (PP) prevent a significant increase in the levels of ALT, AST and ALP. This could be due to the antioxidant activity of these treatments. Silymarin is a mixture of flavonolignans extracted from *Silybum marianum*, which had reported protective effect against various liver diseases (19), including those induced by oxidative stress like cirrhosis, diabetes and liver fibrosis (20-22). Its mechanism of action is still unknown; however, two possible mechanisms proposed are through free radical scavenging and regulation of immune functions modulated by cytokines (23, 24). The decrease in enzyme levels (AST, ALT and ALP) in silymarin group (SI) is probably due to the antioxidant activity of silymarin, demonstrated against DPPH, ROS such as •OH, HOCl and against lipid peroxidation. A similar effect can be attributed to the extract of *P. peruviana* which had demonstrated superoxide radical scavenging activity, inhibition of lipid peroxidation *in vitro* and scavenging of ROS like H_2O_2 (6).

Microvacuolar steatosis found in histopalogical studies is associated with a state of oxidative stress generated, since the trichloromethyl radical (•CCl_3) covalently binds to cellular components such as glutathione reductase, inhibiting the secretion of lipoproteins causing the subsequent steatosis. On the other hand, after the •CCl_3 reaction with molecular oxygen forms the peroxyl radical (•$OOCCl_3$), the process of lipid peroxidation initiates generating disruption of membrane cell of the hepatocytes (25, 26). Furthermore, histopathological findings in liver tissues of rats with silymarin and *P. peruviana*

presented a slight attenuation of liver steatosis, and absence of hepatic necrosis in the treatment with *P. peruviana* (figure 2).

As mentioned before, after CCl$_4$ administration diverse reactive oxygen species (ROS) are generated, which must be neutralized by the antioxidant defense system of the organism like the enzymes SOD and CAT. The role of SOD is the dismutation of the superoxide radical to hydrogen peroxide, which is converted in water by CAT . For this reason, in most events of oxidative stress these two enzymes are affected (27). Previous studies have fully described the alteration; generally, increase in the activity of antioxidant enzymes such as SOD and CAT after administration of CCl$_4$ to Wistar rats (28). Results found in this investigation with *P. peruviana* were similar to those obtained from silymarin and other plants such as *Solanum tuberosum* (29), *Phyllanthus amarus* (30) and *Hippophae rhamnoides* (31).

Recently, it was studied the possibility that free radicals induced not only lipid peroxidation but also oxidation of carbonyl groups of proteins. In this way, it was established that •OH radicals can extract an H of any carbon of amino acids, forming a carbonyl radical which subsequently can be converted to an alkyl peroxyl radical. The radical formed can break peptide bonds, generating hydrolysis and oxidation of other proteins (18). Finally, free radicals can cause significant alterations of cell function, (including death) causing oxidation and inactivation of enzymes and accumulation of other oxidized proteins. The effect of CCl$_4$ on protein oxidation is not fully elucidated, but recent studies demonstrated that in the presence of this toxic the liver shows an increase (about 50%) in the levels of carbonyl groups (32). The effect of silymarin and the extract of *P. peruviana* in the decreasing of carbonyl groups levels could be associated with the antioxidant activity previously described.

Lipid peroxidation results are similar to those reported for other antioxidants such as carotenoids and tocopherols (33) in models of hepatotoxicity induced by CCl$_4$. Clearly, there is a strong relationship between *in vivo* antioxidant activity and *in vitro* inhibition of lipid peroxidation reported for the extracts of *Physalis peruviana* (6).

CONCLUSIONS

In conclusion, the evaluated calyces extract of *Physalis peruviana* has a significant hepatoprotective

activity that could be related to its antioxidant capacity, especially by the inhibition of lipid peroxidation.

ACKNOWLEDGEMENTS

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