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Hepatoprotective Effect of the Ursolic Acid-Oleanolic Acid Mixture Administered Intragastrically in Mice with Liver Damage Induced by Anti-TB Drugs

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Abstract

Objective: The hepatoprotective (HPP) effect of the ursolic acid and oleanolic acid (UA/OA) mixture administered intragastrically (i.g.) against the damage caused by antituberculosis drugs (rifampicin/isoniazid/pyrazinamide, -RIF/INH/PZA-) is described.

Materials and methods: The UA/OA mixture was obtained from the methanolic extract (MeOH) of*Rosmarinus officinalis*. The assay was performed in male Balb/C mice with hepatic damage induced by RIF/INH/PZA, and, as a positive control, silymarin (SIL) was used. The UA/OA mixture was administered by i.g. at 10 and 20 mg/kg during 60 days.

Results: The UA/OA mixture administered at 10 and 20 mg/kg for 60 days favored body weight gain and lower levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). This effect was similar to positive control (SIL). At the histological level, a slight reduction of steatosis in the group that received the UA/OA mixture at 10 mg/kg was observed with respect to the group with hepatic damage and with the UA/OA group at 20 mg/kg. The UA/OA mixture at 10 mg/kg showed a good HPP effect.

Conclusions: The UA/OA mixture administered i.g. favored body weight gain and lowered levels of hepatic enzymes while reducing steatosis before the damage caused by antituberculosis drugs. The best dose was 10 mg/kg administered over 60 days.

Keywords: Oleanolic acid; Ursolic acid; Hepatoprotector; Antituberculosis; Silymarin.

Introduction

Tuberculosis (TB) is one of the ten main causes of death by an infectious agent and represents a worldwide public health problem. Annually, it causes approximately 1.3 million deaths, and 11 million new cases appear; of the latter, between 10-

12 % are HIV-positive (approx. 300,000 cases), this co-infection (TB/HIV) being the main cause of mortality. It is estimated that 1.7 billion people in the world are infected and could develop TB disease. The reduction in TB cases is very slow; in 2020, it only decreased by 30% in the total number of cases, and there was a 20% reduction in incidence [1][2][3].

Between 2018 and 2020, approximately 5 million were new cases of TB; 600,000 were resistant to rifampicin (RIF), approximately 78% were multi-drug resistant (MDR), and only 111,000 people were treated adequately. For 2019, there was an increase of 10% in MDR cases compared to 2018. Currently, there is an increase in TB-MDR and extended drug resistance (TB-XDR). Around 60% of MDR cases are cured, and approximately 20% become TB-XDR [1][2][3][4][5].

The RIF, INH and PZA are three basic drugs to treat TB (sensitive and MDR) and cannot be substituted. These drugs mainly cause hepatotoxicity (HPT), nephrotoxicity, as well as neuropathy, hypersensitivity, nausea, vomiting and gastritis. The HPT incidence depends on the study population, treatment time, age, malnutrition, alcoholism, type 2 diabetes mellitus, rheumatoid arthritis, HIV/AIDS, cancer, etc. [5,6]. The biotransformation of anti-TB drugs is carried out in the liver, and their metabolism generates highly reactive products, which alter the integrity and functionality of the liver, generating liver inflammation, chronic drug hepatitis, hepatic fibrosis, non-alcoholic cirrhosis and even hepatocellular carcinoma, the latter being one of the main causes of withdrawing drugs from the market or is the main cause of liver transplants [6][7][8][9][10]. The metabolism of anti-TB drugs is also influenced by some genetics. For example, it has been reported that there are patients who are slow and fast INH acetylators, which, to a greater or lesser extent, cause liver damage. In slow acetylators, the low amount of the enzyme N-acetyltransferase 2 (NAT2) causes an accumulation of INH, leading to greater liver damage [11][12][13][14]. This can result in fulminant hepatitis HPT, which is one of the primary reasons for treatment abandonment in TB cases (approximately 48% of patients). This situation favors the emergence of drug-resistant strains of TB cases.

Currently, research is being carried out focused on preventing and/or reducing the HPT effect of TB drugs through the use of medicinal plant extracts, natural compounds and/or biological products. Among these, we can describe SIL, resveratrol, quercetin, vitamins E and C, polyphenols, garlic, and others. The inhibition of cytochrome P_{450}, in its isoform CYP 2E1, along with an antioxidant effect, are the most common and beneficial mechanisms of herbal remedies (extracts, biological products and natural compounds) and are the most recommended to protect the liver against liver damage induced by anti-TB drugs [9,10]. Among these HPP substances is the UA/OA mixture, metabolites that are found in different medicinal species and foods.

This mixture has several biological activities such as anti-inflammatory, antinociceptive, antimicrobial, antitubercular, antiviral, antiparasitic, low toxicity, anti-cancer, antitumoral, and antioxidant; it also has HPP effects against the damage caused by carbon tetrachloride, EtOH, D-galactosamine, cadmium, benzene and thioacetamide, among others substance [9,16-20]. It should be noted that there is only one work that described the HPP effect of this mixture administered by subcutaneous route (s.c.) against the hepatic damage caused by the mixture of anti-TB drug (RIF/INH/PZA) in male Balb/C mice. In this assay, the animals were treated with UA/OA at doses of 0.1 and 0.2 mg (dissolved in 100 µL of extra-virgin olive oil) over 11 weeks, administered by s.c. route, and the results showed that the UA/OA mixture (0.1 µg) favored body weight gain (BWG), lowered levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and

did not generate steatosis, respect to the group with hepatic damage [15].

The justification for the present investigation is based on the fact that treatment for sensitive TB or TB-MDR generates several secondary effects (hepatotoxicity and nephrotoxicity, among others), and other drugs cannot be used;

these factors influence treatment abandonment and the appearance of resistant strains. Considering this background, it is necessary to contribute to the search for substances with a protective effect against the hepatic or renal damage these drugs cause. Among these substances is the UA/OA mixture, which has an anti-TB effect on the *in vivo* model and has HPP activity against damage generated by carbon tetrachloride, EtOH, D-galactosamine, cadmium, benzene and thioacetamide. This work described the hepato- and nephroprotective effect of the UA/OA mixture against damage caused by the RIF/INH/PZA mixtures, administered by i.g. route over 60 days.

Materials and Methods

Obtention of UA/OA mixture

The UA/OA mixture was obtained from MeOH (10 gr) extract of*Rosmarinus officinalis (R. officinalis)*; the extract was washed with acetonitrile (CH_3CN, 200 mL), after with chloroform ($CHCl_3$, 200 ml) and finally with MeOH (200 mL); each washing was done three times; they were joined and vacuum concentrated to eliminate the dissolvent at 40°C. To the wash with MeOH, activated carbon (5 mg/mL) was added and agitated for 10 minutes; this process was performed in duplicate to eliminate chlorophylls. Each wash was analyzed by thin-layer chromatography (TLC) using $CHCl_3$:MeOH 9:1 as an elution system. The UA/OA mixture was observed in $CHCl_3$ (3.5 g) and MeOH (2.6 g) washes in high quantities. These samples were subjected to a chemical fractioning in normal-phase column chromatography (NP-CC) using silica gel F254 (200 g). The NP-CC was eluted with dichloromethane (CH_2Cl_2) 100 % and a mixture of CH_2Cl_2:Ethanol (EtOH) (8:2, 1:1, 3:7) and EtOH 100 %. From the fractions eluted with the CH_2Cl_2:EtOH mixture, cream-colored dust was obtained (3.54 g), and it was washed three times with CH_3CN. Through this process, 3.10 g of white dust was obtained, with a melting point of 265-269°C, soluble in MeOH and EtOH. This dust was subjected to Hydrogen Nuclear Magnetic Resonance (^{1}H- RMN) analysis, and the spectra data allowed the identification of the UA/OA mixture. It was compared with what was previously described in the literature [16].

In vivo assay

The present study was experimental, and the male Balb/C mice (24 ±2 g) from the IMSS vivarium were used, which were maintained under standard laboratory conditions according to the Official Mexican Standard (NOM-062-ZOO-1999), modified in 2016. The animals were treated according to the Care and Use Guidelines for laboratory animals of the National Science Academy. The protocol was approved by the local scientific research and bioethics commission 3601 of IMSS (CLIC R-2015-3601-47, proof attached). Food and water were available *ad libitum.*

Hepatoprotector activity

The assay was performed following the methodology described earlier[7,8,21], with some modifications. The hepatic damage was induced with the RIF/INH/PZA mixture (10:10:30 mg/kg) dissolved in carboxymethylcellulose (CMC) at 0.5 % in isotonic saline solution (ISS) and was administered by i.g. route, in a volume <10 mL/kg. The UA/OA mixture at doses 10 and 20 mg/kg and SIL (2.5 mg/kg, as positive control of hepatoprotection) were dissolved in the control Tween 80:CMC (0.5:9.5) and were administered by i.g. route. The groups received the following treatment during 60 days: Group I: CMC, 0.5%; Group II: anti-TB (INH/RIF/PZA, 10:10:30 mg/kg); Group III: anti-TB plus SIL, 2.5 mg/kg; Group IV: anti-TB + UA/OA, 10 mg/kg; Group V: anti-TB + UA/OA, 20 mg/kg.

During the experimental period (60 days), BWG was recorded every third day. Upon ending the period, blood was taken without anticoagulant (by retro-orbital puncture) with a previous fast of 12 hours. After, the animals were sacrificed by cervical dislocation, and the liver, kidney and spleen were extracted to record weight and for histological analysis.

In serum, levels of ALT, AST, alkaline phosphatase (ALP), cholesterol (CHOL), triglycerides (TRIG) and High-density lipoproteins (HDL) were determined. Analysis was performed with automated Selectra II Analyser (Modelo Vitalab 2) equipment using the Brand (Randox LAB) commercial kit.

Histological analysis

This trial was performed in accordance with that previously described by Pérez-González*et al.*[6] and by Gutiérrez-Rebolledo *et al.*[15].

Quantification of parameters of oxidative stress

The activity of superoxide dismutase (SOD), catalase (CAT) and oxidized glutathione (OxG) was determined in the supernatant by colorimetric method. Levels of oxidized proteins (OxP) and oxidized lipids (LOx) were determined in the homogenate without centrifuge. The trials were determined in accordance with that previously described by Pérez-González *et al.* [7,8].

Statistical analysis

The program SigmaPlot ver. 12.5 was used for the analysis of results and graphics. Data are presented as mean of standard error (SEM). GPC was analyzed using ANOVA, followed by post hoc Student-Newman-Keuls (SNK) testing. Results with values of p <0.05 were considered statistically significant. Parameters of biochemistry and oxidative stress were analyzed with one-way ANOVA and post hoc SNK testing.

Results

Obtaining and identification of the UA/OA mixture

From 10 g of MeOH extract of *R. officinalis,* 3.10 gr of white dust was obtained with a melting point of 265-269 °C, behind a chemical fractionation in NP-CC of $CHCl_3$ and MeOH washes. This white dust was identified as a UA/OA mixture according to ^{1}H-RMN data and compared with that previously described[16] and by comparison with the reference factor with standard commercial (Sigma).

Hepatoprotector effect *in vivo* of the UA/OA mixture at 60 days

Figure 1 shows the BWG generated by the UA/OA mixture when it was administered daily by i.g. route for 60 days. Group I (control) showed a slight increase in body weight; this BWG was constant during the treatment period, and at day 60, the increase was 0.85 g. Group II (anti-TB drugs) showed a greater increase than Group I (control), with an increase of 1.1 g vs 0.85 g by day 60. Groups IV and V (anti-TB plus UA/OA at 10 and 20 mg/kg, respectively) showed slight BWG during the treatment period; however, by day 60, these groups showed greater weight increase with regards to group I (control) and group II (anti-TB), this increase being 1.82 g for group IV (UA/OA at 10 mg/kg) and 1.64 g for group V (UA/OA at 20 mg/kg). The weight increase in group III (anti-TB/SIL) was similar to group V (anti-TB + UA/OA at 20 mg/kg), being 1.64 g.

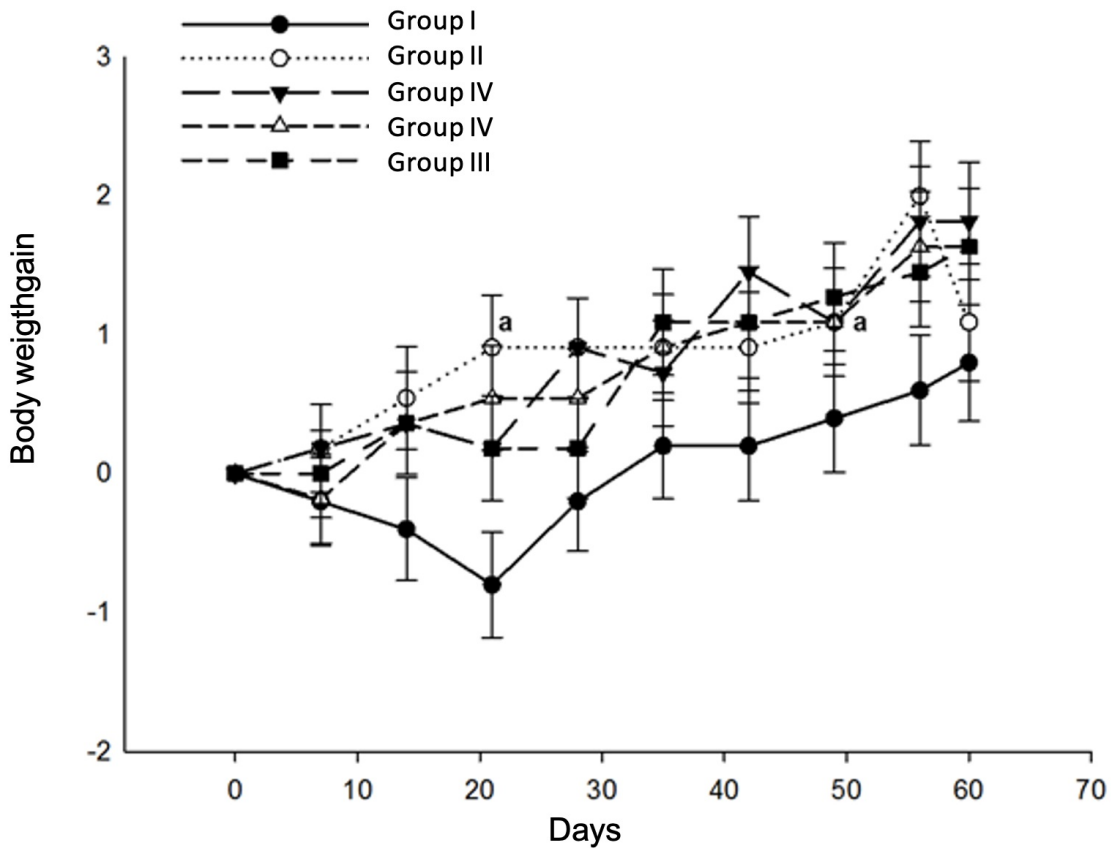

Figure 1. Body weight gain (BWG) of male Balb/C mice with liver damage caused by RIF/INH/PZA and treated with the UA/OA mixture for 60 days.

Data presented as the mean (±) with its standard error (e.s.). Statistical analysis (ANOVA) Two-way Repeated Measures

(RM), Tukey post hoc test ($p<0.05$). Group I (Vehicle); Group II (antiTB); Group III (antiTB/SIL, 2.5 mg/kg); Group IV (antiTB + UA/OA, 10 mg/kg); Group V (antiTB + UA/OA, 20 mg/kg). n=10.

Table 1 describes the relative weight of organs; only the weight of the liver of group II (anti-TB) showed a slight increase (1.37 g) with regards to the weight of the other groups (<1.33 g). The weight of the spleen and kidney was similar in all groups.

Table 1. Organ weights of male Balb/C mice with liver damage caused by RIF/INH/PZA and treated with the UA/OA mixture for 60 days.

Organ	Treatment				
	Group I	Group II	Group III	Group IV	Group V
	Vehicle	anti-TB	anti-TB + SIL	anti-TB + AU/AO	anti-TB + AU/AO
liver (g)	1.29±0.02	1.37±0.14	1.32±0.06	1.31±0.03	1.33±0.14
spleen (g)	0.11±0.02	0.16±0.19	0.10±0.01	0.10±0.01	0.15±0.07
kidneys (g)	0.39±0.01	0.38±0.03	0.43±0.05	0.40±0.04	0.43±0.10

Data presented as the mean (±) with its standard error (s.e.). Group I: CMC, 0.5%; Group II: anti-TB (INH/RIF/PZA, 10:10:30 mg/kg); Group III: anti-TB/SIL, 2.5 mg/kg; Group IV: anti-TB + AU/AO, 10 mg/kg; Group V: anti-TB + AU/AO, 20 mg/kg.kg; n=5.

Blood chemistry results are shown in Table 2. The creatinine level of group II (anti-TB) was elevated (0.84 mg/dL), with regards to group I (control, 0.53 mg/dL), and the levels for groups III (SIL), IV and V (UA/OA at 10 and 20 mg/kg) were similar to group I (0.49, 0.50 and 0.57 mg/dL vs 0.53 mg/dL). In addition, levels of urea were high in group II (anti-TB, 91.1 mg/dL) and slightly less in groups IV and V, which received UA/OA at 10 and 20 mg/kg, with values of 83.26 and 83.28 mg/dL, without reaching the levels of the control group (75.27 mg/dL); only group III (anti-TB/SIL, 71.7 mg/dL) showed a level slightly lower than the control group. Levels of AST for group II (anti-TB) were elevated by 102% compared with the controls (251 IU vs 123 IU), with a statistically significant difference of p=0.004. Group III (anti-TB/SIL 125.67 IU) and groups IV and V (175.0 and 141.25 IU, respectively) showed a slight increase with regards to group I (control, 123 IU), although these levels were less than group II (251 IU). On the other hand, levels of ALT showed similar behavior; these were elevated in group II (anti-TB, 372 IU) and reduced in groups III (SIL, 248 IU), IV and V (248 and 235 IU, respectively), these values being lower than the control (256.75 IU). Regarding levels of ALP and HDL, the following behavior was observed: anti-TB > SIL > UA/UO 20 mg/kg > UA/UO 10 mg/kg >control. CHOL levels showed the following behavior: anti-TB > UA/OA 10 mg/kg > UA/OA 20 mg/kg > SIL > controls) and in levels of triglycerides there were no significant changes.

Table 2. Blood chemistry values of male Balb/C mice with liver damage caused by RIF/INH/PZA and treated with the UA/OA mixture for 60 days.

Parameter	Treatment				
	Group I	Group II	Group III	Group IV	Group V
	Vehicle	anti-TB	anti-TB + SIL	anti-TB + AU/AO	anti-TB +AU/AO
Creatinine (mg/dL)	0.53±0.02	0.84±0.01^{a,c}	0.49±0.02	0.50±0.02	0.57±0.04^{a,c,e}
Urea (mg/dL)	75.27±0.88	91.1±1.1^{a*,c,d,e***}	71.77±0.28	83.26±1.4^{a,e***}	83.28±2.07^{a,e*}
AST (IU)	123.75±13.82	251.0±29.48	125.67±9.65	175.0±18.61	141.25±16.74
ALT (IU)	256.75±10.78	372.0±16.90	248.0±52.32	248.0±14.12	235.00±22.68
ALP (IU)	131.5±13.70	292.0±22.02	192.0±5.66	174.0±35.25	183.5±8.46
CHOL (mg/dL)	38.33±0.41	49.4±0.84	40.67±1.77	44.25±1.19	43.5±2.60
TRIG (mg/dL)	1.26±0.14	1.24±0.22	1.24±0.04	1.23±0.15	1.22±0.12
HDL (mg/dL)	19.13±0.67	26.68±1.99	21.07±1.96	17.37±2.53	19.87±0.88

*Data presented as the mean (±) with its standard error (s.e.). Group I: Vehicle (CMC, 0.5%); Group II: anti-TB (INH/RIF/PZA, 10:10:30 mg/kg); Group III: anti-TB/SIL, 2.5 mg/kg; Group IV: anti-TB + UA/OA, 10 mg/kg; Group V: anti-TB + UA/OA, 20 mg/kg. n= 3. ^{a}vs Group I;^{b}vs Group II;^{c}vs Group IV;^{d}vs Group V;^{e}vs Group III. ***p<0.001. Post hoc Holm-Sidak test n= 5.*

The results of the histological liver analysis are shown in Table 3 and Figure 2. According to the observations, 3/3 of the animals from group I (control) showed slight steatosis. Group II (anti-TB) showed slight hepatic hematopoiesis (2/3), slight lymphoid infiltration (2/3) and moderate steatosis (3/3); in addition, they presented splenomegaly (2/3). The animals of group IV (anti-TB plus UA/OA, 10 mg/kg) presented mild steatosis (2/3) and slight splenomegaly (1/3). Group V (anti-TB plus UA/OA, 20 mg/kg) presented mild hepatic hematopoiesis (1/3), moderate steatosis (2/3) and mild splenomegaly (1/3). Finally, group III (anti-TB/SIL) presented slight lymphoid infiltration (1/3) and moderate steatosis (2/3). It should be noted that steatosis was greater in the anti-TB/SIL group with regard to the anti-TB group.

Table 3. Histological analysis of the liver of male Balb/C mice with damage caused by RIF/INH/PZA and treated with the AU/OA mixture for 60 days.

Group	Hepatic hematopoiesis	Hepatic Microabscesses	Lymphoid infiltrade	steatosis	Centrilobular hydropic degeneration
I Vehicle	-	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-
II anti-TB	+	-	-	++	-/splenomegaly
	+	-	Perivenous/++	++	-/splenomegaly
	-	-	Perivenous/++	++	-
III anti-TB + SIL (2.5 mg/kg)	-	-	+	-	-
	-	-	-	++	-
	-	-	-	++	-
IV anti-TB + AU/AO (10 mg/kg)	-	-	-	-	-
	-	-	-	+	-
	-	-	-	+	-/splenomegaly
V anti-TB + AU/AO (20 mg/kg)	-	-	-	++	-
	-	-	-	++	-
	+	-	-	-	-/splenomegaly

(+) mild; (++) moderate; (+++) intense; (-) negative. n=3. Staining with H&E.

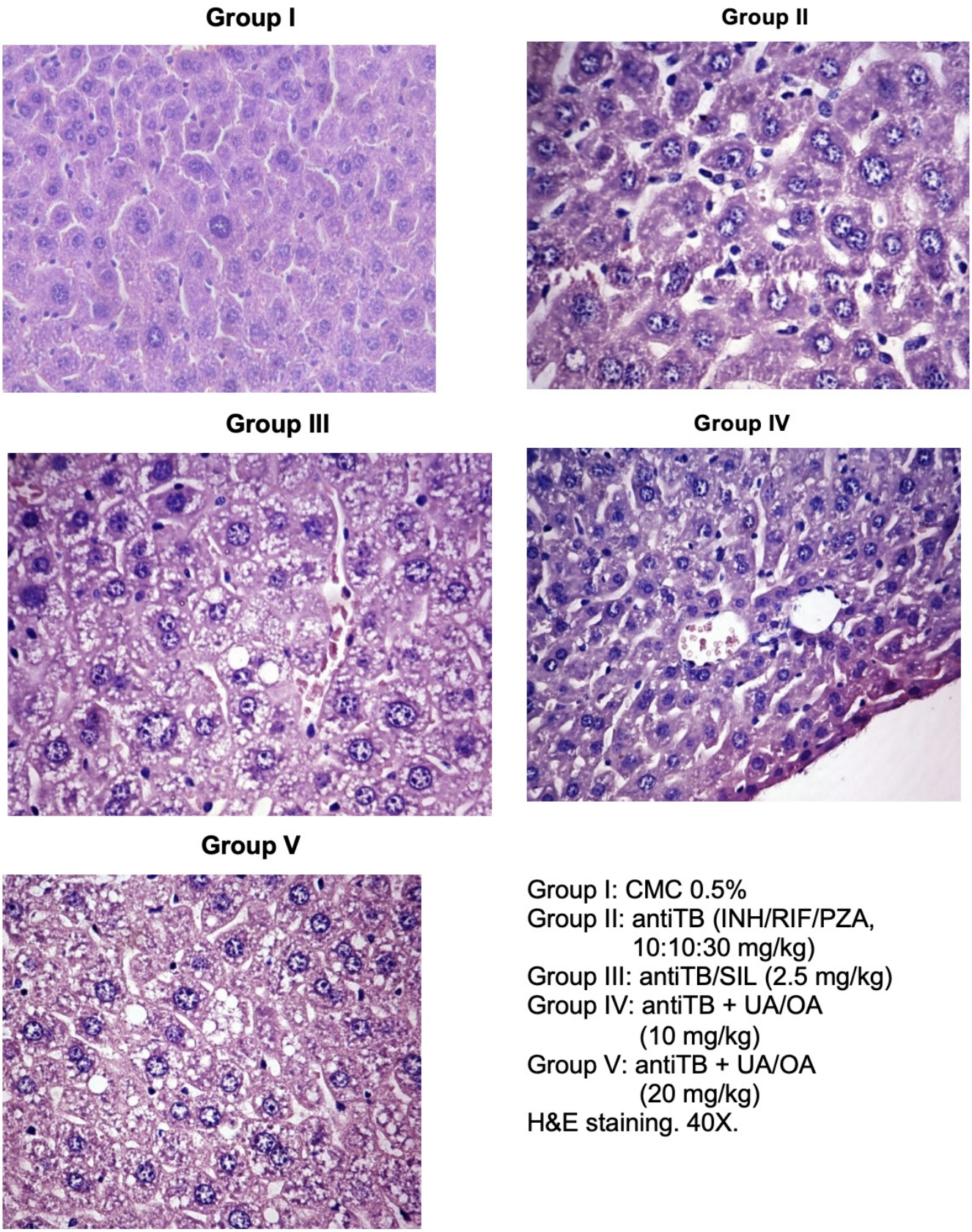

Figure 2. Histological sections of livers from male Balb/C mice with liver damage caused by RIF/INH/PZA and treated with the UA/OA mixture for 60 days.

Discussion

In this investigation, it was observed that the UA/OA mixture (at 10 and 20 mg/kg) and SIL administered by i.g. route during 60 days favored BWG with regard to the anti-TB group and controls. This behavior was similar to that reported previously by Gutiérrez-Rebolledo et al.,[21] when the UA/OA mixture (100 or 200 µg) was administered subcutaneously. It was also observed that the livers of the animals that received only anti-TB (group II) presented high weight with regards to groups I, III and IV.

About the biochemical parameters, the UA/OA mixture reduced levels of creatinine with respect to the anti-TB group, the dose of 10 mg/kg being the best, since the values were similar to the SIL group and slightly less than controls. In addition, the UA/OA mixture (at doses of 10 and 20 mg/kg) reduced the levels of urea and AST compared with the anti-TB group, but without reaching the control levels or of SIL groups. These values were similar to those previously reported [15]. The ALT level was reduced in the UA/OA groups at 10 and 20 mg/kg with regards to controls, and these levels were similar to the group that received SIL. This effect was similar to that observed when it was administered subcutaneously. Levels of ALP and CHOL lowered slightly in all groups without reaching the levels of the control group.

Levels of TRIG were not significantly altered; in contrast, levels of HDL were similar between UA/OA (10 and 20 mg/kg) and controls, but slightly lower than the anti-TB/SIL and anti-TB groups.

The UA/OA mixture reduced steatosis with regard to the anti-TB and anti-TB/SIL groups. In a previous study, it was reported that the UA/OA mixture at a dose of 100 µg/mouse/day, administered by s.c. route favored the BWG, reduced AST, ALT and urea levels, and also reduced steatosis [15]. These results found in this study indicate that the mixture of triterpenes (UA/OA) administered by i.g. route protects the liver damage caused by the RIF/INH/PZA mixture; the HPP effect was similar to that observed when administered by s.c. route. It is also well documented that levels of urea and creatinine increase in cases of HPT caused by anti-TB drugs due to the increase in the metabolism of proteins [21,23,24]; the results found in this study, upon administering the UA/OA mixture (by i.g. route) over two months, it generated a reduction in the levels of this parameter, which indicates that this mixture also has a nephroprotective effect.

It should be noted that the liver is the key organ in the biotransformation of various substances, among which are anti-TB drugs, so the disorders and alterations in this organ are numerous and varied [9,10]. The anti-TB drugs, when metabolized, generate more toxic compounds, altering the functional and structural integrity of this organ while generating inflammation, chronic and non-chronic drug hepatitis, hepatic fibrosis, non-alcoholic steatosis and/or cirrhosis, and, on occasions, even hepatocellular carcinoma [9,10,25]. The combination of basic drugs (RIF/INH/PZA) to treat tuberculosis causes serious liver damage by increasing the levels of AST, ALT and ALP; it favors the development of oxidative stress, lipoperoxidation and choline deficiency, altering the synthesis of phospholipoproteins. It has been found that PZA is the most hepatotoxic [9-11,25,26]. Currently, substances of natural or synthetic origin with hepatoprotective effects are being sought. For example, it has been described that SIL shows this effect *in vivo* and *in vitro* assays against liver damage caused by anti-TB drugs, added to the fact that it also presents antitubercular and antimycobacterial effects [17][18][19]. Other examples of hepatoprotective substances are picroliv, N-acetylcysteine, turmeric, and resveratrol, among other substances, [9,10,30-32]

but these substances have not been evaluated in cases of liver damage caused by anti-TB drugs. In previous studies, it has been reported that the UA/OA mixture has an antimycobacterial effect *in vitro*, an antitubercular effect at 30 and 60 days of administration (in a mouse model of pulmonary TB) and no toxicity at 28 days in healthy Balb/C mice whose DL_{50} was > 2 mg/kg[20,21,35,36]. In addition, this mixture has shown an HPP effect against the hepatic damage caused by carbon tetrachloride, EtOH and D-galactosamine. OA also showed an HPP effect against the damage caused by cadmium, bromobenzene and thioacetamide [20][21]. HPP evaluation of this mixture, which was administered over 11 weeks subcutaneously (using as control olive oil at a dose of 100 and 200 µg/mouse/day) in animals with hepatic damage induced with the RIF/INH/PZA mixture (10:10:30 mg/kg, dissolved in SSI and administered by i.g. route), demonstrated that OA/UA at 100 µg/mouse/day reduced levels of AST and ALT, without significant changes in levels of ALP; it was also noted that at this dose, the mixture of triterpenes reduced steatosis [15].

Previously, it has been described that the $CHCl_3$ and MeOH extracts of *R. officinalis* (leaves) contain the UA/OA mixture [22][23][24][25], suggesting that this medicinal species constitutes a potential source for obtaining the triterpene mixture (UA/OA). It is important to point out that the UA/OA mixture is insoluble in water and was partially soluble in CMC, this being a limitation to be taken into account for later studies. It is therefore important and necessary to develop some pharmaceutical formulations with this mixture to submit to evaluation in this same trial, taking into account its HPP and antitubercular potential.

Conclusions

The UA/OA mixture administered by intragastric route favors body weight gain and lowers levels of hepatic enzymes, while reducing steatosis against the damage caused by anti-TB drugs. The best dose was 10 mg/kg administered by i.g. route during 60 días. This mixture of triterpenes can be obtained from the medicinal species *R. officinalis*.

Statements and Declarations

Author contribution: Jiménez-Arellanes wrote and developed the project, analyzed the results, wrote the manuscript and obtained funding. Siordia-Reyes and Juárez-Vázquez contributed to the development of the project, performed the histological analysis and contributed to the writing of the manuscript.

Interest conflict: The authors declare that they have no conflict of interest in this work.

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