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Acute and subacute toxicity (28 days) of a mixture of ursolic acid and oleanolic acid obtained from *Bouvardia ternifolia* in mice

[Toxicidad aguda y subaguda (28 días) en ratones, de la mezcla de ácido ursólico y ácido oleanólico obtenida de *Bouvardia ternifolia*]

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Abstract
Ursolic acid (UA) and oleanolic acid (OA) are triterpenes that are found in a large number of medicinal plants, one of which is the species *Bouvardia ternifolia*. These compounds have been shown to have around 120 types of biological activity, especially the hepatoprotective, anti-inflammatory and antimycobacterial effects. Despite having a high therapeutic potential, not much information concerning their toxicity is available. This article describes the results of acute and subacute (28 days) toxicity evaluations in Balb/c mice (both sexes) treated with the mixture of UA/OA obtained from *B. ternifolia* at doses of 6.5 and 13 mg/kg. The LD_{50} was >300 mg/kg. During the subacute administration, there was no death of animals and no changes were observed in the growth or weight of the different organs when compared to the control groups. Studies of blood chemistry and blood count showed normal levels in all parameters evaluated. The histopathology of major organs showed no changes or abnormalities. The mixture UA/OA is indeed safe when administered subcutaneously as a single dose of 300 mg/kg or in repeated doses of 13 mg/kg during 28 days.

Keywords: Ursolic Acid; Oleanolic Acid; Acute Toxicity; Subacute Toxicity; *Bouvardia ternifolia*.

Resumen
Los ácidos ursólico (UA) y oleanólico (OA) son triterpenos que se encuentran distribuidos en un gran número de plantas medicinales, una de ellas es la especie *Bouvardia ternifolia*. Estos compuestos han mostrado alrededor de 120 actividades biológicas, destacando los efectos hepatoprotector, antiinflamatorio y antimicobacteriano. A pesar de ser compuestos con un alto potencial terapéutico, no se han documentado muchos datos acerca de su toxicidad. En este artículo se describen los resultados de la evaluación de toxicidad aguda y subaguda (28 días) en ratones Balb/c de ambos sexos, tratados con la mezcla de AU/AO obtenida de *B. ternifolia* a dosis de 6.5 y 13 mg/kg. La DL_{50} fue > 300 mg/kg. Durante la administración subaguda, no hubo muerte de animales, tampoco se observaron alteraciones en su crecimiento ni alteraciones en el peso de los diferentes órganos. Los estudios de biometría hemática y química sanguínea mostraron niveles normales en todos los parámetros evaluados. Los análisis histopatológicos de los principales órganos no presentaron cambios o anormalidades. La mezcla UA/OA es practicamente inocua cuando se administra subcutaneamente en dosis única de 300 mg/kg y 13 mg/kg en dosis repetida (28 días).

Palabras Claves: Acido Ursólico; Acido Oleanólico; Toxicidad Aguda; Toxicidad Subaguda; *Bouvardia ternifolia*.

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List of abbreviations: UA - Ursolic Acid; OA - Oleanolic Acid; NAPRALERT - Natural Products Alert; COX - Cyclooxygenase; MIC – Minimum Inhibitory Concentration; MABA - Microplate Alamar Blue Assay; TB - Tuberculosis; MDR - Multidrug-resistant; XDR - Extensively Drug-Resistant; LC_{50} – Lethal Concentration 50; LD_{50} – Lethal Dose 50; ^{1}H-NMR - Nuclear Magnetic Resonance; TMS - Tetramethylsilane; EI-MS - Electron Impact-Mass Spectra; CC - Column Chromatography; TLC - Thin Layer Chromatography; HEX - n-Hexane; CH_3CN – acetonitrile; $CHCl_3$ – Chloroform; MeOH – Methanol; HPLC - High Performance Liquid Chromatography; OECD - Organization for Economic Cooperation and Development; GHS - Globally Harmonised Classification System; SEM - Standard Error of Mean; ANOVA - Analysis of Variance; WBC - White Blood Cells; RBC - Red Blood Cells; HGB – Hemoglobin; HCT - Hematocrit, MCV - Mean Corpuscular Volume; MCHC - Mean Corpuscular Hemoglobin Concentration; SGOT - Serum Glutamic Oxaloacetic Transaminase; SGPT - Serum Glutamic Pyruvic Transaminase.

INTRODUCTION

Triterpenes, such as ursolic acid (UA) and oleanolic acid (OA), are secondary metabolites present in different plant species (Ovesná *et al*., 2004; Somova *et al*., 2003). To date, around 120 types of biological activity have been discovered for these compounds, according to data recorded in the Natural Products Alert Database (NAPRALERT, 2011), including anti-HIV, antiplasmodial, cytotoxic, antibacterial, anti-inflammatory (lipoxygenase and Cyclooxygenase [COX] inhibitor), antitumoral, hepatoprotective (*in vivo*), and antidiabetogenic activity (Jaki *et al*., 2008; Liu, 1995, 2005).

Previous investigation has shown that UA and OA, obtained from different plant species, exhibit moderate to high *in vitro* antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv (Cantrell *et al*., 2001; Copp and Pearce, 2007; Gu *et al*., 2004; Okunade *et al*., 2004; Wächter *et al*,. 1999; 2001). Regarding this activity, a minimum inhibitory concentration (MIC) of 15 μg/mL or 32 μM has been reported for UA and 30 μg/mL or 64 μM for OA when it was tested by the BACTEC 460 radiospirometric method, and 41.9 and 28.7 μg/mL respectively, when evaluated by the Microplate Alamar Blue Assay (MABA) test (Cantrell *et al*., 2001; Gu *et al*., 2004; Wächter *et al*. 1999, 2001). Recently, it was reported that OA exerts a synergistic effect when was combined with isoniazid, (I) rifampicin (R) or ethambutol (first line antitubercular drugs) (Ge *et al*., 2010). Moreover, the antimycobacterial effect reportedly depends on the purity of each compound; for example, high-purity UA (98.6%) has an MIC value > 256 μg/mL, while UA with 69.6% purity has an MIC = 65 μg/mL (Jaki *et al*., 2008).

On the other hand, it is important to note that the search for compounds with antitubercular activity is urgent because of health problems in which tuberculosis (TB) is associated with the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB strains, which are resistant to R and I, the principal (basic) drugs in the treatment of TB. It is known that first- and second-line anti-TB drugs produce severe toxic effects from prolonged use, causing low adherence to or the abandonment of treatment. For example, I and R cause hepatotoxicity (Yogeeta *et al*., 2007), because they generate the production of oxygen-free radicals, initiate lipid peroxidation and produce oxidative stress. Other types of damage caused by TB drugs include peripheral neuropathy, fever, convulsions, hyperuricemia, photosensitivity, renal toxicity and renal failure.

Despite the diverse biological activity described for UA and OA, to date no data have been documented on acute and subacute toxicity for these compounds. The only data in this regard are the median lethal concentrations (LC_{50}) for UA and OA, tested in *Artemia salina* (Brine shrimp test), with values of 0.10 and 0.95 mg/mL, respectively (Somova *et al*., 2003).

In this paper, we describe the LD_{50} and the subacute toxicity caused by a 28-day administration in healthy Balb/c mice with the mixture of UA/OA obtained from *Bouvardia ternifolia.* The effects of this mixture were determined by measurement of body and organ weight, as well as hematological and biochemical parameters. Histological analyses of the data were carried out.

MATERIALS AND METHODS

General experimental procedures

The chemical characterization of the isolated triterpenoids was determined by nuclear magnetic resonance (^{1}H-NMR, Bruker-Avance F, 300 MHz) using Tetramethylsilane (TMS) as an internal standard in $CDCl_3$. Electron impact-mass spectra (EI-MS) were obtained on a Jeol AX-505 HA mass spectrometer at 70 eV. Melting points (m.p.) were determined on a Fisher-Johns apparatus and are uncorrected. All spectroscopic and spectrometric data of each compound were compared with those previously reported in the literature.

Open-column chromatography (CC) was carried out utilizing silica gel 60 GF_{254} (70-230 mesh, Merck) as the stationary phase. Silica gel 60 F_{254} recoated aluminum plates (0.2 mm, Merck) were employed for analytical and preparative thin layer chromatography (TLC) analysis. n-Hexane (HEX), acetonitrile (CH_3CN), chloroform ($CHCl_3$) and methanol (MeOH) were obtained from Mallinckrodt and J.T. Baker. Spots were visualized by spraying with a 10% aqueous solution of H_2SO_4 followed by heating to 100ºC.

High performance liquid chromatography (HPLC) was carried out with a Waters 600 system controller connected to a photodiode array detector 996, which was programmed to take data from 220 to 380 nm at 2.4-nm resolution. Control of equipment, data acquisition, processing, and handling of chromatographic information were carried out by the Millennium 32 software program (Waters). Analyses were undertaken on a Symmetry column (3.9 × 150 mm, 5-µm particle sizes, Waters). The mobile phase comprised an isocratic CH_3CN:MeOH (80:20) system (HPLC grade, J.T. Baker). The flow rate was maintained constant at 1.8 mL/min for 15 min. Samples were solubilized in MeOH at 1 mg/mL, and a volume of 100 µL was injected.

Plant material

B. ternifolia was collected in Miahuatlán, Oaxaca State, Mexico, in November of 2008. The species was botanically identified by Abigail Aguilar, M.Sc., and a voucher specimen was deposited at the Instituto Mexicano del Seguro Social (Mexico, IMSSM) Herbarium.

Extraction and isolation of mixture of UA/OA

Powdered, air-dried aerial particles were extracted with MeOH. The crude extract was obtained by maceration of plant material. After filtration, the extract was concentrated under low pressure to dryness at 40º C. *B. ternifolia* (0.7 kg) was macerated three times with 18, 12, and 8 L of MeOH, respectively.

The MeOH extract of *B. ternifolia* was fractionated on CC, using HEX, $CHCl_3$ and MeOH, as well as a mixture of these solvents with an increasing degree of polarity. The primary fraction eluted with $CHCl_3$:MeOH (80:20) was fractionated by CC under the same conditions. Thirteen secondary fractions were grouped on the basis of TLC patterns. Afterward, the UA/OA mixture was purified from secondary fractions rich in this mixture. These triterpenoids were detected utilizing TLC and were compared with the commercial references (Sigma).

UA/OA-rich primary or secondary fractions were successively extracted with CH_3CN (three times, 20 mL/g) and $CHCl_3$ (three times, 10 mL/g). In each case, the supernatant was filtered through a 0.45 µm GH Polypro 47-mm filter (Pall) into millipore equipment. The solid residuum was dissolved in MeOH (20 mL/g) and filtered in the same millipore equipment. The MeOH solution was then treated with activated charcoal (Sigma, 5 mg/mL) in an Erlenmeyer flask and shaken for 10 min. Subsequently, this solution was filtered with the millipore equipment and concentrated by evaporation under reduced pressure to dryness at 40° C.

Animals

The experiment was performed on male and female Balb/c mice (body weight, 19-23 g) according to Organization for Economic Cooperation and Development (OECD) Test Guidelines (OECD, 2007). All experiments were performed following the Mexican Official Norm for Animal Care Handing (NOM-062-ZOO-1999). The mice were housed in standard environmental conditions and were allowed free access to food and water, with 12-h light/dark photoperiods. The compounds were dissolved in extra virgin olive oil (Sigma). The concentrations were adjusted to subcutaneous (s.c.) administration at 0.1 mL/mouse.

Acute toxicity (median lethal dose–LD_{50})

According to OECD TC423, a group of 3 mice of both sexes was administered s.c. at single doses of 300 mg/kg. The general behavior of the mice was observed after administration at the 1st, 2nd, 4th, and 6th h, and once daily for 14 days. During this 14-day period, the animals were also observed to detect any signs of toxicity or death. At the end of the experiments, the animals were sacrificed in a CO_2 chamber. Subsequently, their organs (lung, kidney, heart, spleen and liver) were extracted and gross pathological observations were performed. The LD_{50} value was determined according to the Globally Harmonized Classification System (GHS) and to the flow chart for this study (OECD, 2007).

Subacute toxicity (repeated-dose, 28-day toxicity)

According to OECD TG407 (OECD, 2007), mice were randomly divided into four groups of 5 animals

per sex. Group I was the control, group II was the vehicle control, and groups III and IV were s.c. treated with the mixture of UA/OA at 6.5 and 13 mg/kg for 28 days. These doses corresponded to 5 and 10 times the MIC of the mixture against *M. tuberculosis* H37Rv *in vitro*. Toxic manifestations such as signs mortality and body weight changes were monitored daily. At the end of treatment (day 28), the animals were fasted overnight, with water provided *ad libitum.* Subsequently, the mice were anesthetized with xylazine and ketamine. Heparinized blood samples were drawn to determine complete blood count and red blood cell count. Serum from non-heparinized blood was carefully collected for blood chemistry and enzyme analysis.

Histological analyses

All animals were euthanized and their main vital organs were removed and macroscopically analyzed. After macroscopic analysis, representative fragments of spleen, heart, stomach, intestine, skin, lung, kidney and liver were subsequently fixed in a 10% solution of formalin and enclosed in paraffin. Paraffin blocks were prepared after completing tissue processing in different grades of alcohol and xylenes. Sections (5-µm) were prepared from blocks employing microtome and stained with hematoxylin and eosin (H&E). Images were taken using a camera connected to the microscope to examine cellular damage.

Statistical analysis

Results were expressed as mean ± Standard error (SEM). Statistical significance was determined by one-way Analysis of variance (ANOVA) and post-hoc least-significant-difference (Bonferroni post-test).

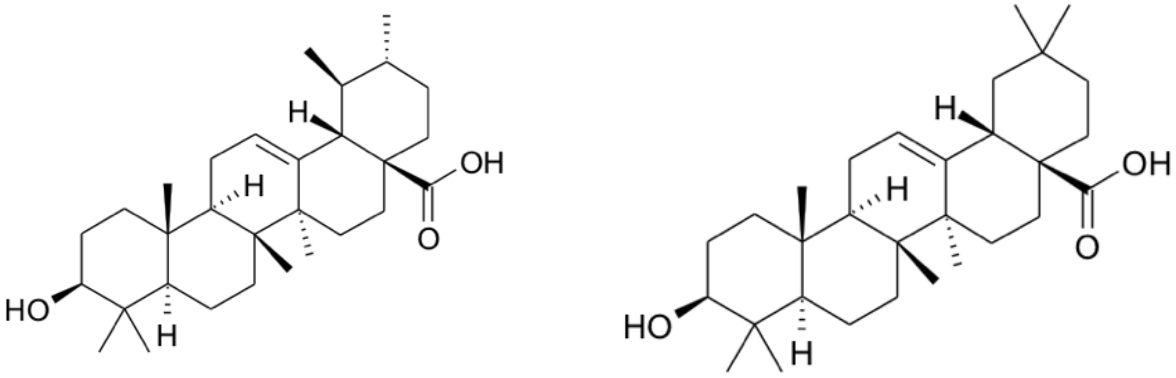
RESULTS AND DISCUSSION

Previous research has indicated that *B. ternifolia* biosynthesizes UA and OA (Jiménez-Ferrer *et al*., 2005; Pérez *et al*., 1998). Thus, it was decided to perform a chemical study of the MeOH extract of this plant. The crude extract (82 g) was obtained by the maceration process from the aerial parts of *B. ternifolia* (700 g of dry material), with an efficiency of 11.7%. After successive chromatographic procedures, 3 g of the mixture of UA **(1)** and OA **(2)** (rate, 2:1) were obtained. This mixture was characterized structurally by ^{1}H-NMR and EI-MS data and compared with the commercial references (Sigma). These UA/OA mixtures were obtained as white powder with an m.p. of 268-270.8°C; this m.p. is similar to those of commercial references (e.g., Sigma, m.p., 276.2-279.5°C). HPLC analysis showed that natural compounds have similar retention times (R_T) to commercial references: the chromatogram had two peaks, with an R_T of 7.21 and 7.67 by *B. ternifolia*, and of 7.22 and 7.65 from commercial references (Figure 1).

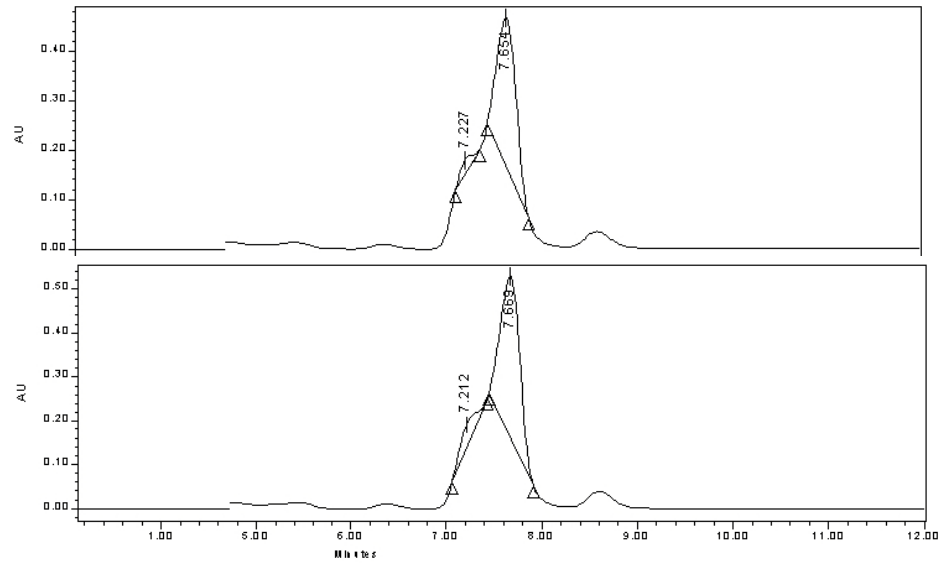
The LD_{50} of the UA/OA mixture obtained from *B. ternifolia* in both sexes of Balb/c mice was >300 mg/kg according to the TG423 method. At this dose, no deaths or hazardous signs of toxicity were recorded in these animals during the 14 day period of observation after acute treatment by the s.c. route. Evaluations at higher doses were not performed since the biologically active concentration employed for each of these compounds is much lower. For example, the ID_{50} for UA is 56 µg/cm^2 and for OA is 132 µg/cm^2, in both cases as an anti-inflammatory agent. For both compounds, the MIC value is ≤50 µg/mL as an antimycobacterial agent, and is 200 µmol/kg as a hepatoprotective agent (Cantrell *et al*., 2001; Gu *et al*., 2004; Ismaili *et al*., 2004; Liu *et al*., 1994; Wachter *et al*. 1999; 2001). A previous report described that the LC_{50} in *A. salina* is 0.10 and 0.95 mg/mL, respectively, for UA and OA. Considering that Logarto-Parra et al. (2001) described the existence of a direct correlation between the LC_{50} determined in *A. salina* and the same value determined in mice, we would expect an LD_{50} >5000 mg/kg.

Regarding the subacute analysis, no signs of toxicity (such as piloerection and alteration in locomotor activity) or mortality were observed in the treated groups compared to the control groups for either sex. Body weights are depicted in Table 1 and no significant variations in this parameter were detected during the treatment period. The organ weights showed no significant difference between the control and the vehicle group (Table 2). The internal organs did not exhibit any gross morphological lesions. The results obtained indicate that the UA/OA mixture does not affect the growth of mice during the treatment period nor does it affect the weight of their principal organs.

Figure 1



UA (1) **OA (2)**



Upper: representative high performance liquid chromatography (HPLC) chromatogram of the mixture of ursolic acid/oleanolic acid (UA/OA) 1:1 (Sigma); RT = 7.22 and 7.65. Lower: representative HPLC chromatogram of the mixture of ursolic acid/oleanolic acid (UA/OA) 2:1 (Bouvardia ternifolia); RT = 7.21 and 7.67.

Table 1
Body weights of mice with the regimen of subacute toxicity of UA/OA

	Body weight (g)			
	Day 0	Day 14	Day 28	Weight gain on day 14
Females				
Control	20.78 ± 0.57	21.43 ± 0.71	21.93 ± 0.70	1.30 ± 0.46
Vehicle	21.02 ± 0.43	22.06 ± 0.16	22.92 ± 0.14	2.18 ± 0.13
UA-OA[a]	20.67 ± 0.25	21.80 ± 0.55	22.62 ± 0.69	1.95 ± 0.55
UA-OA[b]	21.12 ± 0.51	22.11 ± 0.34	22.61 ± 0.28	1.85 ± 0.31
Males				
Control	20.73 ± 0.65	22.30 ± 0.79	24.08 ± 1.45	2.35 ± 0.80
Vehicle	20.23 ± 0.64	23.25 ± 0.38	25.08 ± 0.65	3.02 ± 0.52
UA-OA[a]	20.23 ± 0.64	23.25 ± 0.38	25.08 ± 0.65	3.02 ± 0.52
UA-OA[b]	20.18 ± 0.30	22.57 ± 0.53	24.10 ± 0.91	2.39 ± 0.44

Values are expressed as the mean ± standard error (SEM), *n* = 5; UA = ursolic acid; OA = oleanolic acid. [a]A group was given UA/OA 6.5 mg/kg; [b]A group was given UA/OA 13 mg/kg.

Table 2
Organ weights of mice with the regimen of subacute toxicity of UA/OA

	Organ weights (g)			
	Control	Vehicle	UA/OA[a]	UA/OA[b]
Females				
Spleen	0.45 ± 0.02	0.54 ± 0.07	0.54 ± 0.02	0.53 ± 0.02
Heart	0.51 ± 0.03	0.48 ± 0.09	0.53 ± 0.08	0.52 ± 0.02
Liver	4.24 ± 0.55	4.31 ± 0.12	4.34 ± 0.30	4.23 ± 0.09
Kidney	1.14 ± 0.02	1.13 ± 0.05	1.12 ± 0.08	1.14 ± 0.05
Lung	0.71 ± 0.02	0.66 ± 0.09	0.75 ± 0.02	0.71 ± 0.02
Brain	1.77 ± 0.03	1.81 ± 0.10	1.80 ± 0.08	1.79 ± 0.05
Males				
Spleen	0.41 ± 0.00	0.41 ± 0.04	0.47 ± 0.03	0.51 ± 0.06
Heart	0.63 ± 0.03	0.61 ± 0.06	0.59 ± 0.09	0.63 ± 0.03
Liver	4.71 ± 0.61	4.86 ± 0.36	4.87 ± 0.08	4.99 ± 0.16
Kidney	1.43 ± 0.05	1.40 ± 0.05	1.44 ± 0.02	1.40 ± 0.13
Lung	0.72 ± 0.05	0.72 ± 0.03	0.60 ± 0.03	0.75 ± 0.05
Brain	1.53 ± 0.06	1.50 ± 0.10	1.51 ± 0.04	1.48 ± 0.03

Values are expressed as the mean ± standard error (SEM), *n* = 5; UA = ursolic acid; OA = oleanolic acid;. [a]A group was given UA/OA 6.5 mg/kg; [b]A group was given UA/OA 13 mg/kg.

The status of bone marrow activity and intravascular effects was monitored by hematological examination and the results are reported in Tables 3 and 4. Values of white blood cells (WBC), lymphocytes, neutrophils, eosinophils, monocytes, basophils, red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) exhibited no variation. Consequently, the 28-day administration of the UA/OA mixture at doses of 6.5 and 13 mg/kg did not induce any alteration in hematological or biochemical parameters.

Table 3
Hematological values of mice with the regimen of subacute toxicity of UA/OA

	Group							
	Control		Vehicle		UA/OA[a]		UA/OA[b]	
Females								
RBC × (10^6 /uL)	9.28	± 0.31	9.64	± 0.26	8.83	± 0.73	8.76	± 1.09
HGB (g/dL)	14.80	± 0.32	14.65	± 1.24	16.03	± 0.95	15.35	± 2.06
HCT (%)	45.20	± 1.79	47.33	± 1.85	42.53	± 3.53	41.68	± 4.85
MCV (fL)	48.70	± 0.75	49.08	± 0.64	48.38	± 0.31	47.63	± 0.36
MCHC (g/dL)	32.73	± 1.07	32.35	± 1.53	36.10	± 2.03	36.37	± 2.16
Males								
RBC × (10^6/uL)	9.65	± 0.71	9.71	± 0.67	9.15	± 0.33	8.32	± 0.96
HGB (g/dL)	15.94	± 0.67	16.04	± 1.09	15.95	± 0.35	14.75	± 1.63
HCT (%)	46.34	± 2.61	46.74	± 2.68	43.70	± 1.13	39.80	± 5.66
MCV (fL)	48.08	± 1.19	48.16	± 0.86	47.80	± 0.57	47.75	± 1.34
MCHC (g/dL)	34.40	± 0.65	34.38	± 1.54	36.60	± 0.00	37.05	± 1.20

Values are expressed as the mean ± standard error (SEM), *n* = 5; RBC = Red blood cells; HGB = Hemoglobin; HCT = Hematocrit: MVC = Mean corpuscular volume; MCHC = Mean corpuscular hemoglobin concentration. [a]A group was given UA/OA 6.5 mg/kg; [b]A group was given UA/OA 13 mg/kg.

The results of blood chemistry parameters show that the values of total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides do not present any significant difference between the treated and the control groups (Table 5), indicating that the UA/OA mixture did not cause any alteration in lipid metabolism. Similarly, the glucose levels present no significant difference between the two groups, which indicates that the mixture of compounds did not induce any alteration in carbohydrate metabolism (Table 5). There was no significant difference in levels of uric acid or creatinine, which indicates that the kidney function was not altered. Finally, there was no significant difference between the treated and control groups in relation to serum glutamic oxaloacetic transaminase (SGOT) or serum glutamic pyruvic transaminase (SGPT), indicating a lack of liver damage by the treatment.

To complete the subacute toxicity study, a histological examination was performed on the spleen, kidney, liver, heart, stomach, intestine, skin and lung from UA/OA-treated mice given the 13-mg/kg dose. Spleen slides of mice treated with the UA/OA mixture (Figure 2) demonstrated normal splenic architecture with normal lymphoid follicles and sinuses. These observations corroborate that the mixture of triterpenes did not produce alterations at the hematological level, because the RBC and WBC values were not affected.

Kidney sections from animals treated with the triterpenoids mixture (Figure 2) showed a normal renal cortex with the normal appearance of glomerulus, tubules and renal papilla. Normal renal function was corroborated by the fact that the values of the uric acid and creatinine markers were not different between the study groups (Carvalho *et al*., 2011). Liver sections showed normal hepatic

architecture, hepatocytes, portal triad and central vein (Figure 2). These observations, combined with the serum levels of the transaminases evaluated, are employed to establish the lack of hepatocellular damage. These parameters are essential for recognizing liver damage induced by xenobiotics (Carvalho *et al*., 2011; Silva *et al*., 2011).

Table 4
Differential white blood cell count of mice with the regimen of subacute toxicity of UA/OA

	Group			
	Control	Vehicle	UA/OA[a]	UA/OA[b]
Females				
WBC (x× 10^3/uL)	3.53 ± 1.37	2.73 ± 0.89	5.08 ± 2.52	2.70 ± 1.59
Lymphocytes (%)	84.50 ± 6.61	82.25 ± 12.76	87.50 ± 5.26	85.00 ± 5.16
Neutrophils (%)	13.00 ± 5.03	16.75 ± 11.35	12.50 ± 5.26	13.25 ± 4.11
Eosinophils (%)	0.50 ± 1.00	1.00 ± 2.00	0.50 ± 1.00	1.75 ± 2.06
Monocytes (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Basophils (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Males				
WBC (× 10^3/uL)	6.52 ± 1.47	5.80 ± 3.11	5.20 ± 1.98	2.80 ± 1.41
Lymphocytes (%)	78.00 ± 2.58	81.10 ± 6.23	80.50 ± 4.95	78.50 ± 3.54
Neutrophils (%)	20.50 ± 5.97	18.25 ± 2.36	17.00 ± 2.83	19.00 ± 9.90
Eosinophils (%)	0.40 ± 0.89	0.80 ± 1.10	2.50 ± 0.71	2.20 ± 0.00
Monocytes (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Basophils (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Values are expressed as the mean ± standard error (SEM), *n* = 5; UA = Ursolic acid; OA = Oleanolic acid; WBC = White blood cells; [a]A group was given UA/OA 6.5 mg/kg; [b]A group was given UA/OA 13 mg/kg.

Another organ that analyzed histologically was the heart. In this case, the slides showed normal endocardium and myocardium, as well as normal cardiac muscle architecture. Stomach and intestine sections showed normal mucosa, submucosa, and circular and longitudinal muscles layers. Skin sections exhibited normal epidermis, dermis, stratum corneum, hair follicles, and sebaceous glands, while the lung section demonstrated normal alveolar geometry and normal appearing alveolar septum (Figure 2).

The histological analysis and the hematological and biochemical parameters are essential for an evaluation of the toxicological risk of substances with biological activity, because any changes in one of these parameters in animal studies can offer a high predictive value of possible damage in humans (Olson *et al*., 2000; Silva *et al*., 2011).

Medicinal plants and their derivatives have been employed as an alternative to allopathic medicine in many countries. Despite their widespread use, few scientific studies have been undertaken to ascertain the safety and efficacy of traditional remedies or pure compounds (Silva *et al*., 2011). In the case of TB, it is well known that two of the main antituberculosis drugs (R and I) cause hepatotoxicity (Saravanan and Pugalendi, 2006; Yogeeta *et al*., 2007), an effect that has an influence on treatment adherence. Therefore, an analysis of the toxic effect of the possible antituberculosis drugs should be carried out prior to clinical trials. In the case of the UA/OA mixture, it has been reported that both compounds exert a hepatoprotector effect on *in vivo* models after hepatic damage is induced with ethanol (Saravanan and Pugalendi, 2006), acetaminophen, CCl_4, or D-galactosamine, among others (Liu, 1993; 1995; Lezhi and Xintang, 1992). Likewise, it has been described that OA offers a hepatoprotector effect in patients with tuberculosis who are treated with antituberculosis drugs (Ge *et al*., 2010). Thus, in patients with TB this UA/OA mixture could offer a beneficial hepatoprotector and antimycobacterial effect.

Table 5
Blood chemistry values of mice with the regimen of subacute toxicity of UA/OA

	Group			
	Control	Vehicle	UA/OA[a]	UA/OA[b]
Females				
Uric acid (mg/dL)	1.46 ± 0.11	1.44 ± 0.11	1.50 ± 0.12	1.47 ± 0.57
Creatinine (mg/dL)	1.78 ± 0.09	1.86 ± 0.09	1.86 ± 0.14	1.81 ± 0.11
Triglycerides (mmol/L)	0.98 ± 0.13	0.94 ± 0.13	0.96 ± 0.15	0.74 ± 0.05
HDL cholesterol (mmol/L)	8.12 ± 0.23	8.24 ± 0.23	8.24 ± 0.38	8.28 ± 0.18
Total cholesterol (mmol/L)	10.60 ± 0.45	10.80 ± 0.45	10.80 ± 0.45	10.40 ± 0.55
SGPT (U/L)	32.60 ± 2.51	32.40 ± 2.51	32.60 ± 2.41	35.60 ± 1.52
SGOT (U/L)	117.60 ± 10.13	120.20 ± 10.13	118.60 ± 9.29	124.20 ± 8.73
Glucose (mg/dL)	373.60 ± 24.39	356.80 ± 24.39	374.67 ± 26.08	363.67 ± 18.01
Males				
Uric acid (mg/dL)	1.18 ± 0.16	1.20 ± 0.19	1.26 ± 0.18	1.16 ± 0.21
Creatinine (mg/dL)	1.75 ± 0.09	1.76 ± 0.05	1.71 ± 0.09	1.81 ± 0.05
Triglycerides (mmol/L)	0.68 ± 0.08	0.68 ± 0.08	0.74 ± 0.09	0.72 ± 0.08
HDL cholesterol (mmol/L)	10.82 ± 0.57	10.24 ± 0.47	10.48 ± 0.38	10.54 ± 0.32
Total cholesterol (mmol/L)	13.80 ± 0.84	14.40 ± 1.52	13.20 ± 0.84	13.80 ± 0.45
SGPT (U/L)	34.00 ± 3.00	36.20 ± 2.17	33.60 ± 2.19	34.40 ± 1.67
SGOT (U/L)	128.80 ± 15.99	129.80 ± 11.01	123.00 ± 14.27	121.80 ± 18.51
Glucose (mg/dL)	423.80 ± 12.58	421.80 ± 13.48	420.40 ± 12.66	425.40 ± 16.32

Values are expressed as the mean ± standard error (SEM), $n = 5$; UA = Ursolic acid; OA = Oleanolic acid SGPT = Serum glutamic pyruvic transaminase; SGOT = Serum glutamic oxaloacetic transaminase. [a]A group was given UA/OA 6.5 mg/kg; [b]A group was given UA/OA 13 mg/kg.

CONCLUSIONS

The mixture of UA/OA is practically innocuous when its toxicity is evaluated by the s.c. route in acute (LD_{50} > 300 mg/kg) and subacute (13 mg/kg repeated-dose, 28-day toxicity) administration in Balb/c mice. Chronic toxicity, genotoxicity, and mutagenesis studies are required for further support concerning the safe use of these compounds.

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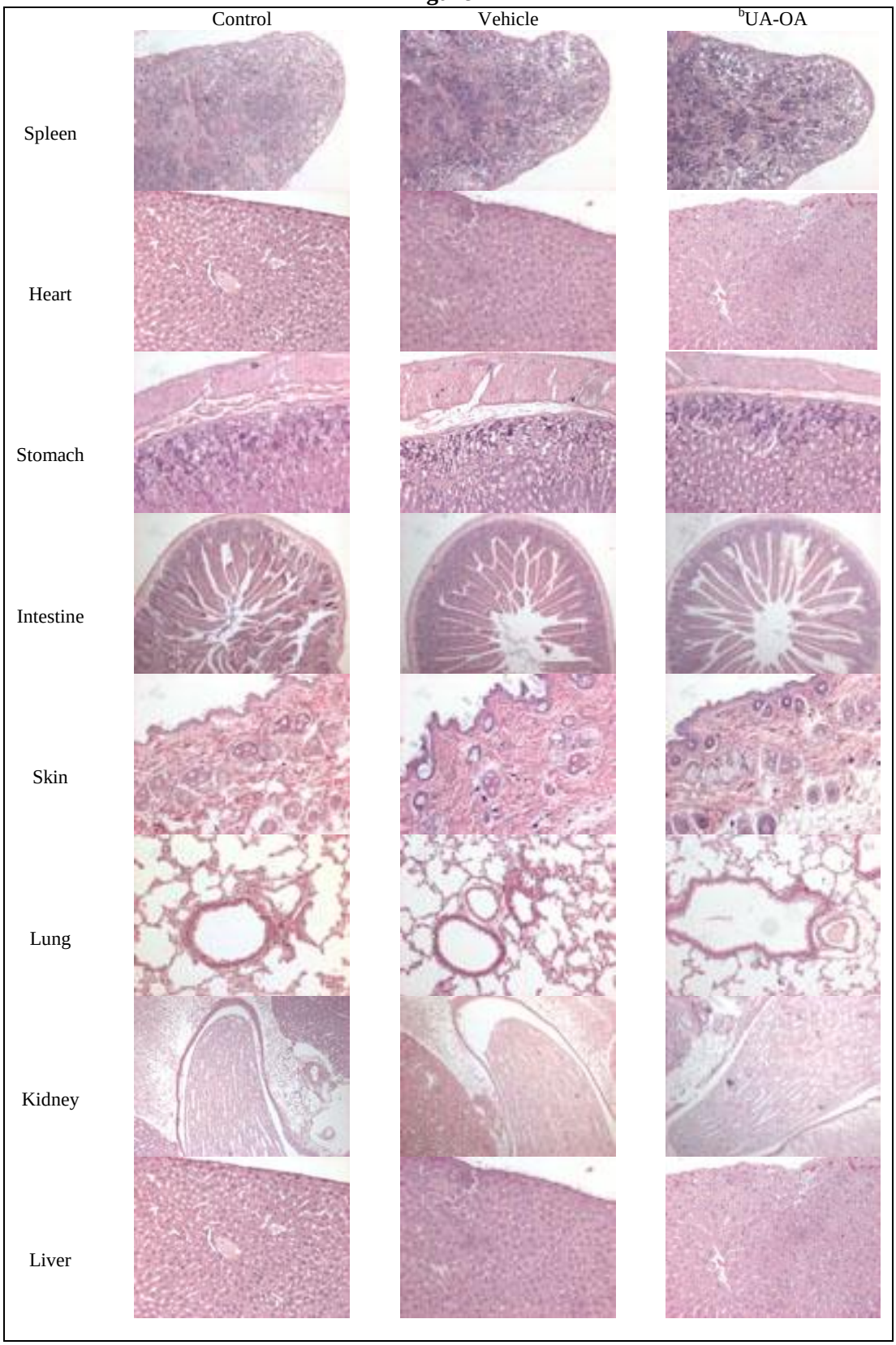
Figure 2


Control
Vehicle
[b]UA-OA
Spleen
Heart
Stomach
Intestine
Skin
Lung
Kidney
Liver

Histologic specimens of tissues (spleen- x100, heart- x100, stomach- x100, intestine- x40, skin- x100, lung- x100, kidney- x40 and liver- x100) collected from mice euthanized on day 28, stained with H&E. UA = Ursolic acid; OA = Oleanolic acid. [b]A group was given UA-OA 13 mg/kg.

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