

Toxicological Evaluation of the Hydroethanolic Seed Extract of *Randia aculeata* (Rubiaceae) in Acute and Sub-acute Oral Toxicity Models

Evaluación toxicológica del extracto hidroetanólico de semillas de *Randia aculeata* (Rubiaceae) en modelos de toxicidad oral aguda y subaguda

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ABSTRACT

Randia aculeata seeds are widely used in traditional medicine; however, information regarding their toxicological safety remains limited. The present study evaluated the acute and subacute toxicity of a hydroethanolic extract of *R. aculeata* seeds in Wistar rats. Acute oral toxicity was assessed following OECD guideline recommendations, while subacute toxicity was evaluated through daily oral administration of the extract at doses of 250, 500, and 1,000 mg/kg body weight for 28 days. Clinical signs, body weight variation, hematological and biochemical parameters, and histopathological changes in liver and kidney tissues were analyzed. No mortality was observed during the acute toxicity assay at doses up to 5,000 mg/kg, indicating that the median lethal dose is greater than this value. Nevertheless, treated animals showed clinical signs of toxicity and significant alterations in liver- and kidney-related biochemical markers. Histopathological examination revealed focal hepatic necrosis and tubular renal damage in treated groups, particularly at higher doses. These findings suggest that although *R. aculeata* seed extract exhibits low acute lethality, repeated exposure may induce hepatotoxic and nephrotoxic effects. The results highlight the need for caution regarding the uncontrolled consumption of *R. aculeata* seeds and support the importance of dose standardization and further toxicological evaluation before considering any therapeutic use.

Keywords: Traditional medicine; Safety assessment; Phenolic compounds; Histopathology.

RESUMEN

Las semillas de *Randia aculeata* son utilizadas de manera tradicional con fines medicinales; sin embargo, la información disponible sobre su seguridad toxicológica es limitada. El presente estudio evaluó la toxicidad aguda y subaguda de un extracto hidroetanólico de semillas de *R. aculeata* en ratas Wistar. La toxicidad aguda se determinó conforme a las

directrices de la OECD, mientras que la toxicidad subaguda se evaluó mediante la administración oral diaria del extracto a dosis de 250, 500 y 1,000 mg/kg de peso corporal durante 28 días. Se analizaron signos clínicos, variaciones en el peso corporal, parámetros hematológicos y bioquímicos, así como cambios histopatológicos en hígado y riñón. No se registró mortalidad en el ensayo de toxicidad aguda hasta la dosis de 5,000 mg/kg, lo que indica que la DL50 es superior a este valor. No obstante, se observaron signos clínicos de toxicidad y alteraciones significativas en biomarcadores hepáticos y renales. El análisis histopatológico evidenció necrosis focal hepática y daño tubular renal en los grupos tratados, especialmente a dosis elevadas. En conjunto, los resultados sugieren que, aunque el extracto presenta baja letalidad aguda, la exposición repetida puede generar efectos hepatotóxicos y nefrotóxicos, lo que subraya la necesidad de un uso cauteloso y de una adecuada estandarización de dosis.

Palabras clave: Medicina tradicional; Evaluación de seguridad; Compuestos fenólicos; Histopatología.

INTRODUCTION

Ethnomedicine, the traditional use of plants and their bioactive constituents for disease prevention and therapy, has garnered renewed scientific attention in recent decades due to its accessibility, affordability, and perceived safety compared to synthetic drugs. Medicinal plants synthesize a wide array of secondary metabolites, including alkaloids, flavonoids, saponins, terpenoids, steroids, glycosides, tannins, and volatile oils that function as natural defenses against environmental stressors and frequently exhibit pharmacological activities of therapeutic relevance (Jamshidi-Kia *et al.*, 2018). Many clinically important drugs were originally derived from such plant metabolites. Nevertheless, herbal remedies are often consumed empirically and without rigorous toxicological evaluation, a practice that can pose significant health risks (Anand *et al.*, 2019).

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In Mexico, *Randia aculeata* L. (Rubiaceae), commonly known as “crucetillo,” is a spiny shrub or small tree native to the Americas and widely used in traditional medicine. Several parts of the fruit, including seeds, are traditionally consumed as aqueous infusions or macerates, and in some cases the crushed or raw seeds are ingested directly. These preparations are employed as antivenoms and as adjunct treatments for chronic conditions of high prevalence, such as diabetes, cancer, inflammation, and pain (Gallardo-Casas *et al.*, 2012). Importantly, the doses used in these traditional practices are highly variable, poorly standardized, and often based on empirical knowledge rather than measured quantities, which complicates safety assessment and increases the potential risk associated with repeated or prolonged consumption (Jamshidi-Kia *et al.*, 2018).

Phytochemical investigations of *Randia* species have identified a variety of bioactive compounds, including 1-nitropyrene, linoleic and palmitic acids, sterols, β -sitosterol, campesterol, oleanolic acid and its acetate, 3- α -L-arabinoside, and mesembriantemoidigenic acid, which have been associated with anti-inflammatory, antioxidant, and antimicrobial activities (Cano-Campos *et al.*, 2011; Cuevas-Juárez *et al.*, 2014; Kandimalla *et al.*, 2016; Juárez-Trujillo *et al.*, 2022). Recent studies have shown that different parts of the fruit seeds, pulp, and shell exhibit diverse biological activities, including anti-inflammatory effects, acaricidal activity against the cattle tick *Rhipicephalus microplus*, antimycobacterial properties, and partial inhibition of venom-induced necrosis in skeletal and cardiac muscle (Pérez-Espinosa *et al.*, 2003; Chaowuttikul *et al.*, 2020; Bravo-Ramos *et al.*, 2023). Despite these reported beneficial effects, several *Randia* species have also been associated with toxic outcomes, particularly affecting organs involved in xenobiotic metabolism such as the liver and kidneys (Juárez-Trujillo *et al.*, 2022). Moreover, although formal clinical documentation is limited, adverse effects, including gastrointestinal discomfort, nausea, and symptoms suggestive of hepatic or renal dysfunction, have been anecdotally reported with the use of *Randia* spp. and other members of the Rubiaceae family used as folk remedies.

Given the high phenolic and triterpenoid content described in *R. aculeata* seeds, excessive or repeated exposure may promote oxidative stress and redox imbalance, mechanisms known to contribute to hepatocellular and tubular epithelial injury (Stickel and Shouval, 2015). Despite its recognized therapeutic potential, oral toxicity studies of *R. aculeata* remain scarce, and virtually no data are available on the safety of its seed extract. This gap is particularly relevant because the seeds contain high concentrations of lipophilic triterpenoids and other metabolites that may accumulate in tissues, potentially producing systemic toxicity. Moreover, the fruit is frequently consumed in large quantities and over prolonged periods by the Mexican population, often without medical supervision (Gallardo-Casas *et al.*, 2012; Juárez-Trujillo *et al.*, 2022). Animal toxicity testing provides essential data to determine safe exposure levels of natural products and to identify target organs susceptible to adverse effects

(Dunnick and Nyska, 2013). Evaluating the seed specifically is especially important, as it is likely to harbor the most potent and potentially the most toxic bioactive constituents.

For these reasons, the present study aimed to assess the toxicological profile of the hydroalcoholic extract of *R. aculeata* seeds (EHRA) in rats using both acute (single-dose) and subacute (28-day repeated-dose) oral toxicity models. Establishing the systemic safety of this traditionally used plant represents a necessary step toward evidence-based recommendations for its medicinal use and the potential development of standardized herbal formulations.

MATERIAL AND METHODS

Botanical Material and Extract Preparation

The fruits of *R. aculeata* were obtained from a local market in Veracruz, Mexico. Their taxonomic identification was verified by a specialist from the Herbarium of the Facultad de Ciencias Biológicas y Agropecuarias, Universidad Veracruzana, who assigned the registration number JLB2 (VER). After collection, fruits were thoroughly washed, and their different components were separated to isolate the seeds which were air-dried at room temperature and ground using an electric laboratory mill (IKA®, Germany). The powdered material was extracted using a hydroethanolic solvent (ethanol: water, 70:30 v/v) at a solid-to-solvent ratio of 1:10 (w/v). Extraction was performed by maceration for 72 h at room temperature with periodic agitation. The extract was filtered through Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator (Büchi®, Switzerland) at 40 °C to remove the solvent. The resulting dry extract was stored at 4 °C until use and reconstituted in distilled water immediately before administration (Bravo-Ramos *et al.*, 2023).

Experimental Animals

Wistar rats (*Rattus norvegicus*) of both genders, aged between 45 and 60 d and weighing 130.5 ± 5.5 g, were obtained from a commercial vivarium. The animals were housed in ventilated cages in a room with a temperature of 25 ± 2 °C and a 12-h light/dark cycle. They were acclimatized to the laboratory conditions for 5 d before the experiments and had access to food pellets and water *ad libitum*. All experimental procedures were conducted in accordance with ethical guidelines and were approved by the Ethics Commission of the Facultad de Medicina Veterinaria y Zootecnia, Universidad Veracruzana (protocol No 07/2021).

Acute Toxicity Study

The acute toxicity assessment was carried out following OECD Test Guideline 425 (Acute Oral Toxicity – Up-and-Down Procedure) (OECD, 2008). Female Wistar rats were used exclusively in this assay, as recommended by the OECD guidelines, since females are generally more sensitive to the toxic effects of chemical substances, allowing a conservative evaluation of acute toxicity. A total of twelve female rats were randomly assigned into two groups ($n = 6$ per group). The EHRA was administered orally via intragastric gavage. On the first day,

a single dose of 2,000 mg/kg body weight was administered to one rat. After a 48-hour observation period, the same dose was administered to five additional females, resulting in six treated animals in total. The control group received an equivalent volume of distilled water by the same intragastric route. Although the extract was prepared using a hydroethanolic solvent, ethanol was completely removed during the concentration process under reduced pressure. Therefore, the dried extract was reconstituted in distilled water prior to administration, which justified the use of distilled water as the vehicle control.

Animals were closely monitored during the first four hours following administration, with special attention during the first 30 minutes, and were subsequently observed daily for a total period of 14 days. Clinical signs of toxicity, changes in physical appearance, and alterations in general behavior were recorded in accordance with the guideline. At the end of the observation period, animals were fasted overnight and anesthetized using inhaled sevoflurane until complete loss of reflexes. Blood samples were collected via cardiac puncture and processed to obtain serum for biochemical analysis and whole blood for hematological parameters, after which euthanasia was performed by intracardiac administration of a lethal dose of sodium pentobarbital. Following euthanasia, major organs were excised, examined macroscopically during necropsy, and preserved for subsequent histopathological analysis.

Subacute Toxicity Test

The subacute toxicity evaluation was conducted in accordance with OECD Test Guideline 407 (Repeated Dose 28-Day Oral Toxicity Study in Rodents) (OECD, 2008). A total of twenty rats of each sex were randomly assigned to four experimental groups. Three of these groups received daily oral doses of the EHRA extract via intragastric gavage at concentrations of 250 mg/kg (G1), 500 mg/kg (G2), and 1000 mg/kg (G3) for 28 consecutive days. The control group (G4) was administered distilled water under the same conditions. Throughout the experimental period, the animals were carefully monitored for any physical or behavioral alterations. At the end of the 28-day treatment, animals were processed following the same procedures described for the acute toxicity study.

Necropsy and Histopathological Analysis

Necropsy was performed on all experimental groups, including both macroscopic and microscopic evaluations of vital organs to identify any potential abnormalities. The heart, liver, kidneys, lungs, spleen, ovaries, uterus, epididymis, and testes were carefully removed, rinsed with cold saline solution, blotted dry, and weighed to determine their absolute organ weights (in grams). The relative organ weight (ROW) for each animal was then calculated using the following formula:

ROW = (Absolute organ weight in grams / Body weight on the day of sacrifice in grams) × 100.

Histopathological assessment was carried out using a semi-quantitative scoring system. Liver and kidney tissue sections (6-µm thick, stained with H&E) were examined for necrosis, inflammation, and cellular degeneration, using a 0–4 scale (0 = absent; 1 = minimal <10%; 2 = mild 10–25%; 3 = moderate 26–50%; 4 = severe >50% of the parenchyma affected) (Dibal *et al.*, 2002). For each sample, five randomly selected, non-overlapping fields were analyzed at 400× magnification from defined anatomical regions. All histological evaluations were conducted blindly by a trained pathologist.

Biochemical and Hematological Analysis

Serum samples were analyzed using the Cobas Integra 400 Plus automatic biochemical analyzer (manufactured by Roche, Germany). The analysis included clinical biochemistry parameters such as total cholesterol, triglycerides, low-density lipoprotein-cholesterol, high-density lipoprotein-cholesterol, glucose, total serum protein, albumin, total bilirubin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, uric acid, urea, and creatinine. Serum electrolytes, including calcium, sodium, potassium, and chlorine, were also determined. Hematological analyses were performed using the XT-4000i analyzer, measuring parameters such as corpuscular volume, mean corpuscular hemoglobin concentration, mean corpuscular hemoglobin, white blood cell count, neutrophils, eosinophils, basophils, lymphocytes, monocytes, and platelet count.

Statistical analysis

Student's t-test was used to analyze the information obtained in the acute toxicity experiment. Statistical significances between control and treated groups in the subacute experiments were determined by one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test. STATISTICA version 12.0 was used for all statistical analyses. Data analyses from both gender groups were analyzed separately. All results are shown as the mean ± standard deviation (SD). Differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

The EHRA evaluated for acute and subacute oral toxicity has been previously characterized by our group. HPLC/MS analysis revealed twelve identified and quantified constituents (µg g⁻¹ plant material): chlorogenic acid (2654.87 ± 22.4), vanillic acid (45.5 ± 2.9), p-coumaric acid (34.5 ± 4.5), caffeic acid (232.4 ± 5.6), rutin (34.6 ± 4.3), quercetin (0.9 ± 2.5), epicatechin (2.3 ± 0.5), 4-hydroxybenzoic acid (1.9 ± 0.45), vanillin (15.4 ± 3.2), 2,4-dimethoxy-6-methyl benzoic acid (2.5 ± 0.9), scopoletin (45.6 ± 3.4), and ferulic acid (13.4 ± 1.2) (Bravo-Ramos *et al.*, 2023). The same phytochemically characterized extract was used in the present toxicity assays.

Acute toxicity study

No mortality was observed in any of the treated animals at the highest administered dose during the 14-day observation period. According to the criteria established in the OECD

Guideline 425 (OECD, 2008), for oral acute toxicity, when no deaths occur at the limit dose, the median lethal dose (LD₅₀) cannot be calculated and is therefore considered to be greater than the highest tested dose. Based on these guidelines, the LD₅₀ of the *Randia aculeata* seed extract was determined to be greater than 5,000 mg/kg body weight.

During the experimental period, the treated group did not show significant changes in body weight. However, there were observed changes in the absolute organ weight of the liver, indicating a potential effect of the extract on liver physiology (Table 1). Clinical signs of toxicity were observed within the first 24 hours following administration of the extract. These signs included reduced locomotor activity, piloerection, transient anorexia, mild tremors, and occasional lethargy. No severe distress, convulsions, or mortality were observed during the observation period. All clinical signs were transient and decreased in intensity after the first 48 hours. Biochemical analysis revealed an increase in the activity of liver enzymes such as AST, ALT, and ALP in the treated group compared to the control group ($p < 0.005$), indicating possible liver damage (Table 2). Hematological analysis showed a significant reduction in the values of red

blood cells (RBC) and hemoglobin (Hb) in the treated group compared to the control group ($p < 0.05$), suggesting an adverse effect on blood parameters (Table 2). Histopathological analysis of the liver revealed focal hepatocellular necrosis accompanied by mild inflammatory infiltrates, indicating structural damage to hepatic tissue in treated animals (Fig. 1). These alterations support the biochemical evidence of liver toxicity induced by the extract. In addition, histopathological examination of kidney tissue showed evidence of renal injury in treated animals, characterized by tubular epithelial degeneration, tubular dilation, and focal areas of acute tubular necrosis, particularly at higher doses (Fig. 2). No comparable alterations were observed in the kidneys of control animals. Overall, these findings indicate that acute administration of *R. aculeata* seed extract at high doses can induce structural damage in both liver and kidney tissues, which is consistent with the alterations observed in hematological and biochemical parameters.

Subacute toxicity test

The second experimental assay involved the daily oral administration of the hydroethanolic extract of EHRA at doses of

Table 1. Organ weights and relative organ weights (g/100 g of body weight) of rats treated orally with hydroethanolic extract of *R. aculeata* seeds.

Tabla 1. Pesos de órganos y pesos relativos de órganos (g/100 g de peso corporal) en ratas tratadas por vía oral con el extracto hidroetanólico de semillas de *R. aculeata*.

Genus/ Organs		Acute toxicity		Subacute toxicity			
		Control	2,000 mg/kg/bw	Control	250 mg/kg/bw	500 mg/kg/bw	1,000 mg/kg/bw
Female							
Kidney	g	0.68 ± 0.06	0.70 ± 0.05	0.72 ± 0.03	0.45 ± 0.03*	0.41 ± 0.04*	0.39 ± 0.02*
	g/100 g	0.55 ± 0.03	0.56 ± 0.04	0.32 ± 0.04	0.33 ± 0.05	0.36 ± 0.02	0.34 ± 0.01
Liver	g	10.2 ± 0.5	14.2 ± 0.5*	9.34 ± 0.4	12.5 ± 0.4*	13.1 ± 0.03*	14.3 ± 0.5*
	g/100 g	6.1 ± 0.4	5.9 ± 0.5	4.19 ± 0.5	4.32 ± 0.05	4.54 ± 0.02	4.76 ± 0.01
Spleen	g	0.35 ± 0.03	0.38 ± 0.05	0.42 ± 0.03	0.48 ± 0.03	0.39 ± 0.02	0.37 ± 0.04
	g/100 g	0.23 ± 0.4	0.24 ± 0.03	0.33 ± 0.05	0.29 ± 0.06	0.29 ± 0.04	0.32 ± 0.03
Heart	g	0.69 ± 0.03	0.72 ± 0.03	0.65 ± 0.04	0.70 ± 0.05	0.69 ± 0.03	0.72 ± 0.01
	g/100 g	0.54 ± 0.02	0.56 ± 0.02	0.56 ± 0.06	0.54 ± 0.03	0.57 ± 0.05	0.62 ± 0.06
Lung	g	1.34 ± 0.04	1.32 ± 0.03	1.19 ± 0.04	1.22 ± 0.04	1.24 ± 0.04	1.32 ± 0.05
	g/100 g	0.89 ± 0.02	0.91 ± 0.02	0.93 ± 0.03	0.87 ± 0.02	0.87 ± 0.05	0.86 ± 0.04
Ovary	g	0.04 ± 0.01	0.05 ± 0.0	0.05 ± 0.01	0.03 ± 0.01	0.04 ± 0.0	0.02 ± 0.0
	g/100 g	0.02 ± 0.01	0.03 ± 0.0	0.03 ± 0.0	0.02 ± 0.0	0.02 ± 0.01	0.04 ± 0.02
Uterus	g	0.34 ± 0.04	0.37 ± 0.05	0.33 ± 0.02	0.34 ± 0.03	0.38 ± 0.03	0.42 ± 0.02
	g/100 g	0.23 ± 0.02	0.24 ± 0.05	0.34 ± 0.01	0.29 ± 0.04	0.33 ± 0.02	0.31 ± 0.03
Male							
Kidney	g	-	-	1.51 ± 0.04	0.74 ± 0.02	0.78 ± 0.03*	0.72 ± 0.02
	g/100 g	-	-	0.64 ± 0.03	0.68 ± 0.03	0.65 ± 0.02	0.72 ± 0.05
Liver	g	-	-	11.22 ± 0.3	13.91 ± 0.05*	13.98 ± 0.4*	14.53 ± 0.3
	g/100 g	-	-	5.42 ± 0.5	5.81 ± 0.03	5.75 ± 0.04	6.12 ± 0.3
Spleen	g	-	-	0.53 ± 0.03	0.48 ± 0.04	0.54 ± 0.05	0.49 ± 0.05
	g/100 g	-	-	0.28 ± 0.02	0.24 ± 0.05	0.25 ± 0.03	0.22 ± 0.05
Heart	g	-	-	1.17 ± 0.4	1.21 ± 0.04	1.32 ± 0.05	1.30 ± 0.02
	g/100 g	-	-	0.76 ± 0.06	0.81 ± 0.06	0.79 ± 0.02	0.78 ± 0.03
Lung	g	-	-	1.73 ± 0.2	1.78 ± 0.05	1.82 ± 0.03	1.72 ± 0.04
	g/100 g	-	-	0.69 ± 0.04	0.71 ± 0.02	0.72 ± 0.03	0.74 ± 0.02
Epididymis	g	-	-	0.42 ± 0.02	0.44 ± 0.03	0.47 ± 0.05	0.44 ± 0.06
	g/100 g	-	-	0.14 ± 0.01	0.16 ± 0.03	0.21 ± 0.02	0.18 ± 0.05
Testis	g	-	-	1.29 ± 0.03	1.27 ± 0.03	1.35 ± 0.03	1.37 ± 0.05
	g/100 g	-	-	0.45 ± 0.05	0.47 ± 0.04	0.42 ± 0.03	0.48 ± 0.03

Values expressed as mean ± S.D.; *P < 0.05 compared with the control group. Student t-test for acute toxicity and ANOVA/Dunnett's test for short-term toxicity

Table 2. Hematological and biochemical parameters were altered after acute administration of the hydroethanolic extract of *R. aculeata* seed 2,000 mg/kg in female rats.

Tabla 2. Parámetros hematológicos y bioquímicos evaluados en ratas hembra en el ensayo de toxicidad aguda (2,000 mg/kg) del extracto hidroetanólico de semillas de *R. aculeata*.

Parameters	Control	2,000 mg/kg
AST (UI/L)	134.7 ± 8.9	205.5 ± 5.6*
ALT (UI/L)	54.6 ± 5.3	102.2 ± 4.8*
ALP (UI/L)	178.7 ± 6.2	185.5 ± 3.9*
Total bilirubin (mg/dL)	0.36 ± 0.2	0.74 ± 0.2*
HGB (g/dL)	13.2 ± 1.5	8.4 ± 0.7*
RBC (x10 ⁶ /μL)	6.75 ± 0.9	4.2 ± 0.4

Data are expressed as mean ± standard deviation (SD). *Indicates statistically significant differences compared with the control group ($p < 0.05$). Statistical analysis was performed using Student's t-test for normally distributed data and the Mann-Whitney U test for non-normally distributed data.

250, 500, and 1,000 mg/kg body weight for a duration of 28 d consecutive. Dose selection was based on the absence of mortality observed in the acute toxicity study, and followed a standard fractionation approach commonly used in repeated-dose toxicity assays, allowing the evaluation of potential cumulative and dose-related toxic effects while avoiding excessive systemic toxicity. The results of this study indicated that EHRA administration induced clinical signs of toxicity, including hyperactivity, asthenia, and piloerection within the first 24 hours of treatment. These signs persisted during the first three days of the experimental period and gradually subsided thereafter. No mortality was recorded throughout the study.

Although no significant changes in body weight were observed among treated groups, alterations were detected in the absolute organ weights of liver and kidneys in both male and female rats receiving all tested doses during the experimental period, suggesting a treatment-related effect on these organs. Biochemical analysis revealed that EHRA

administration at all tested doses resulted in a significant increase in liver function markers, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and total bilirubin (Table 3). In addition, significant elevations were observed in blood urea nitrogen (BUN), creatinine, and electrolyte levels (Na^+ and K^+) compared with the control group ($p < 0.05$), indicating impaired hepatic and renal function. Hematological evaluation showed that rats of both sexes treated with EHRA at all evaluated doses, exhibited significant reductions in red blood cell count (RBC) and hemoglobin (HB) levels relative to control animals ($p < 0.05$) (Table 4), suggesting an adverse effect on erythropoietic parameters. Other hematological variables remained within normal physiological ranges.

Histopathological analysis of liver tissue revealed the presence of necrotic areas in all treated groups, confirming structural hepatic damage (Fig. 2). Likewise, histopathological examination of kidney tissue demonstrated acute tubular necrosis in animals treated with all tested doses, providing morphological evidence of renal injury (Fig. 3); although the severity of lesions increased with dose, the qualitative pattern of tissue damage was consistent across treatment groups.

The most commonly reported toxicities associated with herbal products include hepatotoxicity, nephrotoxicity, cardiotoxicity, and, in severe cases, death. Many plants produce toxic substances as defense mechanisms, which can inadvertently affect humans, resulting in health damage and economic losses (Xie *et al.*, 2014). The presence of several compounds in EHRA likely contributes to most of its pharmacological activities. While these compounds are recognized for their potential pharmacological benefits, it's essential to consider that they may also have adverse effects on normal cells, especially with high consumption (Hussin, 2002; Di Lorenzo *et al.*, 2015; Restani *et al.*, 2016). As demonstrated by Du *et al.* (2013), high doses of chlorogenic acid induced inflammation and oxidative stress in rats, suggesting a shift towards pro-oxidant activity under certain conditions. Similarly, some

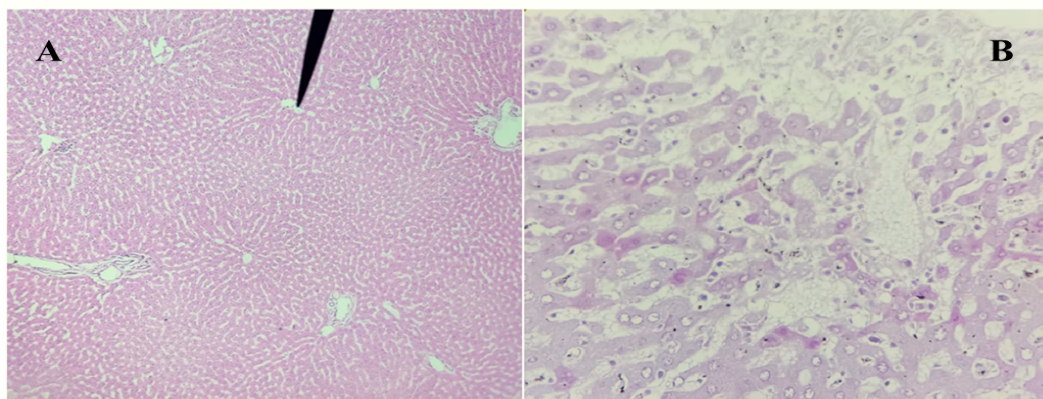


Figure 1. Photomicrograph of liver section from female rats in experimental control (A), and 2,000 mg/kg treated group (B); of acute toxicity test exhibiting diffuse and extensive coagulative necrosis involving the entire liver parenchyma. Hematoxylin and Eosin stain (10x and 40x).

Figura 1. Fotomicrografía de una sección hepática de ratas hembra del grupo control experimental (A) y del grupo tratado con 2,000 mg/kg (B); la prueba de toxicidad aguda muestra necrosis coagulativa difusa y extensa que afecta a todo el parénquima hepático. Tinción con hematoxilina y eosina (10x y 40x).

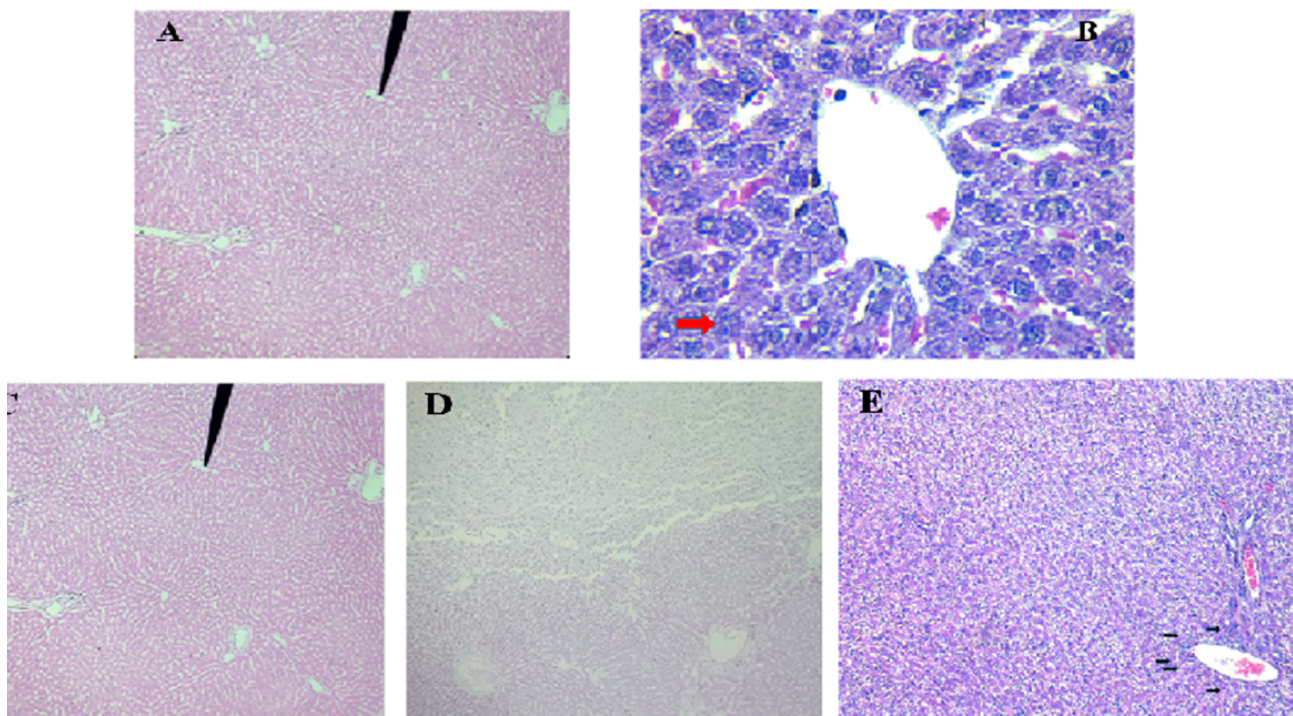


Figure 2. Representative photomicrographs of liver sections from female and male rats in the subacute toxicity study of the hydroethanolic extract of *Randia aculeata*. (A) Female control group showing normal hepatic architecture. (B) Female treated groups (250, 500, and 1000 mg/kg) exhibiting focal hepatocellular necrosis, binucleated hepatocytes (red arrow), and diffusely flocculent cytoplasm. (C) Male control group with preserved hepatic parenchyma. (D) Male treated groups (250, 500, and 1000 mg/kg) demonstrating diffuse and extensive coagulative necrosis involving large areas of the hepatic parenchyma. (E) Higher magnification of a male treated group showing necrosis of isolated hepatocytes, binucleation, and flocculent cytoplasm, more prominent in zone 3 adjacent to the centrilobular vein (black arrows). Hematoxylin and eosin stain; original magnifications 10x and 40x.

Figura 2. Fotomicrografías representativas de secciones de hígado de ratas hembras y machos en el estudio de toxicidad subaguda del extracto hidroetanólico de *Randia aculeata*. (A) Grupo de control hembra con arquitectura hepática normal. (B) Grupos de hembras tratadas (250, 500 y 1000 mg/kg) que exhiben necrosis hepatocelular focal, hepatocitos binucleados (flecha roja) y citoplasma floculento difuso. (C) Grupo de control macho con parénquima hepático preservado. (D) Grupos de machos tratados con 250, 500 y 1000 mg/kg que presentan necrosis coagulativa difusa y extensa que afecta grandes áreas del parénquima hepático. (E) Mayor aumento de un grupo de machos tratados que muestra necrosis de hepatocitos aislados, binucleación y citoplasma floculento, más prominente en la zona 3 adyacente a la vena centrolobulillar (flechas negras). Tinción de hematoxilina y eosina; aumentos originales de 10x y 40x.

studies suggest that chlorogenic acid has anticancer properties, while others indicate that this dietary constituent may cause DNA damage and induce carcinogenic effects, suggesting the double-edged sword property of chlorogenic acid as a drug (Li and Trush, 1994; Wang *et al.*, 2010; Ji *et al.*, 2013). By contrast, other studies have demonstrated protective effects of chlorogenic acid against acetaminophen-induced hepatotoxicity, but these were observed at moderate doses (Hu *et al.*, 2022; Rashidi *et al.*, 2022; Wei *et al.*, 2025). Caffeic acid has also been described as an antioxidant and anti-inflammatory agent, yet at high concentrations, it may disrupt redox balance and potentially aggravate hepatic damage (Espindola *et al.*, 2019; Maity *et al.*, 2022). Ferulic acid exhibits a comparable dual profile, being hepatoprotective under physiological conditions but capable of pro-oxidant activity when present in excess (Ahmadipour *et al.*, 2021; Pandi *et al.*, 2025). Another study found that quercetin administration in rats resulted in increased incidence of renal tubule cell adenomas, suggesting potential nephrotoxic effects at high doses (Hsieh *et al.*, 2010).

Taken together, these findings suggest that the high single dose administered in the acute toxicity test may have created a phenolic overload, leading to oxidative stress and localized hepatocellular injury (Bouayed and Bohn, 2010). This mechanism could account for the observed enzyme elevations, histological necrosis, and increased liver weight. The hematological changes detected in red blood cells and hemoglobin may represent a secondary effect of oxidative stress or damage to erythrocyte membranes, although further studies are needed to clarify this mechanism. Compared with the acute study, the subacute exposure highlights a progressive and dose-dependent organ toxicity, with the kidney showing evident damage in addition to the liver. These findings suggest that repeated exposure exacerbates the effects observed after a single high dose, extending the target-organ toxicity beyond the liver to include the kidneys (Luyckx and Naicker, 2008; De Oliveira *et al.*, 2011; Petejova *et al.*, 2019). On the other hand, in toxicological studies, changes in organ weight can serve as an important indicator of toxicity caused by both chemical and natural drugs (Balogun *et al.*, 2014).

Table 3. Biochemical parameters after subacute administration of hydroethanolic extract from *R. aculeata* seeds in rats.**Tabla 3.** Parámetros bioquímicos en ratas tras el ensayo de toxicidad subaguda con el extracto hidroetanólico de semillas de *Randia aculeata*.

Genus/ Variable	Control	250 mg/kg	500 mg/kg	1,000 mg/kg
Female				
AST (UI/L)	75.50 ± 7.79	145.22 ± 5.42*	154.19 ± 4.32*	243.56 ± 5.67*
ALT (UI/L)	33.58 ± 3.73	65.45 ± 4.56*	69.98 ± 6.53*	110.45 ± 3.56*
ALP (UI/L)	99.36 ± 6.47	134.23 ± 6.43*	142.45 ± 6.76*	156.09 ± 5.45*
Total bilirubin (mg/L)	0.16 ± 0.02	1.23 ± 0.05*	1.12 ± 0.03*	1.79 ± 0.03*
Total proteins (g/L)	6.37 ± 0.09	6.87 ± 1.21	6.54 ± 1.34	6.35 ± 1.12
Albumin (g/L)	4.23 ± 0.10	3.89 ± 1.52	3.65 ± 1.45	4.21 ± 1.67
Total cholesterol (g/L)	60.77 ± 5.50	64.45 ± 4.53	63.23 ± 3.49	62.71 ± 2.63
Triglycerides (g/L)	105.20 ± 4.50	107.56 ± 6.57	108.87 ± 5.67	106.65 ± 5.43
HDL cholesterol (g/L)	60.33 ± 4.43	58.65 ± 5.46	62.01 ± 4.30	61.15 ± 3.67
Glucose (g/dL)	107.00 ± 3.29	110.34 ± 4.54	108.56 ± 4.43	106.76 ± 45.67
Blood urea nitrogen (mg/dL)	33.02 ± 2.46	48.54 ± 3.65*	47.56 ± 6.43*	52.45 ± 5.94*
Creatinine (mg/L)	3.9 ± 0.67	6.12 ± 1.23*	6.78 ± 1.12*	6.67 ± 1.45*
Uric acid (mg/L)	12.72 ± 1.56	11.98 ± 2.45	12.87 ± 3.43	11.45 ± 3.45
Na ⁺ (mmol/L)	134.28 ± 1.60	156.45 ± 3.45*	166.4 ± 4.54*	165.23 ± 3.58*
K ⁺ (mmol/L)	5.02 ± 0.38	8.34 ± 2.34*	8.87 ± 1.23*	8.11.11 ± 1.87*
Ca ²⁺ (mg/L)	102.03 ± 3.34	103.45 ± 3.56	101.32 ± 2.26	102.34 ± 3.56
Cl ⁻ (mmol/L)	98.77 ± 2.40			
Male				
AST (UI/L)	142.42 ± 12.13	252.45 ± 6.32*	256 ± 4.56*	258.45 ± 4.62*
ALT (UI/L)	56.32 ± 6.25	78.65 ± 5.67*	78.45 ± 3.45*	88.67 ± 4.92*
ALP (UI/L)	189.27 ± 16.44	223.45 ± 4.5*	232 ± 6.5*	233 ± 6.8*
Total bilirubin (mg/L)	0.52 ± 0.22	2.55 ± 0.15*	2.45 ± 0.26*	2.87 ± 0.23*
Total proteins (g/L)	67.97 ± 4.11	68.65 ± 3.48	69.12 ± 5.54	68.43 ± 3.75
Albumin (g/L)	42.10 ± 5.39	41.32 ± 2.34	42.42 ± 4.32	42.54 ± 3.45
Total cholesterol (g/L)	0.64 ± 0.06	0.69 ± 0.14	0.66 ± 0.21	0.72 ± 0.32
Triglycerides (g/L)	0.72 ± 0.05	0.69 ± 0.06	0.74 ± 0.09	0.71 ± 0.07
HDL cholesterol (g/L)	0.28 ± 0.04	0.26 ± 0.03	0.30 ± 0.04	0.27 ± 0.05
Glucose (g/L)	124.43 ± 4.54	126.45 ± 3.45	126.32 ± 3.26	125.64 ± 4.31
Blood urea nitrogen (mg/dL)	47.00 ± 5.34	66.78 ± 4.56*	68.65 ± 3.35*	67.65 ± 5.43*
Creatinine (mg/L)	4.15 ± 0.90	7.53 ± 1.23	6.98 ± 0.76	7.98 ± 0.87
Uric acid (mg/L)	11.75 ± 1.54	10.74 ± 2.45	12.87 ± 3.76	12.11 ± 4.32
Na ⁺ (mmol/L)	137.75 ± 2.13	221.24 ± 6.78	219.45 ± 5.56	222.43 ± 4.5
K ⁺ (mmol/L)	5.06 ± 0.45	12.98 ± 2.45	12.36 ± 2.36	12.43 ± 1.23
Ca ²⁺ (mg/L)	99.47 ± 2.71	101.36 ± 3.56	99.32 ± 2.98	98.34 ± 4.32
Cl ⁻ (mmol/L)	97.53 ± 2.59	97.77 ± 2.32	98.34 ± 1.23	98.43 ± 2.1

Data are presented as mean ± standard deviation (SD). * Indicates a statistically significant difference versus the control group ($p < 0.05$), determined by one-way ANOVA followed by Dunnett's multiple comparison test.

In this study, rats of both genders treated with doses of 500 and 1,000 mg/kg exhibited a significant increase in the absolute weights of the liver and kidney. These changes, coupled with observed histopathological alterations, suggest toxicological significance, particularly at the medium and higher doses tested (Al-Medhtiy *et al.*, 2022; Gamde *et al.*, 2023). Considering that liver and kidney are crucial organs involved in the metabolism of foreign substances, and that they showed notable abnormalities in terms of absolute weight, it is plausible to hypothesize that EHRA could induce toxic effects on the vital organs of the treated groups in the subacute experiment. Previous toxicological assays have indicated that changes in hematopoietic system cells serve as a more reliable indicator for predicting human toxicity when

data is extrapolated from animal models (Olson *et al.*, 2000). In the subacute study conducted, significant alterations in red blood cells (RBC) and hemoglobin levels were observed. Various mechanisms could be responsible for liver cell damage, potentially resulting from the intake and accumulation of high concentrations of xenobiotics. It is hypothesized that this process increases the production of reactive oxygen species (ROS) and electrophilic metabolites, leading to glutathione (GSH) depletion within hepatocytes.

The consequent oxidative imbalance promotes covalent binding of reactive intermediates to membrane proteins, lipids, and nucleic acids, ultimately triggering hepatocyte necrosis (Jaeschke *et al.*, 2012; Gu and Manautou, 2012). This direct damage to the cell membrane leads to the rupture of

Table 4. Hematological parameters after subacute administration of hydroethanolic extract from *R. aculeata* seeds in rats.**Tabla 4.** Parámetros hematológicos en ratas tras el ensayo de toxicidad subaguda con el extracto hidroetanólico de semillas de *R. aculeata*.

Genus/ Variable	Control	250 mg/kg	500 mg/kg	1,000 mg/kg
Female				
Erythrocytes (x10 ³ /μL)	7.35 ± 0.31	5.12 ± 0.4*	5.21 ± 0.2*	4.92 ± 0.5*
Hemoglobin (g/dL)	14.56 ± 0.36	10.21 ± 0.3*	10.42 ± 0.4*	9.39 ± 0.5*
Hematocrit (%)	46.40 ± 0.89	45.31 ± 1.26	44.97 ± 1.54	44.76 ± 1.5
MCV (fL)	57.38 ± 2.03	56.52 ± 0.63	57.12 ± 1.16	56.2 ± 1.33
MCH (pg)	19.02 ± 0.20	18.24 ± 0.93	19.34 ± 1.27	18.88 ± 1.0
MCHM (g/dL)	34.26 ± 0.93	34.54 ± 1.23	35.43 ± 1.47	35.12 ± 0.98
RDW (%)	14.72 ± 0.66	15.24 ± 0.95	14.39 ± 1.23	15.43 ± 1.1
Leukocytes (x10 ³ /μL)	11.26 ± 1.87	11.35 ± 1.22	12.11 ± 1.34	12.11 ± 1.4
Lymphocytes (%)	69.00 ± 5.24	72.12 ± 1.35	70.32 ± 1.67	68.35 ± 1.23
Neutrophils (%)	25.40 ± 5.18	26.78 ± 1.11	27.87 ± 1.73	25.76 ± 1.22
Monocytes (%)	6.40 ± 1.52	5.98 ± 1.32	6.75 ± 1.69	5.89 ± 1.96
Eosinophils (%)	1.40 ± 1.14	1.45 ± 0.97	1.38 ± 1.11	1.23 ± 1.29
Basophils (%)	0.40 ± 0.05	0.45 ± 0.17	0.37 ± 0.09	0.42 ± 0.13
PLT (x10 ³ /μL)	1258.00 ± 97.34	1278 ± 84.45	1265 ± 75.98	1270 ± 84.45
Males				
Erythrocytes (x10 ³ /μL)	8.56 ± 0.34	6.12 ± 0.43*	6.23 ± 0.32*	5.98 ± 1.21*
Hemoglobin (g/dL)	17.96 ± 0.32	14.23 ± 1.11*	13.45 ± 0.98*	13.21 ± 1.14*
Hematocrit (%)	51.80 ± 1.30	50.21 ± 0.92	51.32 ± 1.23	49.45 ± 2.34
MCV (fL)	54.48 ± 2.25	53.65 ± 2.44	52.45 ± 2.54	53.87 ± 2.21
MCH (pg)	18.92 ± 0.20	17.90 ± 1.54	19.34 ± 2.43	18.45 ± 1.98
MCHM (g/dL)	32.50 ± 0.43	33.67 ± 2.34	32.45 ± 1.87	32.56 ± 2.34
RDW (%)	11.90 ± 0.33	12.34 ± 1.43	12.67 ± 1.24	12.34 ± 2.67
Leukocytes (x10 ³ /μL)	12.50 ± 3.10	11.33 ± 2.56	11.87 ± 2.34	11.98 ± 2.98
Lymphocytes (%)	70.20 ± 5.67	71.43 ± 2.45	70.56 ± 2.23	69.89 ± 3.43
Neutrophils (%)	21.80 ± 4.82	19.89 ± 4.78	20.34 ± 3.78	19.92 ± 2.45
Monocytes (%)	5.60 ± 0.89	5.43 ± 1.23	5.21 ± 1.45	4.89 ± 1.87
Eosinophils (%)	2.00 ± 1.41	1.87 ± 0.97	1.93 ± 1.15	1.76 ± 1.2
Basophils (%)	0.40 ± 0.32	0.37 ± 0.09	0.34 ± 0.12	0.33 ± 0.19
PLT (x10 ³ /μL)	1179.0 ± 83.34	1195 ± 87.32	1198 ± 94.32	1199 ± 89.34

Data are presented as mean ± standard deviation (SD). * Indicates a statistically significant difference versus the control group ($p < 0.05$), determined by one-way ANOVA followed by Dunnett's multiple comparison test

the cell wall and the subsequent release of cytosolic components into the extracellular space (Nelson, 1995; Tejeda-Cifuentes, 2010; Li *et al.*, 2015; Abou Seif, 2016). Furthermore, the increased activity of AST and ALT enzymes in the experimental groups serves as a sensitive marker of hepatocyte injury (Ramaiah, 2011). Biochemical parameters such as urea and creatinine are commonly linked to kidney function, and the increased serum values in the experimental groups could be associated with renal failure (Gowda *et al.*, 2010). Elevated serum levels of bilirubin are associated with primary biliary cirrhosis and hepatic cholestasis (Thapa and Walia, 2007). Total protein levels provide a rough measure of protein status and can reflect functional alterations in kidney and liver functions (Tatefuji *et al.*, 2014). In the subacute study, significant variations were observed in serum total protein levels, albumin, and bilirubin in the treated groups compared to the control group. These findings, along with the hematological parameters and liver marker enzymes, indicate that EHRA may have adverse effects on the erythropoietic system and

organs involved in xenobiotic detoxification. Additionally, histopathological examination of the kidney revealed acute tubular necrosis. The proximal tubule is particularly susceptible to xenobiotic-induced injury due to its high metabolic activity and the expression of basolateral organic anion and cation transporters (OATs and OCTs), which facilitate the intracellular accumulation of toxic compounds.

Following uptake, metabolic bioactivation may generate reactive oxygen species (ROS) and electrophilic intermediates that deplete intracellular glutathione and induce oxidative stress. These reactive species can interact with membrane lipids, proteins, and nucleic acids, promoting lipid peroxidation, mitochondrial dysfunction, ATP depletion, and ultimately tubular epithelial cell necrosis (López-Novoa *et al.*, 2011; Perazella, 2018; Hall and Schuh, 2014). As a result, the tubules lose their ability to reabsorb electrolytes, leading to uremia and subsequent hydroelectrolytic imbalances affecting other organs (Perazella and Wilson, 2016; Negi *et al.*, 2018).

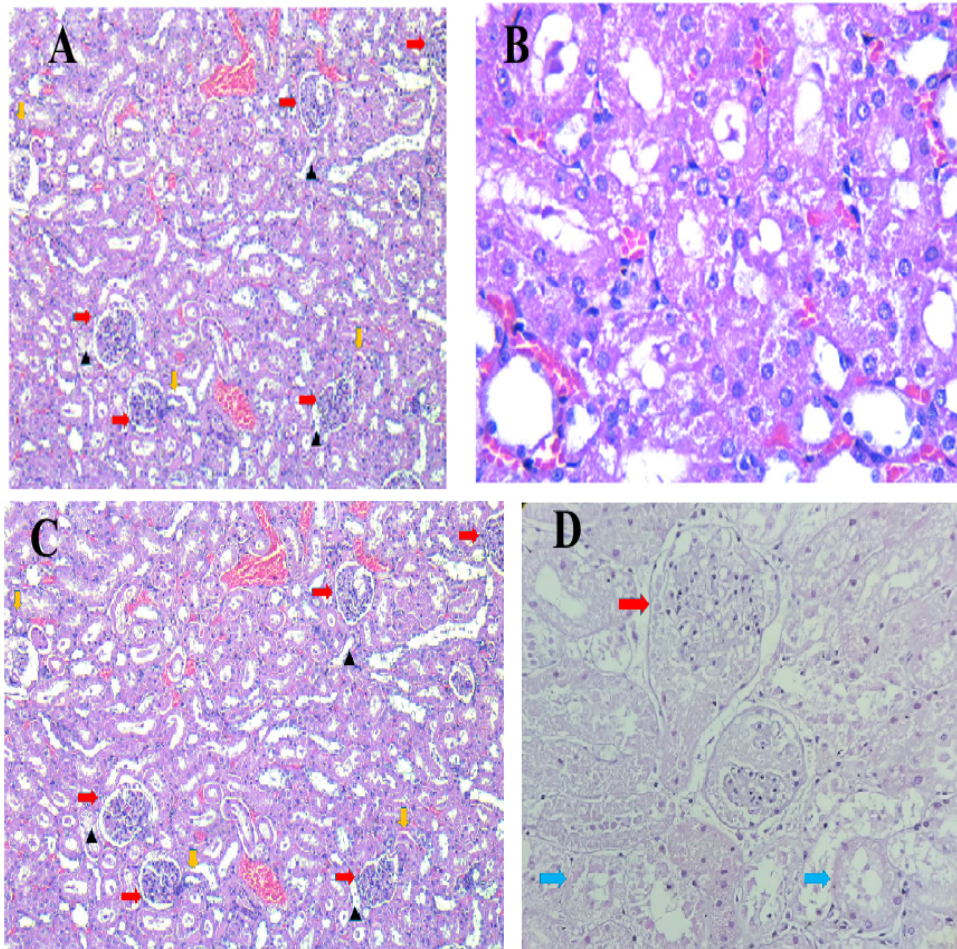


Figure 3. Representative photomicrographs of kidney sections from female and male rats in the subacute toxicity study of the hydroethanolic extract of *Randia aculeata*. (A) Female control group showing normal renal histoarchitecture, with intact glomeruli (black triangle) and proximal (red arrow) and distal tubules (yellow arrow) without histological alterations. (B) Female treated groups (250, 500, and 1000 mg/kg) exhibiting diffuse and extensive acute tubular necrosis accompanied by marked chronic inflammatory infiltrate in the interstitium, consistent with acute pyelonephritis. (C) Male control group showing preserved renal architecture. (D) Male treated groups (250, 500, and 1000 mg/kg) demonstrating diffuse and extensive acute tubular necrosis with associated interstitial inflammatory infiltration. Hematoxylin and eosin stain; original magnifications 10x and 40x.

Figura 3. Fotomicrografías representativas de cortes histológicos de riñón de ratas hembra y macho incluidas en el ensayo de toxicidad subaguda del extracto hidroetanólico de *Randia aculeata*. (A) Grupo control hembra, con arquitectura renal conservada; se observan glomerulos íntegros (triángulo negro) y túbulos proximales (flecha roja) y distales (flecha amarilla) sin alteraciones histológicas. (B) Grupos de hembras tratadas (250, 500 y 1000 mg/kg), que muestran necrosis tubular aguda difusa y extensa, acompañada de infiltrado inflamatorio crónico severo en el intersticio, compatible con pielonefritis aguda. (C) Grupo control macho, con histoarquitectura renal preservada. (D) Grupos de machos tratados (250, 500 y 1000 mg/kg), en los que se observa necrosis tubular aguda difusa y extensa asociada a infiltrado inflamatorio intersticial. Tinción con hematoxilina y eosina; aumentos originales de 10x y 40x.

The hypothesis that phenolic overload promotes redox imbalance is based on the observed enzyme elevations and histological necrosis; however, no direct quantification of oxidative stress indicators (ROS levels, glutathione depletion, lipid peroxidation products, or antioxidant enzyme profiles) was undertaken. Moreover, to clarify whether the hepatic and renal lesions reported here represent true necrosis rather than apoptosis, subsequent work should incorporate molecular endpoints capable of distinguishing these pathways.

Assessment of cleaved caspase-3 and caspase-9 activation, together with the Bax/Bcl-2 expression ratio, by Western blotting or immunohistochemistry would identify classical apoptotic signaling, while TUNEL staining could reveal DNA fragmentation. In contrast, evidence of high-mobility group box-1 (HMGB1) release, lactate dehydrogenase (LDH) leakage, and the absence of caspase activation would support a necrotic mechanism. Integrating these assays would provide a mechanistic framework to confirm the predominant mode

of cell death in the liver and kidney. This represents a key limitation and warrants targeted biochemical analyses in follow-up experiments to substantiate the proposed pathway. Moreover, because the actual amount of *R. aculeata* seed consumed in traditional remedies is poorly documented, the relationship between our experimental doses and human exposure remains uncertain.

Establishing typical consumption patterns will be essential for translating these toxicological findings into public health recommendations. To fully situate these findings within the broader toxicological context, it is worth emphasizing that the dual behavior of phenolic metabolites is protective at physiologically relevant concentrations yet damaging when accumulated, mirroring patterns reported for other botanicals rich in secondary metabolites. The hepatic and renal alterations observed here therefore not only expand the toxicological profile of *R. aculeata* but also illustrate a recurring challenge in herbal toxicology: the fine balance between pharmacological benefit and metabolic overload.

CONCLUSIONS

The EHRA exhibits low acute systemic toxicity under the conditions evaluated. Nevertheless, repeated administration and higher doses are associated with significant hepatic and renal injury, accompanied by mild hematological alterations. The observed adverse effects are consistent with mechanisms involving phenolic compound-mediated oxidative stress, direct cellular injury, and cumulative metabolic burden, supporting a dose-dependent toxicological profile. These findings highlight the need for careful consideration of dosage, duration of exposure, and the potential combined activity of phenolic metabolites before proposing any therapeutic or nutraceutical use. In light of the increasing consumption of *R. aculeata* in Mexico in recent years, establishing well-defined safety margins is particularly relevant. Further mechanistic investigations, especially those focused on bioactivation pathways, redox cycling, mitochondrial involvement, and long-term tissue adaptation, are essential to better characterize the underlying processes driving toxicity. Such studies will be critical for refining risk assessment, defining safe exposure limits, and supporting evidence-based recommendations for the future application of EHRA.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest

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