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# ACUTE A SUB-CHRONIC TOXICITY AND IMMUNOMODULATORY ACTIVITY OF AN AQUEOUS EXTRACT OF *EUPHORBIA RESINIFERA* IN RODENTS

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**Keywords:**

Acute and sub-acute toxicity, Histopathological assay, Immunomodulation, Moroccan medicinal plant, *Euphorbia resinifera* Berg

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**ABSTRACT:** *Euphorbia resinifera* Berg. (ER) an endemic Moroccan plant has been used to treat different diseases. In spite of its toxicological potential, no scientific report until now was made to evaluate *in-vivo* neither the toxic effect nor its immunomodulatory activity. The objective was to evaluate the safety of an aqueous extract (AE) of *ER in-vivo* and its effect on the biochemical, immunological and histopathological parameters. The AE was administrated in single oral dose (5g/kgbw) for the acute toxicity tests given daily by gavages at 0.1, 0.5, 1, 2.5, or 5 g/kg for 28 consecutive days. Serum was analyzed for creatinine, urea, alanine aminotransferase, and aspartate aminotransferase. Histopathological examination of liver, kidneys, and spleen was done at the end of the study. The immunological effect was tested by the evaluation of antibodies production and the level of the delayed type hypersensitivity reaction. No signs of toxicity or deaths were observed in mice treated by a single dose. In the sub-acute toxicity tests, no visible toxic effects were observed at 0.1-1 g/kg doses. However, at 2.5 and 5 g/kg doses, the mice showed some behavioral signs of toxicity. This was confirmed by the histological and biochemical evaluations. When tested against SRBC, there was a significant increase of "haemagglutinating antibody titer" and in "delayed-type hypersensitivity" response in mice treated by ER at 1 g/kg bw (4 and 1.5 times respectively $p<0.05$). Higher than 2.5 g/kg, the ER can cause liver and kidney toxicity. The immune-stimulatory effect was induced by an inflammatory reaction.

**INTRODUCTION:** *Euphorbia resinifera* Berg. (Euphorbiaceae) is an endemic Moroccan spurge plant known as "Zaqqum" in local language [1]. It is characterized by the unique structure of their flowers and their latex resin and has been used in traditional folk medicine since the time of recorded history under the name of Euphorbium [2].

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One of their therapeutic uses in folk medicine is for their anticancer activities. It has been reported that the Moroccan patients mixed the aerial parts with honey to treat general cancer [3]. The fresh latex of this plant is used for poisonous punctures, bites, and dental pains. The women also use a mixture containing the resin of *Euphorbia* likes abortive in spite of its dangers [4]. In Algeria, *Euphorbia resinifera* L. is also used to treat cancer, rheumatism, cyst, and snakes bite poisoning [5]. It contains a high concentration of resiniferatoxin, a strong irritant diterpene that is an ultrapotent capsaicin analog [6] used to induce inflammation or to desensitize pain neurons [7].

Euphorbia genus is rich in tannins, volatile compounds, phytosterols, diterpenes and triterpenes [8, 9, 10] which have been found to have moderate cytotoxic, antitumor, antibacterial, anti-inflammatory, anticancer and anti-HIV activities [11]. A phytochemical investigation of roots, stems, and flowers of methanolic and ethyl acetate extracts of *Euphorbia resinifera* revealed the presence of saponins, polyphenols, flavonoids, tannins, terpenoids, coumarins and cardiac glycosides [6].

The presence of phenol compounds indicated the higher antioxidant activity of root. Antibacterial activity was also evaluated and showed that the extracts of all the part of *Euphorbia resinifera* were active against *S. aureus* and *B. subtilis* strains [12].

The fresh or dried latex of *Euphorbia resinifera* cause several inflammations in the digestive membrane with gastrointestinal ulcers, arrhythmia, convulsions, hematuria and can even cause death by asphyxiation [13].

In spite of this toxicological potential, no scientific report until now was made to evaluate neither the toxic effect of aqueous extract *in-vivo* nor its immunomodulatory activity. This present work was undertaken in the aim to determine the toxic effect of aqueous extract of *Euphorbia resinifera in vivo* and to investigate its effect on the biochemical, immunological and histopathological parameters after acute and sub-acute orally administered to mice.

## MATERIALS AND METHODS:

**Plant Material:** The aerial part of *Euphorbia resinifera* Berg was collected in March 2014 from "Bzou," an area of Beni-Mellal city in Morocco and was authenticated by Professor Leila EL GHAZI, a plant taxonomist, at the Department of Biology, Faculty of Sciences; Hassan II University of Casablanca. A voucher specimen of the plant sample was deposited (N°61353) in the Herbarium of the National Scientific Institute of Rabat.

**Preparation of the Aqueous Extracts of Aerial Parts of *Euphorbia resinifera* Berg:** The stems of the plant were air dried, and then pulverized. The aqueous extract was prepared by adding 500 mL of distilled water to 50 g of ER powder and heating the mixture under reflux for 60 °C for 1 h in a round-bottom flask then the boiled decoction was centrifuged, filtered, and then concentrated in a rotary vacuum evaporator at 40 °C. The extracted was stored at −20 °C until use. For oral administration (gavages), the crude extract was dissolved in water at the desired concentration which was prepared on the day of the experimental studies.

**Preliminary Phytochemical Screening:** The aqueous extract was screened for alkaloids, flavonoids, tannins, and saponins as described before [14, 15]. Tests were based on the visual observation of color changes or formation of a precipitate after the addition of specific reagents.

**Animals:** Young Adult Male mice (30-35 g), were purchased from the animal house of the Department of Biology, Faculty of Sciences, University Mohammed V; Rabat, Morocco. The animals were kept in plastic cages in environmental conditions (22–24°C, 12 h: 12 h dark/light cycle) with frequent air changes and allowed to drink water *ad libitum* and standard pellet diet. Mice were deprived of food but with access to water 16-18 hour prior to the experiments. An adaptation period of 2 weeks was allowed before each experiment.

**Toxicological Evaluation:**

**Acute Toxicity Test:** The assessment of acute toxicity was performed according to the World Health Organization (WHO) guideline (WHO 2000) and the Organization of Economic Co-operation and Development (OECD) guideline for testing of chemicals 420 (OECD 2001) in accordance with the internationally accepted principles for laboratory animal use and care (EEC Directive of 1986; 86/609/EEC).

The aqueous extract was administrated in a single oral dose at 5 g/kg body weight while the control group received a saline solution (NaCl 0.9%). Signs of toxicity and mortality have been followed at the first, second, fourth, and sixth hours after oral administration; then once daily for 14 days (Ha 2010).

**Sub-Acute Toxicity Test:** Mice were divided into 6 groups containing seven animals per group and housed in separate cages during the study. The aqueous extract was given orally by gavages to different groups daily at a dose of 0.1, 0.5, 1.0, 2.5

or 5 g/kg body weight for 28 consecutive days, while the control group received the vehicle only. The animals were closely monitored daily for general behavior and toxicity signs throughout the experimental period [16]. At the end of the 28th day treatment period, the mice were sacrificed, and the blood samples and organs collected. Blood was collected in tubes without anticoagulant, allowed to clot before centrifugation (3000 rpm at 4°C for 10 min) to obtain serum, which was analyzed for biochemical parameters.

**Measurement of Biochemical Parameters:** Serum was analyzed for creatinine, blood urea, nitrogen concentrations, and the activity of liver enzymes: alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT). They were determined enzymatically by standard methods with a biochemical automat (Konelab 20 Thermo).

**Histopathological Examination of Liver, Kidneys, and Spleen:** Freshly dissected mice's liver, kidneys and spleen were cut carefully to a small slice and fixed in buffered formaldehyde solution (10%), dehydrated in ascending series of ethanol solutions, then embedded in paraffin. Then 4-5 μm thick sections of each tissue were prepared and stained with hematoxylin-eosin and examined under a light microscope; photomicrographs of the samples were recorded and interpreted by a pathologist.

**Immunomodulatory Effect of the Aqueous Extract of ER:**

**Antigen:** Fresh Sheep Red Blood Cells (SRBC) was washed three times in a large volume of phosphate buffered saline (PBS) by repeated centrifugation at 2000 t/min for 10 min. The washed SRBC was adjusted to a concentration of approximately $10^9$cells/ml and used for immunization [17].

**Hemagglutination Titer (HT) Assay:** HT assay was performed using the procedure described by Benzakour *et al*. [18] Two groups with 7 mice in each were challenged with 0.2 ml of 30% SRBCs suspension in day 0 by intra-peritoneal injection. The treated group received daily by oral gavages, the aqueous extract (1 g/kg body weight) 3 days before and 7 days after the immunization. The second group was considered as control. On day 7, blood was collected from each mouse, and the serum recuperated after overnight incubation at 4 °C, then centrifuged, incubated for 30 min at 56 °C and stored at –20 °C until use. The antibody levels were determined by hemagglutination technique using the method described by Bin Hafeez *et al*. [19] Briefly, 25 μl of 1% SRBC in physiological solution were added to serum serially diluted in 25 μl PBS two-fold in 96-well microplates. The mixture was incubated at room temperature for 2 h. The reciprocal of the highest dilution of the positive test serum agglutination was taken as the antibody titer.

**Delayed Type of Hypersensitivity Response (DTH):** After an intraperitoneal injection of 0.2 ml of 30% of an SRBC suspension on day 0, animals (n=7 per group) were challenged subcutaneously with 1% an SRBC in the right hind footpad 7 days later. The left footpad injected with the same volume of normal saline served as control. Increase in footpad thickness was measured 24 h after the immunization. Two groups were used to determine the degree of DTH: the first was treated by 1 g/kg body weight of aqueous extract of *ER* three days prior the immunization and continued 7 days after the challenge. In the same conditions, the second group received PBS and was considered as the negative control.

**Statistical Analysis:** All values were expressed as the mean ± standard error of measurement and the statistical significance between control and experimental groups were analyzed using Student's t-test. Statistical significance was assigned at p values <0.05.

**RESULTS:**

**Chemical Constituents:** The phytochemical screening of the aqueous extract of *Euphorbia resinifera* used revealed the presence of flavonoids and tannins, and there was no saponins or alkaloids.

**Acute and Sub-Acute Toxicity of Aqueous Extract of *E. resinifera* in Mice:** There were no signs of toxicity or deaths observed in mice treated by a single oral administration of the aqueous extract of ER at 5 g/kg bw during 14 days of observations. In the sub-acute toxicity test, there were no animal deaths in any of the treated groups, and no visible toxic effects were observed at 0.1;

0.5 and 1 g/kg doses of oral administration of the aqueous extract. Indeed for 2.5 and 5 g/kg doses, the mice showed some behavioral signs of toxicity after 15 days of administration of *E. resinifera* extracts such as hypoactivity, anorexia, asthenia, ataxia, diarrhea and urination, and these signs remained until the end of experimentation **Table 1**.

**TABLE 1: SUB-ACUTE TOXICITY OF AN AQUEOUS EXTRACT OF *E. RESINIFERA* ADMINISTERED ORALLY TO MICE**

| The dose of ER-extract (g/kg BW) | Mortality Dead /Treated Mice | Toxic symptoms |
|---|---|---|
| 0.1 | 0/7 | None |
| 0.5 | 0/7 | None |
| 1.0 | 0/7 | None |
| 2.5 | 0/7 | Hypoactivity, anorexia, asthenia, ataxia |
| 5.0 | 0/7 | Hypoactivity, anorexia, asthenia, ataxia, diarrhea, and urination |

The aqueous extract of *ER* dissolved in distilled water was administered orally; each dose was administered to groups of 7 mice. All the treated animals were carefully examined for 28 days for any signs of toxicity (behavioral changes and mortality). D/T: dead/treated mice; none: no toxic symptoms were seen during the observation period

**Evaluation of Biochemical Parameters:** For the serum biochemical analysis, we noted that the ALAT, ASAT, Creatinine, and Urea levels did not show any differences among treated and control groups at the doses of 0.1 and 0.5 g/kg. From the dose of 1 g/kg body weight, we noted significant dose-dependent increases in ALAT and ASAT the principal indicators of liver toxicity [20], especially at the higher dose of 5 g/kg. A slight increase in urea and creatinine serum levels suggests a possible potential toxicity effect on the renal tissue **Table 2**. These serological results were confirmed by the histological observations **Fig. 1**.

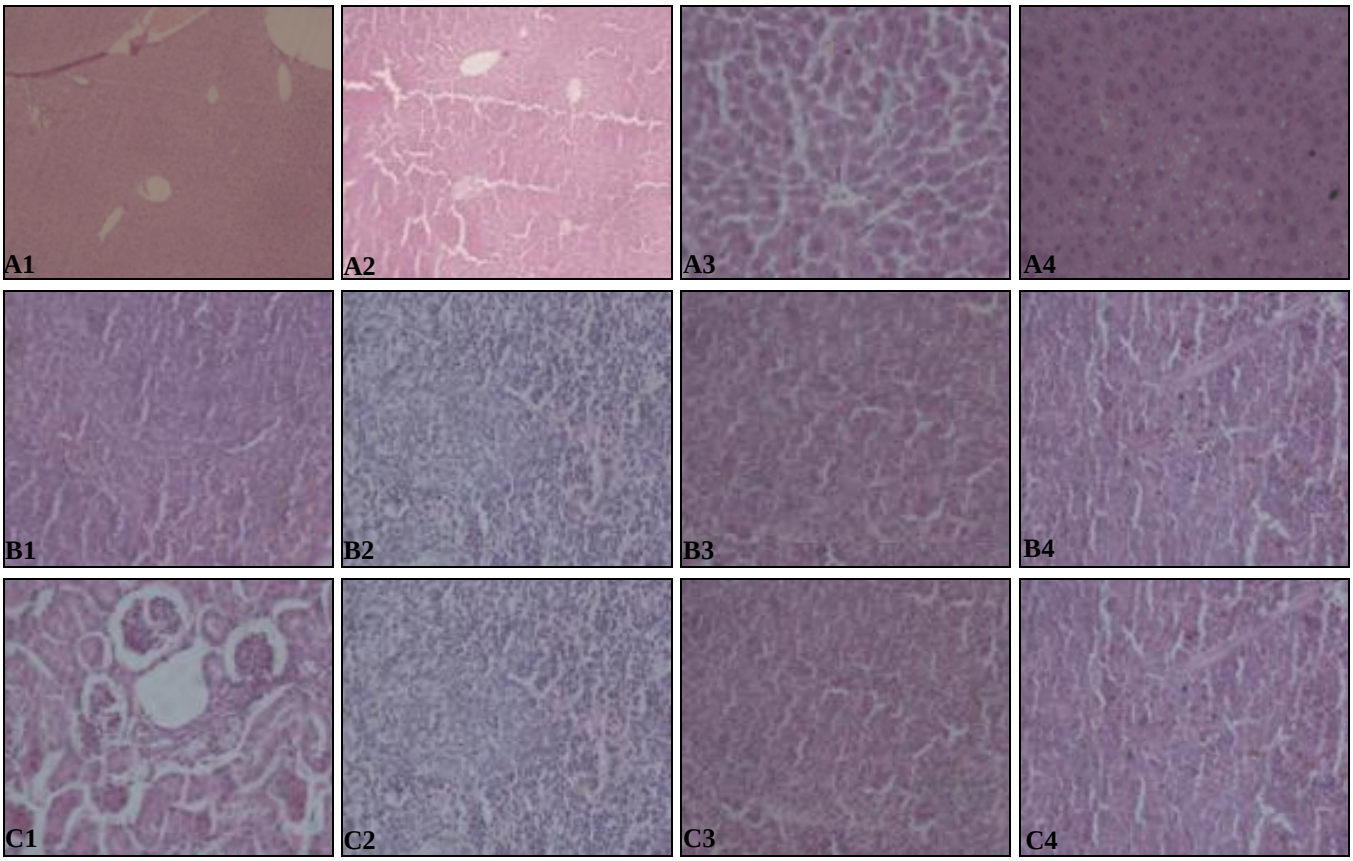


**FIG. 1: HISTOLOGICAL OBSERVATIONS OF LIVER, SPLEEN AND KIDNEY TISSUES AFTER SUB-CHRONIC TREATMENT WITH THE EUPHORBIA RESINIFERA EXTRACTS. (A) LIVER SECTIONS OF NORMAL CONTROL RATS (1), RATS TREATED WITH 1 g/kg (2); 2.5 g/kg (3) AND 5 g/kg (4) OF EXTRACT FOR 28 DAYS. (B) SPLEEN SECTIONS OF NORMAL CONTROL RATS (1), RATS TREATED WITH 1 g/kg (2) 2.5 g/kg (3) AND 5 g/kg (4) OF EXTRACT FOR 28 DAYS. (C) KIDNEY SECTIONS OF NORMAL CONTROL RATS (1), RATS TREATED WITH 1 g/kg (2) 2.5 g/kg (3) AND 5 g/kg (4) OF EXTRACT FOR 28 DAYS.**

**TABLE 2: EFFECT OF ORAL ADMINISTRATION OF *EUPHORBIA RESINIFERA* EXTRACTS ON SERUM BIOCHEMICAL PARAMETERS**

| The dose of ER-extract (g/kg BW) | ALAT | ASAT | Creatinine | Urea |
|---|---|---|---|---|
| 0.0 | 16.4 ± 1.94 | 13.6 ± 4.09 | 11.04 ± 2.34 | 0.33 ± 0.08 |
| 0.1 | 15.9 ± 1.74 | 13.2 ± 3.65 | 10.95 ± 1.85 | 0.32 ± 0.06 |
| 0.5 | 16.8 ± 2.30 | 14.6 ± 4.02 | 9.88 ± 1.32 | 0.35 ± 0.05 |
| 1.0 | 18.8 ± 4.20 | 17.4 ± 5.02 | 8.33* ± 1.06 | 0.28 ± 0.03 |
| 2 .5 | 22.2* ± 3.11 | 29.2* ± 4.96 | 11.67 ± 1.48 | 0.35 ± 0.06 |
| 5.0 | 24.0* ± 5.09 | 30.6* ± 10.54 | 13.69 ± 1.57 | 0.42 ± 0.06 |

The aqueous extract of the plant ER was given daily by the oral route to groups of mice (n = 7 per group) at the following doses: 0g/kg (control), 0.1 g/kg, 0.5 g/kg, 1g/kg, 2.5 g/kg and 5 g/kg for 28 days. Data are expressed as mean ± S.E.M.; (*) P<0.05; (**) P<0.01 versus the control group of three independent experiences. (ALAT: Alanine aminotransferase; ASAT: Aspartate aminotransferase; AE: Aqueous extract; ER: *Euphobia resinifera*).

**Histopathological Examinations:** Histopathological examination revealed no detectable alteration in kidney or spleen tissues between the controls and the treated mice for the doses lower than 2.5 g/kg of the aqueous extract of E.R **Fig. 1**. But for the dose of 5 g/kg we observed for the kidney tissues, high interstitial inflammatory lymphocytes infiltrate and for the spleen an accentuated hemosiderin.

For the liver tissue, some low hepatocytes lesions have been observed for the doses of 0.5 and 1 g/kg and the highest dose of 5 g/kg a massive steatosis centrilobular microvacuolar with nucleus duplication of the hepatocyte.

**Effects of Plant Extract on Humoral and Cellular Immunity Parameters:** As shown in **Fig. 2**, a significant increase (4 times) in the titer values of antibody in the treated group (2048) with no toxic dose (1 g/kg) compared to control (512) ($p<0.05$). ER aqueous extract at a dose of 1g/kg **Fig. 3** increased the Delayed-type hypersensitivity (DTH) reactivity response (37%) as compared to control animals (25%) ($p<0.05$).

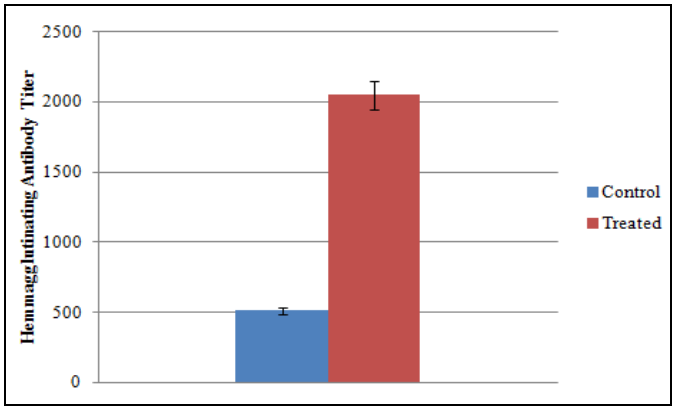


**FIG. 2: EFFECT OF ORAL ADMINISTRATION OF *EUPHORBIA RESINIFERA* AQUEOUS EXTRACT ON ANTIBODY PRODUCTION IN MICE.** Two groups of seven animals for each were immunized with a 30% erythrocyte suspension in PBS, were inoculated by gavages with an extract concentration (1 g/kg) in PBS or an equal volume of PBS (Control). The animals received a daily dose of the extract. The animals were bled 7 days post-immunization and the hemagglutinating antibodies were determined. The day of immuni-zation with erythrocytes was considered day 0. Titers were expressed as the reciprocals of the maximal dilution that produced positive heamagglutination. Statistical signi *vs.* control: $p<0.05$.

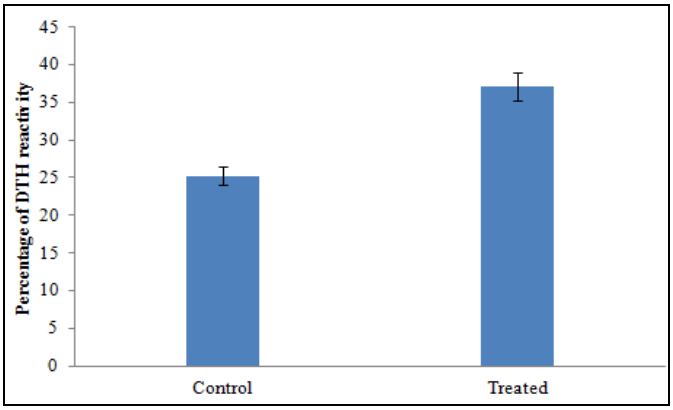


**FIG. 3: EFFECT OF ORAL ADMINISTRATION OF *EUPHORBIA RESINIFERA* AQUEOUS EXTRACTS ON THE DTH RESPONSE IN MICE.** Mice sensitized with an intraperitoneal injection of 30% erythrocyte suspension in PBS on day 0. The mice received a daily oral dose (1g/kg) of *ER* extract (treated) or an equal volume of PBS (control) on days -3, +7. On day +7, the DTH reaction was elicited with a sc injection of 1% erythrocyte suspension in PBS into the right hind footpad. The left hind footpad was referred as control. Footpad swelling was measured on day +8. The difference between the means of right and left hind footpad thickness gave a degree of footpad swelling or DTH reactivity. Statistical significance versus control: $p<0.05$.

**DISCUSSION:** Euphorbiaceae is one of the large flowering plant families with great importance, commonly used for their medicinal value and therapeutic potentials. However, no scientific validation of quality, safety, and efficacy of the most popular plant formulae have done before any therapeutic recommendations [21] such as acute and sub-acute toxicity which is currently performed in animals [22] to provide guidelines for selecting a 'safe' dose.

Acute toxicity studies with a range of doses have to be conducted first to select proper dose(s). The present investigation shows that an aqueous extract of the aerial parts of ER at the dose below 2.5 g/kg given by oral route doesn't have any effect of toxicity. Daily oral administration of ER aqueous extract for 28 days did not produce any obvious symptoms of toxicity or mortality up to the highest dose level of 2.5 g/kg. There was also no change in animal behavior, and the body weight gains were not significantly different in the treated rats as compared to the controls. In the present study, we report that the aqueous extract of ER at doses of 0.5-2 g/kg was not toxic but at 2.5 g/kg produced toxic effects in mice after 4 weeks of treatment by oral gavages. Because kidney and liver toxicity has been reported following the use of phytotherapeutic products [23], biochemical parameters evaluation is important. As markers of kidney activity, determinations of creatinine and urea were critical.

There were no significant differences of glucose, creatinine, and urea levels in the groups treated with the herbal extract at doses 0.5-2.5 g/kg. At a dose of 5 g/kg, a slight increase in urea and creatinine serum levels suggests a possible potential toxicity effect on the renal tissue confirmed by high interstitial inflammatory lymphocytes infiltrate. Among the parameters evaluated, ASAT and ALAT are considered markers for liver function. ALAT is located primarily in the cytosol of hepatocytes, and this enzyme is considered a more sensitive marker of hepatocellular damage than ASAT.

ASAT is an enzyme found in the cytoplasm and mitochondria in different tissues, chiefly in the heart and skeletal muscles, liver, kidneys, pancreas, and erythrocytes [24]. The increase of these two enzymes values suggested hepatocellular damages by the aqueous extract of *ER*. The present study showed that some low hepatocytes lesions had been observed for the doses of 0.5 and 1 g/kg and for the highest dose of 5 g/kg a massive steatosis centrilobular microvacuolar with nucleus duplication of the hepatocyte have been observed.

For the spleen tissues, an accentuated hemosiderin was observed. Hemosiderin is most commonly found in macrophages and is especially abundant in situations following hemorrhage, suggesting that its formation may be related to phagocytosis of red blood cells and hemoglobin.

Euphorbia genus is rich on tannins, volatile compounds, phytosterols, diterpenes and triterpenes [25, 9] which there been found to have moderate cytotoxic activity. A phytochemical investigation of roots stems and flowers of methanolic and ethyl acetate extracts of *Euphorbia resinifera* revealed the presence of saponins, polyphenols, flavonoids, tannins, terpenoids, coumarins, and cardiac glycosides [6, 26].

In this study, the preliminary phytochemical analyses of the extracts of the aerial parts of ER have shown the presence of flavonoids and tannins, and there was no saponins or alkaloids like described by Benmehdi *et al*., [5] for ER from Algeria. Some studies have noted changes in total alkaloid substances in response to nitrogenous fertilizer [27]. The component(s) of the ER extract, which caused toxicity; both in the acute and sub-chronic dose studies are not known. Since in acute toxicity studies, a product is considered non-toxic if no deaths are registered and no clinical signs of toxicity are observed at doses at or below 2.5 g/kg [28], the highest dose of ER-extract which did not cause any toxicity was 2.5 g/kg suggesting that it is relatively non-toxic.

The lymphocytic infiltrates were very pronounced in all tissues examined, suggesting an immuno-stimulating effect induced the toxicity observed. So, we tested the immunomodulatory effect of the aqueous extract of ER in mice. It has equally shown a stimulatory effect on both humoral and cellular immune responses to SRBC in mice. In the HA titer test, the plant showed a significant enhancement of antibody responsiveness to SRBC in mice as a consequence of both pre and post-plant treatment, which indicates the enhanced responsiveness of B-lymphocytes involved in antibody synthesis. This was confirmed by an anti-bovine sera albumin response tested by a liquid-based immunoprecipitation analysis (data not shown).

In the DTH response, which directly correlates with cell-mediated immunity (CMI), was found to be the highest at the dose tested (1 g/kg) in the extract. An increase in DTH response indicates a stimulatory

effect of the plant, which has occurred on the lymphocytes and accessory cell types required for the expression of this reaction [29].

When challenged by the antigen, they are converted to lymphoblast and secrete a variety of molecules including proinflammatory lymphokines, attracting more scavenger cells to the site of reaction [30]. Increase in both, HA titer and DTH response indicated that *ER* potentiates humoral as well as the cellular immunity. This plant also is a rich source of terpenoids which may act as immuno-modulatory, which could justify the high number of lymphocytes infiltrates found in tissues examined [31, 32].

**CONCLUSION:** In conclusion, at the doses consumed empirically in traditional Moroccan medicine, *Euphorbia resinifera* appears to be relatively nontoxic. However, at higher doses than 2.5 g/kg, it can cause liver and kidney toxicity. The immunostimulatory effect observed was a consequence of a toxic effect of the extract that induces an inflammatory reaction. Since, the toxicity studies in experimental animals cannot always be totally extrapolated to humans [21] and a reasonable estimate of the self-administered dose is difficult to make, and given the widespread traditional use of this plant, recommendations are necessary to protect the population from possible toxic effects of the plant.

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**DECLARATION OF INTEREST:** The authors report no declarations of interest.

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