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Hepatoprotective Activity of *Rubia tinctorum's* Extract against CCl_4 Induced Hepatic Injury in Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Authors FM, YZ, HB and NE designed the study, planned, and executed the experiments, analyzed the data and drafted the manuscript. Authors MAL, JL, AIH, AB and AC designed the study and gave substantial contributions in analysis and interpretation of data. All authors provided intellectual input and participated in acquisition of data. All authors read and approved the final manuscript.

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ABSTRACT

This study investigated the hepatoprotective activity of *Rubia tinctorum's* extract against CCl_4 induced liver damage in rats. Twenty four rats were divided into four groups. Group I received normal saline (10 ml/kg) and group II (normal control) was treated with vehicle (olive oil 1 ml/kg), Group III (CCl_4 control) received 1 ml/kg CCl_4 mixed with equal volume of olive oil and Group IV

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(test group) received simultaneously the extract (1 g/kg) + CCl_4 for 15 days. At the end of the experiment (2 weeks), blood and liver samples were collected for biochemical and histopathological analysis. The present study revealed that CCl_4 significantly increased *(P<0.001*) hepatic markers (ASAT, ALAT, GGT, ALP and direct bilirubin) activities, but these effects were decreased by the treatment of rats with *Rubia tinctorum's* extract. Histopathologically, the potential hepatoprotective (the hepatoprotective potential of the plant) activity of the plant was also revealed by its regenerative action on CCl_4 induced liver tissues injury.
This finding indicates that *Rubia tinctorum* L has a potent hepatoprotective effect against CCl_4-induced liver damage and improved the biochemical and histopathological results.

Keywords: Rubia tinctorum L; hepatoprotective activity; CCl4; biochemistry; histopathology.

1. INTRODUCTION

The liver is the largest internal organ and the largest gland in the body; it performs divers functions, including glycogen storage, lipid and protein synthesis, bile salt production, hormone production, and detoxification [1]. It has been shown before that oxidative stress and inflammation are leading causes of liver diseases. Carbone tetrachloride (CCl_4) administration can induce chronic liver injury in rats. It is therefore considered as the experimental model of choice for liver injury [2,3].

Carbon tetrachloride (CCl_4) has been widely used to investigate the pharmacological benefits of hepatoprotective agents [4,5,6]. The CCl_4 treatment was used extensively to investigate hepatoprotective activity on various experimental animals. The mechanism of CCl_4 hepatotoxicity has been thoroughly studied since the 1970s, by using *in vivo* models of acute and chronic CCl_4 poisoning, perfused livers, and isolated or cultured hepatocytes [7,8]. CCl_4-induced toxicity is a multifactorial process involving the generation of free radicals [8,9,10]. CCl_4 is biotransformed by cytochrome P450 enzyme family, with subsequent generation of highly toxic trichloromethyl ($CCl_3^{\bullet}$) and trichloromethylperoxyl (CCl3OO●) free radicals that in turn induce damages by interfering with proteins, lipids and DNA. These free radicals bond covalently to cellular macromolecules and causes peroxidative degradation of lipids membrane of the adipose tissue. This leads to the formation of lipid peroxides, which in turn yield products like MAD (Malondialdehyde, lipid peroxidation products), which causes loss of integrity of cell membrane and damage to hepatic tissue [11].

It has been reported before that antioxidants prevent oxidative damage caused by free radicals and can thereby reduce the risk of liver diseases [12]. Medicinal plants have been used extensively for decades for the treatment of many diseases. Indeed, natural products continue to be important sources for the development of many drugs to treat a wide variety of diseases such as cancer and liver disease among others [13].

Rubia tinctorum L is a plant belonging to Rubiaceae family that historically originated from Caucasus and Near East. This plant is widely distributed in southern and southeastern Europe, in central Asia, and in the Mediterranean area [14] including the north of Africa. It is commonly known as "El foua" in Morocco. The roots of *Rubia tinctorum* L are the source of a natural dye and they have been used to dye textiles in many parts of the world since ancient times [15]. It was long accepted for use as a food additive in Japan and Korea, and widely applied for a variety of foods/ drinks [16]. Major constituents of *Rubia tinctorum* L are anthraquinones, such as purpurin [17] lucidin, alizarin [18,19,20] and their primeverosides, e.g., lucidin-3-0-primeveroside and ruberythric acid [21].

Extracts from *Rubia tinctorum*'s root have been used as a traditional medicine to cure various ailments and also used against kidney and bladder stones in the orient and the occident [22]. For example, studies have shown that the dried roots are useful in alleviating dropsy, paralysis, jaundice, amenorrhea and visceral obstructions [23]. Also the plant was applied for treatment of rheumatic disorders in Europe [24]. Other finding demonstrates that *Rubia tinctorum* can be source of potent antioxidants for treatment of diseases such as cancer [25] and has antibiotic and anti-inflammatory activities [26]. In one of the previous studies in Japan, acute and sub-acute toxicity tests of madder root were surveyed on the mice and no toxic effects was determined [27]. Furthermore, it is used as an ingredient of a native recipe being prescribed by traditional healers to cure chronic diseases

and liver trauma [28]. However, so far no scientific study has been reported regarding the hepatoprotective property of the root extract of *Rubia tinctorum* L. Therefore, the aim of the present study was to explore the potential of its *in vivo* hepatoprotective activity against CCl_4 induced hepatotoxicity. The hepatoprotective activity *in vivo* was studied on rat liver damage induced by CCl_4 by monitoring biochemical, hematological parameters and histological changes.

2. MATERIALS AND METHODS

2.1 Animals

Male and female Sprague-Dawley rats weighing about 200 - 250 g were purchased and housed in the animal facility of the faculty of Sciences Semlalia, Marrakesh, Morocco. They were acclimatized at laboratory conditions for a week before starting the experiments. The experiment was conducted in accordance to internationally accepted standard guidelines for use of animals. The rats have free access to food and water and were kept under a controlled 12 h light/dark cycle at 22 ± 2°C. All the experiments were performed in the morning according to current guidelines for the care of the laboratory animals and the ethical guidelines for the investigation of experimental pain in conscious animals [29], in accordance with the provisions for animal care and use described in the Scientific Procedures on Living Animals ACT 1986 (European Council directive: 86⁄609/EEC).

2.2 Plant Material

Rubia tinctorum L was collected from Marrakech region (Morocco). The plant materiel was botanically classified and authenticated for their correct botanical identification by Professor Mohamed Ouhammou. A voucher specimen of plant has been deposited at the herbarium of Semlalia sciences faculty, Cadi Ayyad University.

2.3 Preparation of the Extract of *Rubia tinctorum* L

The dried roots of *Rubia tinctorum* L were collected in June 2015 from Marrakech region (Morocco). The roots were coarsely powdered (102.9 g) and packed into soxhlet column and extracted with 70% v/v ethanol in water at 75-79°C for 15 h. The extract obtained was evaporated at 45°C, then dried and stored in airtight container. The yield of the extract was 23%. The extract was prepared daily, just before administration, by dissolving it in distilled water.

2.4 Pharmacological Materials

Carbon tetrachloride (CCl_4) was obtained from a PROLABO (R.P. NORMAPUR® EMB usine de 45-briare, Lot 89083. Paris). Chloral hydrate crystallized ≥ 98.0% (T) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.5 Induction of Hepatic Injury (Experimental Protocol)

To study the protective effects of *Rubia tinctorum*'s extract on carbon tetrachloride mediated hepatotoxicity, 24 adult rats were randomly allocated to four groups of 6 rats each. Hepatic injury was induced in rats by oral administration of (1 ml/kg) CCl_4 mixed with the equal volume of olive oil every 72h during 15 days. Group I received normal saline (10 ml/kg) and group II (normal control) was treated with vehicle (olive oil 1ml/kg) daily for 15 days. Group III (CCl_4 control) received 1ml/kg CCl_4 mixed with equal volume of olive oil every 72h during 15 days. Group IV (test group) received simultaneously the extract (1g/kg) daily and CCl_4 every 72 h for 15 days.

Animals were anaesthetized with Chloral hydrate (10mg/kg) 24 h after the last treatment and blood samples (1-2 ml) were collected from jugular vein in two tubes from all rats, one with the anticoagulant ethylene-diamine-tetra-acetat (EDTA) to measure the hematological parameters and the other without any additive. Serum was separated by centrifugation (4000 rpm, for 10 min) and used to estimate the biochemical parameters.

The jugular vein is located in the sternoclavicular junction. To escape destruction of these small veins a security microperfuseur butterfly epicranien G23 with adapter for sampling was inserted in the caudocephalic direction and blood was flowed slowly. All animals were operated correctly only 3 to 4 mm of needle was inserted into the jugular vein. This region appears in blue color when we kept the rat in hyper extended position with a neck shave.

2.6 Measurement of Biochemical and Hematological Parameters

At the end of the experiment, 1.5 ml blood samples from each animal were taken in tube containing no anticoagulant for collection of serum after centrifuged at 3000 rpm for 5 min at 4°C. Serum was analyzed for the activity of liver enzymes: alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), alkaline phosphatase (ALP), gamma glutamyltransferase (GGT) and Direct bilirubin (D.Bil). They were determined enzymatically by standard methods with a biochemical automat (Cobas c 311 analyzer Roche Diagnostics GmbH D-68298, Mannheim Germany).

2.7 Histopathological Examination of the Liver Tissue

After blood collection, all animals were dissected, and the liver was carefully excised. Small slices of this freshly harvested tissue was fixed in buffered formaldehyde solution (10%), dehydrated by serial ethanol solution, diaphanized with ethanol–benzene and enclosed with paraffin. Micrometer sections cut by a microtome were stained with hematoxylin–eosin and examined under a light microscope.

2.8 Statistical Analysis

All data are expressed as mean ± standard error of measurement (SEM); *P*-values less than 0. 05 were considered to be significant. Comparisons among different groups were performed by analysis of variance using One-way ANOVA test. Significant difference between control and experimental groups were assessed by student's t-test and Tukey's test.

3. RESULTS

The present study aimed to show the potential hepatoprotective activity of *Rubia tinctorum*'s extract in carbon tetrachloride induced hepatotoxicity.

3.1 Hepatoprotective Effect on Weight

Rats treated with CCl_4 developed hepatic damage. Liver weight has decreased significantly in CCl_4 induced rats when compared to normal rats (5.15 g) due to the development of necrosis. *Rubia tinctorum* given with CCl_4 for 15 days significantly prevented the formation of liver necrosis and thereby the increase of the liver weight (7.10 g) (Table 1).

Table 1. Liver weight of control and experimental animals

Groups	Liver weight (g)
Control	5.15 ± 0.16 [a]
Olive oil	4.48 ± 0.79*
CCl_4+ olive oil	6.43 ± 0.44*
Extract+ CCl_4+ olive oil	7.10 ± 0.15*

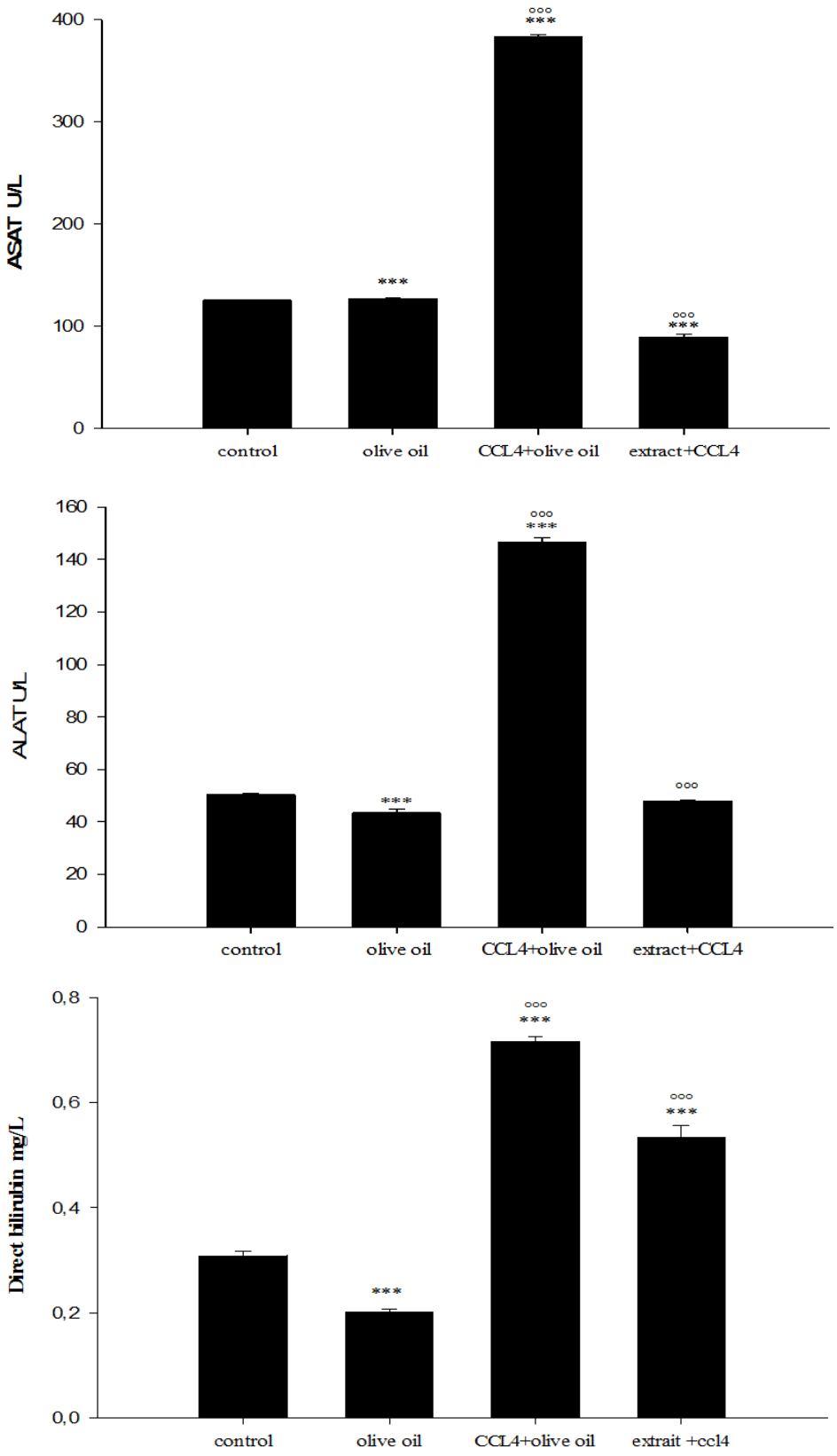
[a] *Values are expressed as mean ± SD.*
* *Significantly different from the control at the level of P< 0, 05. (Student's t-test).*
Number of animals (N) = 6

3.2 Effects of *Rubia tinctorum*'s Extract on Liver Function Biomarkers

Biochemical assays of ASAT, ALAT, ALP, GGT and direct bilirubin were estimated in serum samples as the liver function biomarkers. These results are given in Fig. 1. The CCl_4 treatment remarkably affected the liver specific enzymes. A significant ($p < 0.001$) increase in serum ASAT, ALAT, ALP, GGT and direct bilirubin activities (350 U/L, 140 U/L, 500 U/L, 7 U/L, 0,7 mg/L respectively) of CCl_4 treated rats was shown. This result suggests that these hepatic biomarkers were elevated in the serum due to release of the enzymes from damaged liver. However, a significant decrease ($p < 0.001$) was recognized in the respective serum activities of rats given *Rubia tinctorum*'s extract + CCl_4 compared with CCl_4 treated group.

3.3 Histopathological Finding of *Rubia tinctorum*'s Extract/CCl_4-Treated Group

Histopathological examination of the liver sections of normal control animals (Group I) and (Group II) showed normal hepatic cells with well preserved cytoplasm, prominent nucleus and nucleolus and well brought out central vein (Fig. 2A,B). The liver sections of CCl_4-intoxicated rats (Group III) showed massive fatty changes, necrosis, ballooning degeneration, histiocytes and Kuppfer cells around the central vein and the loss of cellular boundaries (Fig. 2C). The histological architecture of liver sections of rats treated with extract (Group IV) showed a more or less normal lobular pattern with a mild degree of fatty change, without necrosis almost comparable to the normal control (Fig. 2.D).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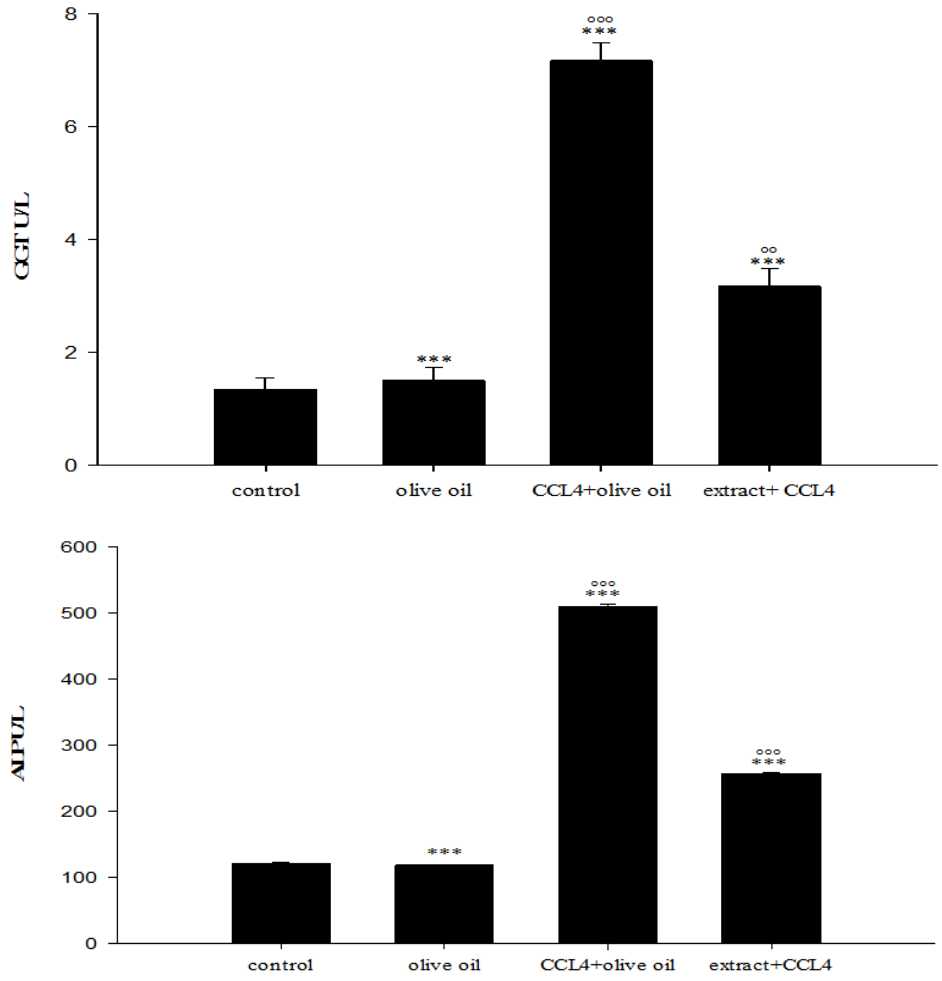
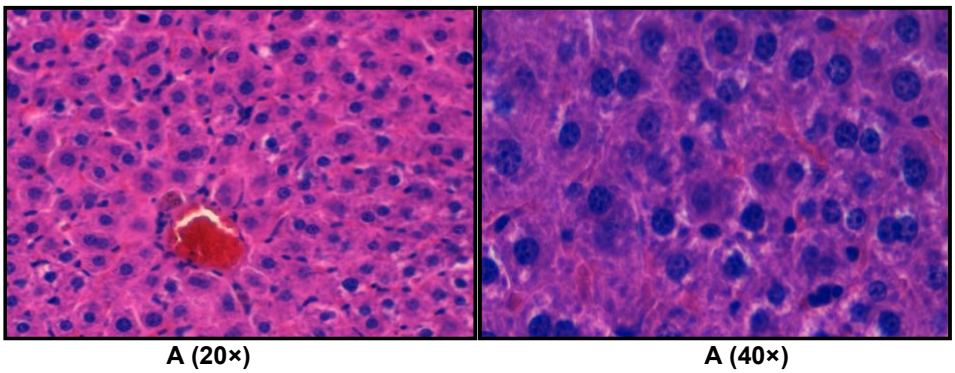


Fig. 1. Effect of the ethanol extract of *Rubia tinctorum* on some biochemical parameters (Alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), alkaline phosphatase (ALP), gamma glutamyltransferase (GGT) and direct bilirubin (D.Bil) of rats after CCl_4 administration

Data are expressed as mean ± S.E.M (N=6); (***) P<0.001 as compared with normal control group. (°°°) P< 0.001, (°°) P < 0.01 as compared with olive oil group



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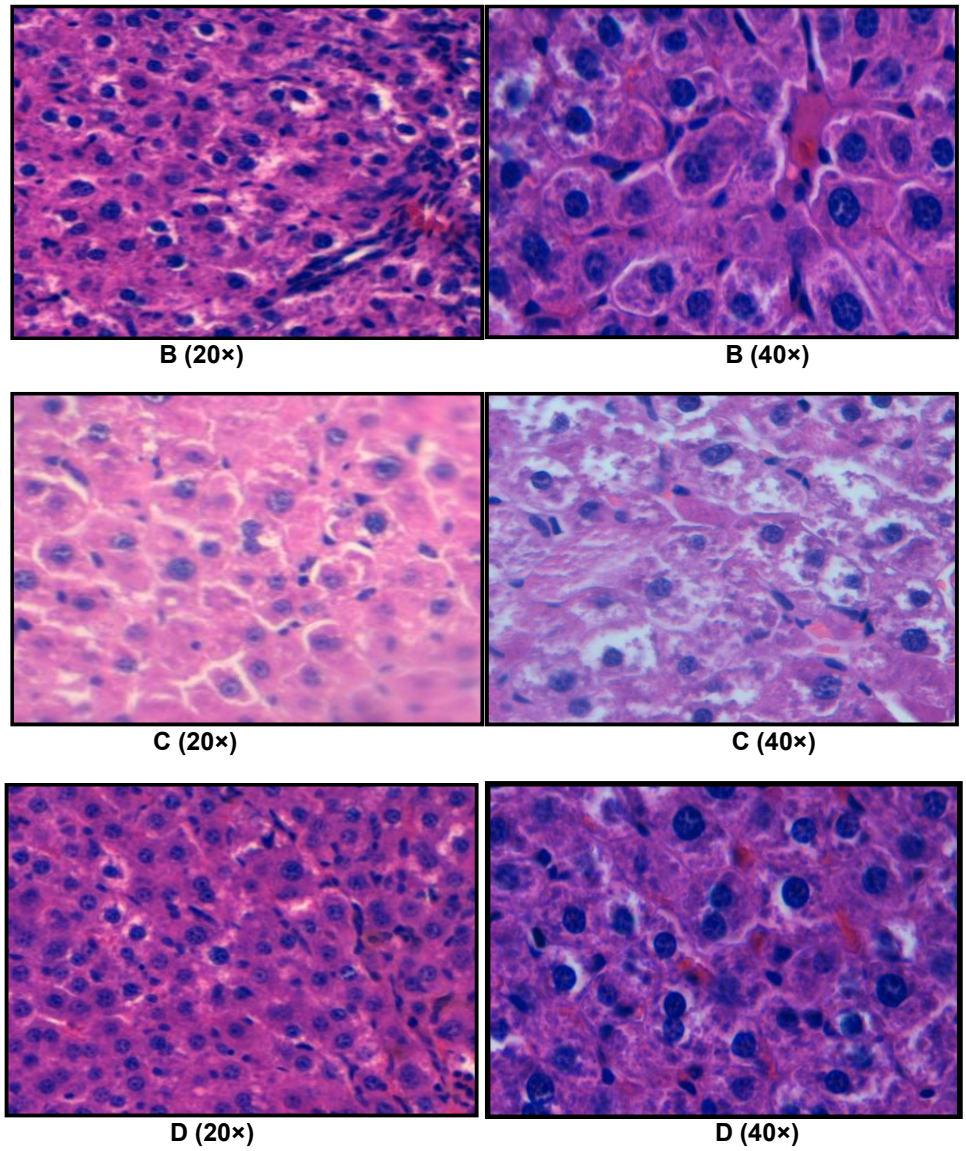

Fig. 2. Histology of liver changes in CCl_4 intoxicated and *Rubia tinctorum* treated rats

(A) Liver section of control rats and (B) Liver section of rats treated with olive oil showing normal hepatic cells with well-preserved cytoplasm; well brought out central vein; prominent nucleus and nucleolus. (C) Liver section of CCl_4 treated rats. (HE 40×) cholestase with biliary intracytoplasmic inclusion. (HE 20×) massive fatty changes, necrosis, ballooning degeneration. (D):Liver section of CCl_4 and extract treated rats. (HE 40×) normal lobular pattern with a mild degree of fatty change, without necrosis almost comparable to the normal control

4. DISCUSSION

In the present study serum hepatic enzymes, ASAT, ALAT, ALP, and GGT were markedly increased (*P<0.001*) in rats with the CCl_4 treatment compared to control group. The raise of hepatic markers have been associated to the liver damage as affirmed by Dkhil et al. [30]. Estimating the serum level of such enzymes provides a reliable image for structural integrity of liver cells. These enzymes are typically located in cytoplasmic area of the cell and are released into circulation in case of cellular damage [10,31,32,33]. Zimmerman et al. [34] showed that the carbon tetrachloride (CCl_4) induced increase of serum ALAT and ASAT levels which source from cell membrane and mitochondrial damages in liver. Other data indicating that these enzymes activities were significantly elevated after CCl_4 treatment [35,36,37,38]. In consistent, Atawia et al. [39] demonstrated that increase of serum ALAT and ASAT activities is a constant finding

following CCl_4 treatment in different experimental animal models. CCl_4 induced rats showed elevated serum ALP levels, which confirms the disorder of liver function. ALP is principally engaged in metabolite transport across cell membranes found in a decreasing order of abundance in placenta, ileal mucosa, kidney, bone, and liver. The elevated level of serum ALP is associated mainly with liver cell damage [40].

Results of the treatment with *Rubia tinctorum*'s extract was found to protect the structural integrity of hepatic cells by significantly decreasing the levels of cytosolic enzymes (ASAT, ALAT, ALP and GGT) activities induced by CCl_4 treatment rats. This finding is in agreement with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes [11].Many previous studies that showed administration of several antioxidant mixtures or herbal medicines significantly declined the levels of serum marker enzymes in CCl_4 treated animals as mentioned by Go et al. [41].

The normalization of elevated levels of serum enzymes, as observed after the treatment with *Rubia tinctorum*'s extract, is an indication of the stabilization of plasma membranes and the reversal of hepatic tissue damage caused by CCl_4. This *Rubia tinctorum*'s extract stabilizing effect on plasma membranes can explain the regain of normal serum activities of liver enzymes in CCl_4-induced liver damage after the treatment. We attribute the reason behind this to the antioxidant activity of *Rubia tinctorum*'s extract, which blocks, at least in part, the effects of released free radical metabolites of CCl_4 that leads to lipid peroxidation and hence membrane destabilization and eventually liver cell injury.

Direct bilirubin levels were significantly increased in the CCl_4 treated rats as compared to the control group. Treatment with the extract lead to a significant decrease (*P<0.001)* in their levels.

Bilirubin, a major breakdown product of haemoglobin rise when there is liver damage. Reduction of direct bilirubin levels after treatment by *Rubia tinctorum*'s extract show its protective effect against CCl_4 induced liver injury. This result is in line with the reports of some authors which have worked with different extracts such as *Cassia tora, Phyllanthus amarus, Zizphus maurtiana* [42].

The biochemical results were also confirmed by histological observations. The changes mostly include hepatocellular necrosis, fatty accumulation, inflammatory cells infiltration and other histological manifestations which were also consistent with the reports of other authors [43].

To the extent of our knowledge, the reviewed literature showed that no research has been reported on hepatoprotective proprieties of roots of *Rubia tinctorum* L. The present biochemical and histopathological findings of our extract showed a perfect development in ameliorating CCl_4 induced hepatic cells damage.

5. CONCLUSION

In conclusion, *Rubia tinctorum* L has a potent hepatoprotective effect against CCl_4-induced liver damage and improved the biochemical and histopathological results. Further studies are in progress for better understanding of the mechanism of action and to evaluate the efficacy of *R. tinctorum* on liver organelle that are possibly damaged during experimental hepatitis.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This study was carried out according to a protocol approved by the Cadi Ayyad University ethical committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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