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Original Article

Hepatoprotective effects of hydroethanolic extracts of *Crocus sativus* tepals, stigmas and leaves on carbon tetrachloride induced acute liver injury in rats

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ABSTRACT

Introduction: The present study investigated the hepatoprotective effects of stigmas, tepals and leaves of *Crocus sativus* on carbon tetrachloride (CCL4) induced liver injury in rats.

Methods: Hydroethanolic extracts of Crocus sativus (stigmas, tepals and leaves) were administrated daily for 14 days by oral gavage. In the present study, 30 male rats divided into five groups were treated as 1: normal rats gavaged with distilled water; 2: intoxicated rats gavaged with distilled water and injected with CCL4; 3: rats treated with stigmas extract and injected with CCL4; 4: rats treated with tepal extract and injected with CCL4; 5: rats treated with leaf extract and injected with CCL4. Bodyweight and the relative liver weight were determined. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), total cholesterol, triglycerides, bilirubin direct and total, total protein, albumin, urea and creatinine measured in plasma. Malondialdehyde (MDA) was quantified in liver homogenate.

Results: The experimental data showed that the stigmas and tepals extracts significantly prevented weight body loss and improved the relative liver weight. They significantly protected against elevation of ALT, AST, direct bilirubin, total bilirubin, LDH, ALP, creatinine and MDA. Also, they enhanced significantly total proteins and albumin compared to the CCL4 control group. Moreover, leaves reduced ALT, AST, total bilirubin, LDH and MDA significantly.

Conclusion: In conclusion, these results suggest that tepals, stigmas, and leaves extracts of Crocus sativus have hepatoprotective effects on CCL4 induced liver injury in rats.

Keywords:
Hepatoprotective effects
Liver injury
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Introduction

The liver is an essential organ of the human body. It is involved in multiple functions, such as metabolism, protein synthesis, enzyme secretion and detoxification (Vuda et al., 2012; Koyama and Brenner, 2017). Carbon tetrachloride (CCL4) is a well]-known toxin to induce hepatotoxicity in laboratory animals (Ingawale et al., 2014). Intoxication of CCL4 linked to its metabolites trichloromethyl and trichloromethyl peroxyl formed by cytochrome P450 enzymes (Chiu et al., 2018). These radicals reactive cause a hepatic inflammation (Callewaert et al., 2004; Lin et al., 2014), lipid peroxidation (Basu, 2003), fatty liver and cell necrosis (Recknagel et al., 1989). Protein carbonylation and enzyme disorder are also induced by CCL4 application (Recknagel et al., 1989a). Such perturbations are related to the high affinity of CCL4 metabolites for attachment to cell membrane proteins and lipids (Debnath et al., 2013).

Liver disease remains a global health problem (Ahsan et al., 2009). Unfortunately, conventional drugs used as pharmacotherapy for hepatic disease are expensive and associated with undesirable side effects. Extracts of medicinal plants are considered effective, safe and inexpensive for the treatment of liver disease (Jaishree and Badami, 2010). The *Crocus sativus* (saffron) belongs to the Iridaceae family. High quantities of organic saffron residues are produced during the processing of stigmas. About 350kg of tepals, 1500kg of leaves are necessary to obtain just 1kg of dry stigmas (Smolskaite et al., 2011). Currently, these by-products are not used and are very little studied. *Crocus sativus* stigmas have long been used in traditional medicine, cosmetics and as a food additive for coloring and flavoring (Melnyk et al., 2010).

Various studies have shown that saffron stigmas have antioxidant properties (Farahmand et al., 2013), antitumor (Samarghandian and Borji, 2014), anti-inflammatory, antidepressant (Lopresti and Drummond, 2014), antitussive (Hosseinzadeh and Ghenaati, 2006), hypolipidemic (Sheng et al., 2006) and could improve memory and learning abilities in rats (Ghadami and Pourmotabbed, 2009; Papandreou et al., 2011). Various beneficial properties of tepals extracts have been reported, including free radical scavenging (Goli et al., 2012; Zeka et al., 2015; Tuberoso et al., 2016), an antidepressant (Moshiri et al., 2006), antinociceptive and anti-inflammatory (Hosseinzadeh and Younesi, 2002; Kumar et al., 2012). They also have chelating metal, cytotoxic and antifungal activities (Zheng et al., 2011; Sánchez-Vioque et al., 2012; Serrano-Díaz et al., 2013).

Smolskaite et al. (2011) reported that saffron leaves considered as a source of bioactive components. Extract of saffron leaves have antioxidant and metal chelating activities (Sánchez-Vioque et al., 2012; Lahmass et al., 2017b). They also have an anti-aging and anti-proliferative effect (Sánchez-Vioque et al., 2016; Lahmass et al., 2017a). It has been reported in our laboratory that *Crocus sativus* by-products have an anti-hyperglycemic effect and improve the control of diabetes complications such as liver and kidney dysfunction (Ouahhoud et al., 2019). In addition, the presence of bioactive molecules with potent antioxidant properties in *Crocus sativus* stigmas, tepals and leaves prompted us to design the present study. In this current work, we investigate a comparative research on hepatoprotective effect of hydroethanolic extracts of tepals, leaves and stigmas of *Crocus sativus* on CCL4-induced acute liver injury in rats.

Materials and methods

Plant materials

The stigmas and tepals collected from a farm in Taliouine (30° 31′ 54″ North, 7° 55′ 25″ west-south of Morocco) and therefore the leaves were harvested from a farm in Oujda placement (34° 41′ 12″ North, 1° 54′ 41″ west-east of Morocco) which the authentic corms received from Taliouine. Saffron of each farm was cultivated without any chemical treatment. The saffron by-products were harvested between October and November (2016). Plant identification has been confirmed by Professor Fennane Mohammed, from Scientific Institute in the capital of Morocco. The plant have been deposited under the voucher number (HUMPOM210) at the Herbarium of University Mohammed first in Oujda, Morocco.

Chemicals

CCL4 purchased from Sigma chemicals, USA. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), bilirubin direct and total, total protein, albumin, total cholesterol, triglycerides, urea

and creatinine kits were purchased from Roche, France. All other chemicals used in this study were of high quality and analytical grade.

Hydroethanolic extracts preparation

Stigmas and the other parts of the plant (*Crocus sativus*) were separated manually. At room temperature, fresh tepals or dried and ground stigmas or leaves were dynamically extracted by maceration under agitation for 24h in the dark, with ethanol/water 80/20 (v/v) at a plant solvent. Then, the mixture was filtered (0.45µm) and the marc recuperated for the second extraction. The protocol repeated three times and the total filtered solvent phase dried at 37°C on a rotatory evaporator. Finally, the resulting dried extracts were kept as stable at -20°C until use.

Animals

Adult male Wistar rats (weight 150-280g) were housed in animal house of the Department of Biology, University of Mohammed First, Morocco and were maintained at constant temperature of 21±2°C during 12h light/dark cycle. They fed on a commercial diet (dry rat pellets supplies produced by SONABETAIL Society, Oujda, Morocco) and water throughout the experiments.

This study was following the international standard guidelines for the car and use of laboratory animals.

Experimental procedure

Hydroethanolic extracts of *Crocus sativus* (stigmas, tepals and leaves) were administrated daily for 14 days by oral gavage. In the present study, 30 male rats divided into five groups were treated as follows: Group 1: normal rats gavaged with distilled water; Group 2: intoxicated rats gavaged with distilled water and injected with CCL4; Group 3: rats treated with stigma extract and injected with CCL4; Group 4: rats treated with tepal extract and injected with CCL4; Group 5: rats treated with leaf extract and injected with CCL4.

Except for the standard control group, all animals of other groups were received CCL4 intraperitoneally at a dose of 1ml/kg body weight (CCL4 dissolved in 25% olive oil; v/v) at the 7th and 14th days of treatment to induce experimental liver disease. Rats weighed before and after the treatment. All animals treated and observed daily for two weeks.

After twelve hours of final intraperitoneal injection of CCL4, all rats were anesthetized and blood samples were collected from abdominal aorta in Heparin tubes (VACUETTE®). Then, whole blood centrifuged at 3000g for separation of plasma. Approximately 2ml of plasma from every rat has been conserved at -20°C until biochemical assays. After blood collection, the liver was removed and deposited in physiological water, dried on filter paper and weighed. The liver stored at -20°C for the determination of malondialdehyde (MDA).

Biochemical assays

ALT, AST, ALP, LDH, bilirubin direct and total, total protein, albumin, total cholesterol, triglycerides, urea and creatinine were determined with autoanalyzer from Roche diagnostics (COBAS INTEGRA®).

Determination of MDA

Lipid peroxidation was determined according to the thiobarbituric acid (TBA) protocol (Buege & Aust, 1978) with slight modifications. The liver (1g) was homogenized in 5ml of PBS buffer (pH = 7.4) and centrifuged at 14500rpm during 15min. Then, 2ml of the reagent (0.375% TBA and 15% trichloroacetic acid dissolved in 0.25N hydrochloric acid) was added to 1ml of the supernatant. Then, samples were putted in water bath regulated on 100°C for 30min and centrifuged at 4750rpm during 5min. The absorbance was measured at 535nm and the concentration of MDA was calculated using a molar attenuation coefficient 1.56x. The results expressed in nanomoles of MDA produced per milligram of tissue.

Statistical analysis

Statistical analysis of data realized by one-way ANOVA and groups were compared by Tukey's multiple comparison post-test using GraphPad Prism 5.0 statistical software. The data were expressed as mean±SEM for six rats in each group and with significance levels of $P<0.05$, $P<0.01$ and $P<0.001$.

Results

Effect of stigmas, tepals and leaves extracts on the body weight and the relative liver weight

The effects of stigmas, tepals and leaves extracts on the body weight gain and the relative liver weight shown in Figure 1. The CCL4 group showed a significant weight loss ($P<0.001$) compared to the normal control group. Stigmas (50mg/kg/day) and tepals (250mg/

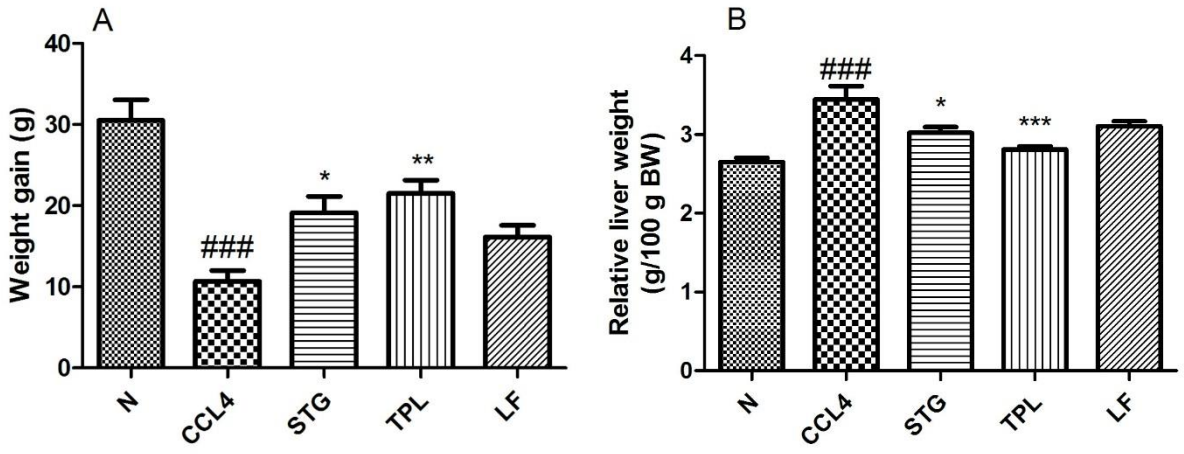

FIGURE 1. Effect of *Crocus sativus* stigmas (STG, 50mg/kg), tepals (TPL, 250mg/kg) and leaves (LF, 250mg/kg) on the body weight gain (A) and the relative liver weight (B) in CCL4-intoxicated rats. The results are expressed as mean±SEM (n=6). $^{###}P<0.001$ compared with normal control group (N); $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with CCL4.

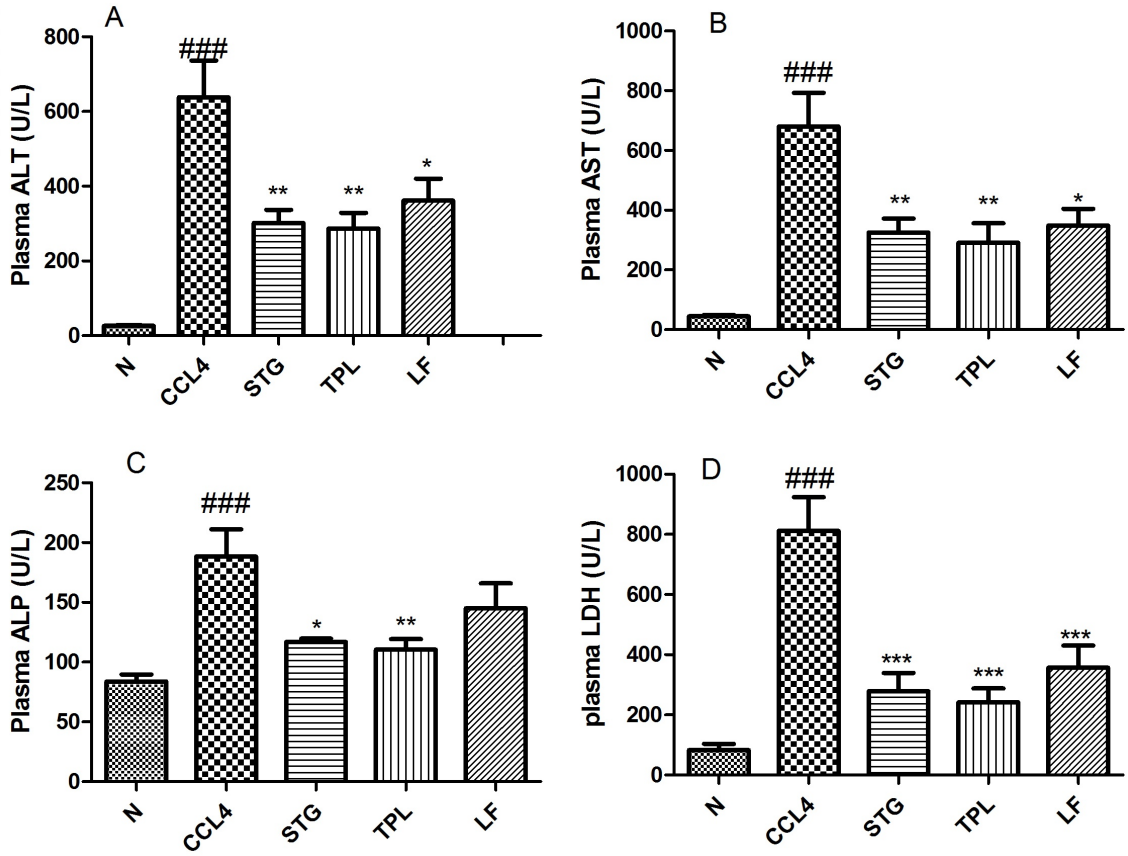

FIGURE 2. Effect of *Crocus sativus* stigmas (STG, 50mg/kg), tepals (TPL, 250mg/kg) and leaves (LF, 250mg/kg) on plasma levels of (A) alanine aminotransferase (ALT), (B) aspartate aminotransferase (AST), (C) alkaline phosphatase (ALP) and (D) lactate dehydrogenase (LDH) in CCL4-intoxicated rats. The results are expressed as mean±SEM (n=6). ###P<0.001 compared with normal control group (N). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with CCL4.

kg/day) extracts significantly protect against weight loss in CCL4 rats ($P<0.05$ and $P<0.01$, respectively) compared to the CCL4 untreated rats. While there was no significant effect on the body weight gain in the group treated with leaves extract (250mg/kg/day). The CCL4 group showed a significant increase ($P<0.001$) of relative liver weight compared to the normal group. The treatment with stigmas and tepals extracts decreased significantly the relative liver weight ($P<0.05$ and $P<0.001$, respectively). However, there was no

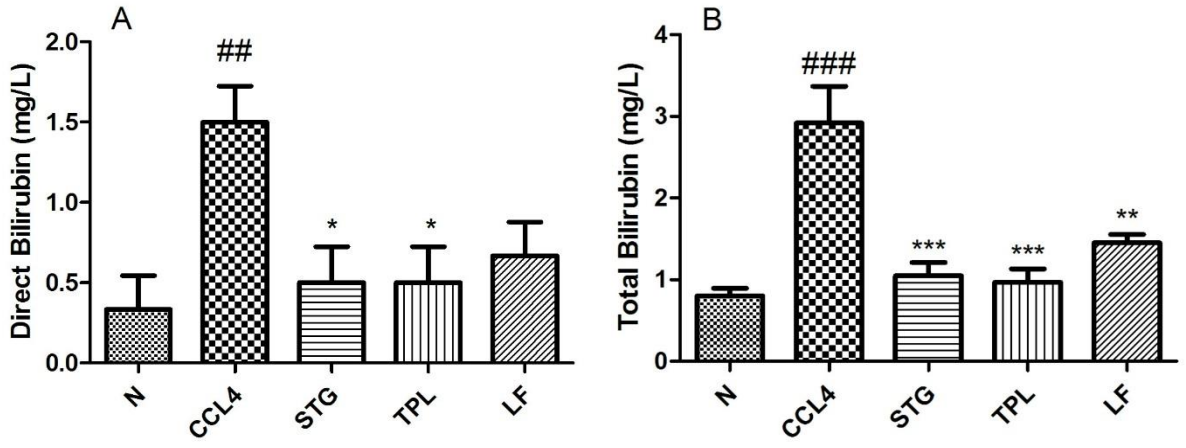

FIGURE 3. Effect of *Crocus sativus* stigmas (STG, 50mg/kg), tepals (TPL, 250mg/kg) and leaves (LF, 250mg/kg) on plasma levels of direct bilirubin (A) and total bilirubin (B) in CCL4-intoxicated rats. The results are expressed as mean±SEM (n=6). $^{###}P<0.001$; $^{##}P< 0.01$ compared with normal control (N). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with CCL4.

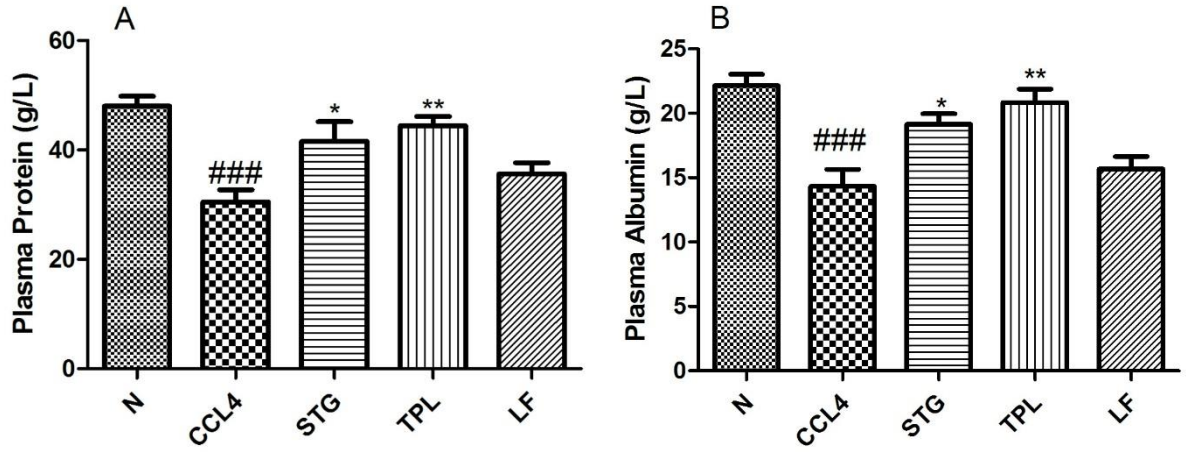

FIGURE 4. Effect of *Crocus sativus* stigmas (STG, 50mg/kg), tepals (TPL, 250mg/kg) and leaves (LF, 250mg/kg) on plasma levels of total protein (A) and albumin (B) in CCL4-intoxicated rats. The results are expressed as mean±SEM (n=6). $^{###}P<0.001$ compared with normal control group (N). $^{*}P<0.05$, $^{**}P<0.01$ compared with CCL4.

significant effect on the relative liver weight in the group treated with leaf extract.

Effect of stigmas, tepals and leaves extracts on plasma levels of ALT, AST, ALP and LDH

Figure 2 shows the plasma levels of ALT (A) and AST (B) in normal and experimental animals in each group. The CCL4 group showed a significant increase ($P<0.001$) in the level of plasma ALT and AST compared with the standard control group. The administration of stigmas, tepals and leaves extracts significantly decreased plasma ALT ($P<0.01$, $P<0.01$ and $P<0.05$, respectively) and plasma AST ($P<0.01$, $P<0.01$ and $P<0.05$, respectively).

The level of plasma PAL and LDH levels in control normal, control CCL4 and treated CCL4 animals showed in Figure 2 (C and D, respectively). The treatment of rats with CCL4 induced a significant increase in plasma ALP and LDH ($P<0.001$). In contrast, stigmas and tepals extract significantly decreased plasma ALP ($P<0.05$ and $P<0.01$, respectively) compared to the CCL4 group. Although the leaves extract group did not present a significant decrease. Then, daily administration for 14 days of stigmas, tepals and leaves extracts significantly reduced the plasma LDH level ($P<0.001$) compared to the CCL4 group.

Effect of stigmas, tepals and leaves extracts on plasma levels of direct bilirubin and total bilirubin

Figure 3 summarizes the plasma levels of direct bilirubin (A) and total bilirubin (B) in normal, CCL4 untreated and CCL4 treated rats. The treatment of

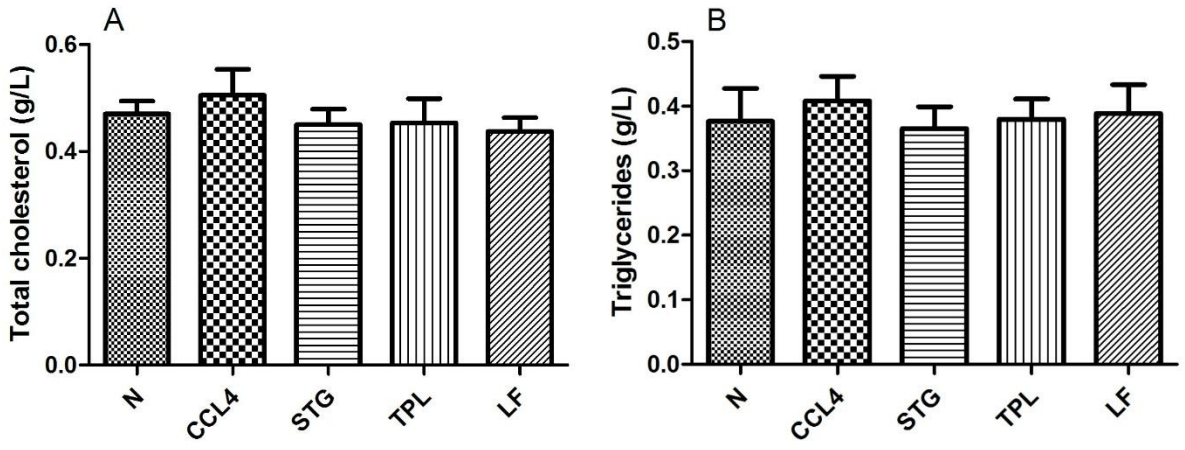

FIGURE 5. Effect of Crocus sativus stigmas (STG, 50mg/kg), tepals (TPL, 250mg/kg) and leaves (LF, 250mg/kg) on plasma levels of total cholesterol (A) and triglycerides (B) in CCL4-intoxicated rats. The results are expressed as mean±SEM (n=6).

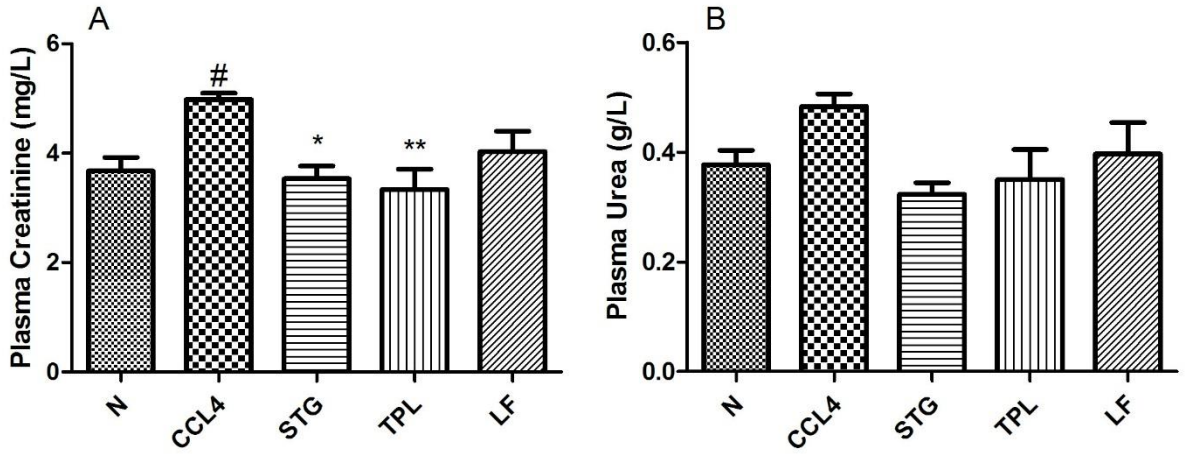

FIGURE 6. Effect of *Crocus sativus* stigmas (STG, 50mg/kg), tepals (TPL, 250mg/kg) and leaves (LF, 250mg/kg) on plasma levels of creatinine (A) and urea (B) in CCL4-intoxicated rats. The results are expressed as mean±SEM (n=6). $^{\#}P<0.05$ compared with normal control group (N). $^{*}P<0.05$, $^{**}P<0.01$ compared with CCL4.

rats with CCL4, induced a significant elevation in direct plasma bilirubin and total bilirubin ($P<0.01$ and $P<0.001$, respectively). The height of plasma direct bilirubin significantly slowed down after the daily administration of stigmas and tepals extracts ($P<0.05$). Besides, stigmas, tepals and leaves extracts significantly decreased total plasma bilirubin ($P<0.001$, $P<0.001$ and $P<0.01$, respectively) compared to the CCL4 group.

Effect of stigmas, tepals and leaves extracts on plasma levels of total proteins and albumin

The levels of plasma total proteins and albumin in control normal, control CCL4 and treated CCL4 animals showed in Figure 4. The treatment of rats with CCL4, induced a significant decrease in plasma total proteins and albumin levels ($P<0.001$). The administration for 14 days of stigmas and tepals extracts significantly protected against the decrease of the total plasma proteins and albumin levels compared to the CCL4 group ($P<0.05$ and $P<0.01$ respectively). However, there was no significant effect on the total plasma proteins and albumin levels in the group treated with leaf extract.

Effect of stigmas, tepals and leaves extracts on plasma levels of cholesterol and triglycerides

The plasma levels of total cholesterol and triglycerides represented in Figure 5. Results showed that intraperitoneal injection of CCL4 to the rats did not influence the plasma levels of total cholesterol and triglycerides significantly compared with the control group. Furthermore, there was no significant effect on plasma levels of total cholesterol and triglycerides in

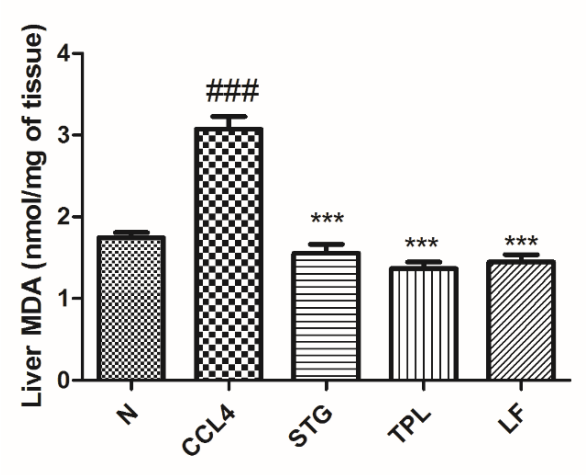

FIGURE 7. Effect of Crocus sativus stigmas (STG, 50mg/kg), tepals (TPL, 250mg/kg) and leaves (LF, 250mg/kg) on liver levels of MDA in CCL4-intoxicated rats. The results are expressed as mean±SEM (n=6). $^{###}P<0.001$ compared with normal control group (N). $^{***}P<0.001$ compared with CCL4.

stigmas, tepals and leaves extract groups compared with the CCL4 control group.

Effect of stigmas, tepals and leaves extracts on plasma levels of creatinine and urea

On day 14, the plasma levels of creatinine and urea in normal animals and animals injected with CCL4 in each group shown in Figure 6. Creatinine in plasma increased in untreated CCL4 rats compared with the normal group ($P<0.05$). The administration of the stigmas and tepals extracts significantly decreased plasma creatinine level ($P<0.05$ and $P<0.01$, respectively). However, there was no significant decrease in the leaves extract group. There was no significant elevation on the plasma urea level between normal and CCL4 control rats. Also, there was no significant decrease in plasma urea levels in the stigmas, tepals and leaves extract groups compared with the CCL4 control group.

Effect of stigmas, tepals and leaves extracts on liver MDA levels

The liver MDA levels in control normal, control CCL4 and treated CCL4 animals showed in Figure 7. The injection of rats with CCL4, induced a significant elevation in liver MDA levels ($P<0.001$); while a significant decrease in liver MDA levels observed in the stigmas, tepals and leaves extract groups ($P<0.001$).

Discussion

The present study illustrated the protective propriety of stigmas, tepals, and leaves of *Crocus sativus* against CCL4 induced hepatic damage in rats. CCL4 intoxication is a standard model for assessing the hepatoprotective activity of compounds (Recknagel et al., 1989; Manibusan et al., 2007). CCL4 causes rapidly severe hepatic necrosis at single exposure and the pathological damage induced in CCL4 treated animals is similar to the symptoms of cirrhosis in humans (Lin et al., 2014). Hepatotoxicity of CCL4 related to its metabolites trichloromethyl and trichloromethyl peroxyl which are formed by cytochrome P450 enzymes (Chiu et al., 2018). These radicals reactive cause hepatic inflammation (Callewaert et al., 2004; Lin et al., 2014), lipid peroxidation (Basu, 2003), hepatic steatosis, cell necrosis (Recknagel et al., 1989), protein carbonylation and enzyme disorder (Recknagel et al., 1989). Consequently, various species of oxygen radicals are present, including superoxide, hydroxyl radicals and hydrogen peroxide, which can be attached to DNA, proteins and phospholipids, resulting in damage to cell membrane structure and organs (Lin and Huang, 2000).

The results of this study showed that the extract of tepals and stigmas prevented significantly weight loss and enhanced relative liver weight. It significantly protected against elevations of ALT, AST, direct bilirubin, total bilirubin, LDH, PAL, creatinine and MDA. In addition, they significantly improved total protein and albumin compared to the CCL4 control group. Moreover, the leaves significantly reduced ALT, AST, total bilirubin, LDH and MDA. Various studies indicated that the relative liver weight was a sensitive indicator of hepatotoxicity in a CCL4-induced liver

damage model (Uemitsu et al., 1986; Wu et al., 2008). An increase in liver weight in CCL4 control rats is likely due to the fat vacuole accumulation revealed by hematoxylin and eosin staining (Wu et al., 2008). The CCL4 group showed a significant loss weight and a significant elevation in relative liver weight compared to the normal control group. While stigmates and tepal extract significantly protected against weight loss and significantly decreased relative liver weight in CCL4 rats compared to the CCL4 group.

Elevated ALT, AST, ALP and LDH levels are specific indices of liver damage (Thabrew et al., 1987).The present data showed a significant elevation in the plasma levels of ALT, AST, ALP, and LDH in CCL4 group, indexing the damage on hepatic cells (Lee et al., 2007; Kale et al., 2012). However, the plasma ALT, AST and LDH activities significantly declined by treatment with tepals, stigmas and leaves extracts. Also, the plasma ALP was significantly reduced by treatment with tepals and stigmas extracts. Bilirubin is the product of heme catabolism, produced during the destruction of old or abnormal erythrocytes. Its elevation considered a clinical index of the binding, hepatobiliary disease and excretory capacity of liver cells (Martin, 1992; Vuda et al., 2012). Our data revealed a significant elevation of direct bilirubin and total bilirubin in plasma rat on exposure to CCL4. Whereas, administration of tepals, stigmas and leaves extracts restored the levels of total bilirubin in plasma rat. Then, our extracts may reflect protection against the hepatic damage caused by CCL4.

In this study, total protein and albumin levels in plasma were also measured. The decrease in total protein and albumin levels may be due to liver dysfunction. Albumin synthesized in the liver, and its measurement employed to control the liver dysfunction (Friedman et al., 1980). Reduced level of total protein and albumin were observed in CCL4 group, which indicate the liver dysfunction. While the treatment with tepals and stigmas significantly restored the protein content. Our study showed that CCL4 had no effect on increasing plasma levels of total cholesterol and triglycerides. Moreover, there was no significant effect on total cholesterol and triglyceride plasma levels in the tepals, stigmas and leaves extract groups compared to the CCL4 group.

These results are in agreement with those of Byun et al. (2018) which found that CCL4 administration had no significant effect on plasma total cholesterol and triglyceride levels. Urea and creatinine are considered an indicator of renal dysfunction (Perrone et al., 1992; Burtis et al., 1999). This finding indicates that CCL4 control rats increased plasma creatinine levels significantly. However, this marker was decreased in hepatotoxic rats treated with tepals and stigmas extracts. While, there was no significant increase in plasma urea levels in CCL4 rats.

Studies have shown that lipid peroxidation is the first result of CCL4-induced liver injury and considered as an indicator of oxidative damage (Basu, 2003; Chiu et al., 2018). MDA is the final product of polyunsaturated fatty acids peroxidation and usually employed as a biomarker of this process (Janero, 1990). In this study, the injection of rats with CCL4 caused a significant elevation in liver MDA levels. While, the levels of hepatic MDA significantly attenuated by the administration of tepals, stigmas and leaves extracts.

Antioxidant compounds have been reported to prevent oxidative damage to the liver and may avoid the risk of liver disease (Bertolami, 2005). Various studies have shown that saffron stigmas, tepals and leaves have antioxidant properties. The stigmas are rich in hydro-soluble carotenoids, mainly crocin and crocetin, while the tepals and leaves are rich in polyphenols and flavonoids compounds (Sánchez-Vioque et al., 2012; Farahmand et al., 2013; Tuberoso et al., 2016). The hepatoprotective effect of the tepals and leaves extract may be due to polyphenols and flavonoids compounds. While the action of stigmas probably due to hydro-soluble carotenoids.

The reinstatement of serum enzyme levels (ALT, AST, ALP and LDH) to normal levels in CCL4 group treated with hydroethanolic extract of different parts of *Crocus sativus* may indicate prevention of the leakage of intracellular enzymes by stabilizing the hepatic cell membrane. The hepatoprotective effect of *Crocus sativus* by-products may be due to the improvement of non-enzymatic antioxidant, which plays an important role in maintaining the body's antioxidant defense mechanism, conjugates with free radicals to protect the integrity of cell membranes (He et al., 2012). Also, the increase in the enzymatic antioxidant levels such as superoxide dismutase and catalase can be considered as an efficient defense mechanism to prevent and scavenge the free radical (Bansal et al., 2005).

In addition, hydroethanolic extracts of stigmas, tepals

and leaves may protects against hepatic fibrosis via multiple protective mechanisms such as the inhibition of CCL4 metabolic activation by blocking the CYP450 enzymatic system, the anti-inflammatory effect of principals compounds, the increase of beneficial restorative macrophages (CD11bhi F4/80int Ly-6Clo) after liver injury, the elevation of MMP9 (type of matrix metalloproteinases) level to regulate extracellular matrix reconstructing after liver injury and the reduction of hepatic stellate cells activation which can transformed into myofibroblast-like cells (Sun et al., 2018). However, the exact protective mechanism(s) of these by-products of *Crocus sativus* is unknown.

Conclusion

In conclusion, this suggests that tepals, stigmas and leaves extracts from *Crocus sativus* have a hepatoprotective effect on CCL4-induced hepatotoxicity in rats. In addition, the hepatoprotective effect of these *Crocus sativus* by-products could be due to the presence of bioactive molecules with antioxidant properties, which could exert various health benefits.

Acknowledgments

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Conflict of interest

None of the authors had a conflict of interest.

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