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RESEARCH PAPER

Hepato-defensive effect of Cysteine, Ursodeoxycholic acid and Silymarin on histopathological and some biochemical parameters of Wister albino rat intoxicated by CCl4

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A B S T R A C T:
The liver is an essential organ in the body for detoxification drugs and toxic substances. The present study was designed to investigate the hepatoprotective effects of L-Cysteine, Ursodeoxycholic acid (Ursoflor), and Silymarin against carbon tetrachloride-induced liver injury in rats. Twenty adult male rats were used in this work; they were randomly divided into five groups (4 rats/group). The first group served as the control group and received 0.5 ml of normal saline orally daily; the second group injected intraperitoneally with CCl_4 (1.5 ml/kg B.W. twice a week), the third group were treated orally with the silymarin at dose of (200 mg/kg B.W. in normal saline orally + CCl_4 daily, the fourth group was treated with Ursoflor drug at dose of (50 mg/kg B.W. in 0.5 normal saline orally+CCl_4 daily. While the fifth group was treated with cysteine at dose of (50mg/kg B.W. in normal saline by oral gavage) +CCl_4. at the end of this study, the biochemical assessment showed alterations in enzyme activity which indicates the hepatocyte injury due to CCl_4 toxicity, The production of ROS indicated by elevated levels in MDA and Peroxynitrite was noticed, which improved partially by the antioxidant effect of L- Cysteine, Ursodeoxycholic acid, and Silymarin, these results further backed by histological examination of the liver showed that CCl_4 caused severe injury to the liver including a high number of fat droplets deposition in the cytoplasm of the hepatocyte, clear vascularization appeared and disorganization of the liver cells were determined, on the other hand, Silymarin has showed good protection against these injurious effects of CCl_4.

KEY WORDS: L-Cysteine, Ursoflor, Silybum marianum, carbon tetrachloride, hepatotoxicity
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1. INTRODUCTION:

The liver has many vital bodily functions, including metabolism and digestion; it is also responsible for detoxifying drugs, xenobiotics, and other harmful chemicals that enter the body (Wang et al., 2014). According to many studies, liver diseases kill between 18000 and 20000 people yearly (Fatma and Uphadhyay, 2015). Hepatotoxicants are exogenous substances that cause liver harm, such as an overdose of some pharmaceutical products (acetaminophen, nimesulide, antitubercular medications like isoniazid, rifampicin, etc.) or chemical products such as (alcohol, CCl_4, beta galactosamine, thioacetamide, etc.)(Pandit et al., 2012).

Synthetic medications are quite efficient, but they have many negative side effects. Natural antioxidants and hepatoprotective plant products are seen to be the greatest alternative to manufactured hepatoprotective medications since they come from nature and have fewer adverse effects(Madrigal-Santillán et al., 2014).

Silybum marianum (milk thistle) Silymarin is made from the fruits of Silybum marianum (L.)

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which belongs to the Asteraceae family and has widely been used as a hepatoprotective, cardioprotective, neuroprotective, anti-inflammatory, and anti-carcinogenic medicine in herbal medicine (Ahmed et al., 2020). Silymarin, the mixture of flavonolignans including silibinin, silydianin, and silychristin acts against different biological toxins and poisons that have radical scavenging, anti-oxidative, chelating, anti-apoptotic properties and regulate the inflammatory responses(Fanoudi et al., 2020). Silymarin is commonly recommended and self-administered as an extra and alternative hepatoprotective medication because of its antioxidant and chemoprotective actions on the liver(Testino et al., 2013). Silymarin has been widely used to treat fatty liver, nonalcoholic fatty liver disease, viral hepatitis and drug-induced liver injury because it is effective in restoring liver function and regeneration of liver cells(Gazak et al., 2007, Abenavoli et al., 2018).

Carbon tetrachloride (CCl_4) has been known to have a highly hepatotoxic effect leading to cirrhosis and many harmful effects (Demiroren et al., 2014). This harmful effect on the liver occurs due to the CCl_4 metabolites (i.e., toxic trichloromethyl and trichloromethyl peroxy radicals) inherent in the cytochrome P450 system (Ustyol et al., 2017). Among the various mechanisms involved in the hepatotoxic effect of carbon tetrachloride, one is oxidative damage through free radical generation(Al Amin and Menezes, 2020). Liver enzymes, including Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) activities at baseline before inducing any xenobiotics are considered to be accepted in limit values. These transaminases will be elevated during the induction of harmful compounds, which will be accepted as hepatic injury(Guo et al., 2019).

Ursodeoxycholic acid (UDCA) is a naturally-occurring bile acid that exist in small quantities in human bile. Ursodeoxycholic acid suppresses the synthesis and secretion of cholesterol by the liver and inhibits intestinal absorption of cholesterol. Ursodeoxycholic acid (UDCA) is a secondary bile acid with exciting benefits as a drug proven to be safe and can reduce inflammation. The UDCA is the drug most widely used as a therapeutic agent for the treatment of hepatopathies (Feng et al., 2013, Mohammed Saif et al., 2012). The mechanism of UDCA in improving liver function is not precise yet (Yoon et al., 2018). However, the distinct physiological and physicochemical properties compared to the other bile acids play a crucial role because of their anti-inflammatory properties and protection (Niu et al., 2019, Işık et al., 2017, Ko et al., 2019, Talebian et al., 2020). The UDCA offers therapeutic potential in a variety of cholestatic liver disorders.

L- Cysteine is a clinically effective amino acid in glutathione (GSH), with the sulfhydryl group (SH) as a scavenger for free radicals (Gould and Pazdro, 2019). Silymarin is a polyphenolic extracted from Silybum marianum (Kabiri et al., 2013). The plant is used to treat hepatic injury and oxidative stress induced by CCl_4 in rat livers (Hermenean et al., 2015). Increasing investigations are held on the benefit of using various plant extracts and products as hepatoprotective agents as antioxidants to minimize the toxicity in such rats (Muriel and Rivera-Espinoza, 2008, Vitaglione et al., 2005). This study aimed to determine the ameliorative effect of L-cysteine and ursoflor and also prove silymarin protection of liver tissue against liver toxicity caused by CCl_4.

2.MATERIALS AND METHODS

2.1.Chemicals

Ursodeoxycholic acid (ursoflor drug) is obtained from a local pharmacy, while silymarin, CCl_4, and L-cysteine are obtained from Sigma-Aldrich.

2.2.Animals

The University of Salahaddin's Ethical Committee authorized this work with reference number: 918-761-0484. Twenty adult male Wister rats (238-248 g) were maintained in the animal house under a controlled light/dark cycle (12hr/12hr), controlled temperature (21±3ºC), and unrestricted access to standard rat food and water.

2.3.Experimental design

The Experimental animals were kept and experimented on in the animal house of the Biology department/College of Education/ University of Salahaddin-Erbil. The experiment was carried out for a period of one month; the groups were organized as follows:

1. Group (1) Control group: (0.5 ml, normal

saline by oral gavage).
2. Group (2) CCl_4 group (1.5 mg\kg B.W. in olive oil) injected intraperitoneally twice a week
3. Group (3) Silymarin group (200 mg\ kg B.W. in normal saline by oral gavage) + CCl_4
4. Group (4) Ursoflor drug groups (50mg/kg B.W. in normal saline by oral gavage) + CCl_4
5. Group (5) Cysteine group (50mg/kg B.W. in normal saline by oral gavage) + CCl_4

The doses of all compounds used were calculated according to the literature (Al-Salmi et al., 2019).

After the last treatment, the animals were fasted overnight. Later on, all the rats were euthanized with general anesthesia (xylazine and ketamine hydrochloride), which were administered intraperitoneally to take the blood. Blood samples were collected from the heart vessels into the non-heparinized bottles and centrifuged for 10 minutes at 3000 rounds per minute (rpm) to separate the serum. The serum was separated into clean dry tubes using a micropipette and kept frozen until enzyme assay analyses and other biochemical parameters were analyzed.

2.4.Histological studies

During the dissection, the abdominal region was opened, and the liver was excised, then cut into small pieces (0.5 cm^3 thick) and saved in the saline formalin. Following was the process of histological preparation, the organs fixed in formal saline fixative for 24-48 hrs. To harden the tissue, and inactivates enzymes that might otherwise degrade the tissue, then dehydrated by a series of alcohol solutions in ascending concentrations up to 100%, Then cleared in xylene three times each for one hour, infiltrated in paraffin wax also three times each for ½ hr. in the oven at 60 C^o and lastly emerged in wax to form a paraffin-embedded block of the sample. Using Microtome, we obtained at 5µm thick sections. The sections were stained with hematoxylin and eosin(Ozturk et al., 2012).

2.5.Biochemical analysis

2.5.1.Determination of liver function

The activity of **(Aspartate transaminase)** AST and (**Alanine transaminase)** ALT were assessed using diagnostic RANDOX kits (Randox laboratories Ltd; UK). The activity of these two enzymes was measured calorimetrically at 546 nm using a spectrophotometer.

2.5.2.Evaluation of oxidative stress

2.5.2.1.Evaluation of Malondialdehyde

Malondialdehyde, produced by the breakdown of polyunsaturated fatty acids, is a helpful indicator of peroxidation reactions. The thiobarbituric acid method of Buege and Aust (1978) was used to measure MDA, which reacts with thiobarbituric acid to yield a pink color. Absorbances were determined at 532 nm.

2.5.2.2.Evaluation of Peroxynitrite

The amount of peroxynitrite in the blood was measured using the technique described by Beckman et al. (1992). The phenol nitration caused by peroxynitrite was detected spectrophotometrically at 412 nm.

2.6.Statistical analysis

GraphPad Prism 7 was used to analyze data statistically using two-way ANOVA, followed by Dunnett's comparison test to compare treatment and control groups. At a p-value of 0.05, a statistically significant difference was considered. The data were reported in the form of a Mean ± Standard error.

3.RESULTS

3.1.Biochemical Evaluations

Table (1) shows the activity of ALT and AST enzyme in control and experimental groups, it was observed that treatment with CCl_4(group 2) led to increased ALT levels, and this increase was highly significant when compared to the control group and treatment groups, Although the treatment groups (3,4,5) also demonstrated an elevation in ALT enzyme in comparison with the control group. The activities of AST followed the same pattern as ALT enzyme, treatment with CCl_4 increased AST when compared to the control groups, and the difference was significant; treatment with the antioxidants in groups (3, 4, 5) led to decreased AST levels when compared to group (2).

Table (2) shows the results of malondialdehyde and peroxynitrite in the serum of all studied groups. The value of Groups (2), (3), (4), and (5) was statistically higher than Group (1). Also, Groups (3), (4), and (5) showed a significant reduction in MDA values compared to Group (2). Also, peroxynitrite shows a significant increase in values in Groups (2) and (5) compared to Group (1). On the other hand, Groups (3) and (4) showed

non-significant elevated values compared to Group (1). A significant reduction in Peroxynitrite for Groups (3) and (4) compared to Group (2) was found. While a non-significant decrease in Group (5) compared to Group (2) was depicted.

3.2Histological investigations

Microscopic evaluation of liver tissues showed normal architecture of the hepatic lobules, normal central vein, and hepatic plate (Fig. 1 A & B). In the CCl_4 treated groups, the normal structure of lobules was destroyed in which fat droplets, hepatocellular necrosis, dilation of the hepatic veins, and vacuolization were seen (Fig. 2 A & B).

4.DISCUSSION

In the present study, oral administration of L-Cysteine, Ursodeoxycholic acid, and Silymarin were used for one month to treat Wister albino rats intoxicated with CCl_4. From table (1), the injury of hepatic cells was proven by the elevation of ALT and AST activities. In groups (3), (4), and (5), a significant reduction in the activities of both enzymes compared to Group (2) was observed, which could attribute to the anti-inflammatory properties of the three used agents as treatment which possess antioxidant potential thus induced hepato-protective property (Mruthunjaya and Hukkeri, 2007) and (Krishna et al., 2010). The present study results agree with Kotb (2012), who found a 35% drop in ALT and a 33% drop in AST activities after treatment with Ursodeoxycholic acid.

The results of table (2) also demonstrated the protective effects of utilized compositions for hepatic cells indicating the antioxidant properties of the three used compositions as treatment. L-Cysteine showed the highest protective effect due to its antioxidant activity. Hence the SH is involved in the function of many compounds as an in vivo antioxidant, i.e., glutathione (Bouhalit and Kechrid, 2018). The protection effect of hepatic cells is not yet achieved, possibly due to the short treatment time with these antioxidants. This time is not enough to stabilize hepatic cells, which need an alteration in lipid peroxidation for about three months (Ruutu et al., 2002).

The finding of this study indicates a significant alteration in the levels of lipid peroxidation markers malondialdehyde and peroxynitrite in the liver due to CCl_4 metabolite radicals which were improved by administration of L-cysteine, Ursodeoxycholic acid, and silymarin. Accordingly, the assessment of the level of the enzyme markers ALT and AST are more specific to the liver and are better parameters for detecting liver injury. The elevation in their activities and the inverted values after administration of the antioxidant substances are well-known for improving liver injury (Menakapriya et al., 2017).

Microscopic evaluation of liver tissues showed normal architecture of the hepatic lobules, normal central vein, and hepatic plate in the control group. However, in the CCl_4 treated groups, the normal structure of lobules was destroyed in which fat droplets, hepatocellular necrosis, dilation of the hepatic veins, and vacuolization were observed.

Previous studies on the effect of Viscum album, misoprostol, and vinpocetine showed the same results for the preventive ability of these hepatoprotective compounds compared to silymarin on the damaged liver caused by oral administration of CCl_4(Abdel-Salam et al., 2010, Salam et al., 2009, Abdel Salam et al., 2007) .

Other studies demonstrated that CCl_4-induced liver damage in monolayer cultures of rat primary hepatocytes (Friedman, 1999) by inhibiting the secretion of VLDL by liver cells which is not due to an increase in cytoplasmic unbound calcium. Three processes are involved in the mechanism of CCl_4 toxicity. Primarily, in the hepatocytes, the cytochrome P450 metabolizes CCl_4, resulting in very reactive CCl_4. Then, the CCl_4-induced immune reaction plays a significant role. Finally, the hepatocytes, like hepatic and sinusoidal endothelial cells, stellate cells, and Kupffer cells, are activated, causing them to produce cytokines that aid in hepatic fibrogenesis. Therefore, the reduced expression of inflammatory responses mediated by cytokines may be linked to the development of liver fibrosis (Koziel, 1999).

The hepatotoxicity of CCl_4 and the preventive effects of Silymarin have been studied extensively (Shenoy et al., 2001). The current findings from figure 3 showed sections from the experimental group treated with cysteine; the sections showed normal central veins, hepatic cells, and hepatic plates except for some fat droplets, which may be due to the short experiment time. On the other

hand, figure 4 demonstrated that the Ursoflor drug has fewer protective effects than our plant extract from silymarin in the present study, in which more fat droplets were shown in the sections of the Ursoflor drug plus the CCl_4 group.

A study by Lee et al. (2007) utilized a different model to test the hepatoprotective effect of silymarin and tea seed oil against CCl_4 -induced liver damage, which caused lymphocytic infiltration in the central vein and fatty degeneration, necrosis, cytoplasmic vacuolization, and mitosis in the hepatic cells. Treatment with tea seed oil and silymarin histologically showed a preventive effect on CCl_4-induced hepatoxicity (Lee et al., 2007). A previous study by Merlin and Parthasarathy (2011) demonstrated that silymarin showed significant hepatoprotective activity against CCl_4-induced hepatotoxicity in rats. In the studies of Papackova et al. (2018) and (Yilmaz et al., 2005), Silymarin is also used to alleviate liver toxicity brought on by substances apart from CCl_4.

A study by (Bahmani et al., 2015) cleared that silymarin is improving the generation of liver tissue by upregulation of protein synthesis. In addition to enhancing protein synthesis, silibinin also induces a rise in ribosome and DNA synthesis. Silibinin promotes protein synthesis and ribosome production in the liver, most likely through physiological modulation of RNA polymerase I. It was that silymarin reversed the negative changes in the liver tissue induced by the N-nitroso alkyl compound, diethylnitrosamine, by improving antioxidant capacity. Many substances, such as ethanol, have been found to harm the liver, whereas silymarin has been demonstrated to protect the liver (Song et al., 2006) and as an anti-tuberculosis drug (Eminzade et al., 2008). In line with the current outcomes of this study, silymarin exerts substantial impacts in experimental animal studies; nevertheless, Its usefulness in human liver toxicity has yet to be demonstrated (Corchete, 2008)

Alternatively, a study by Corchete (2008) demonstrated that silymarin side effects are uncommon in humans, and Serious toxicity has seldom been documented; as a result, toxicological investigations should be conducted.

Table (1): Mean ± SD of activity of ALT and AST enzymes in all studied groups

Parameters	ALT (U/L) Mean±SE	P	P*	AST (U/L) Mean±SE	P	P*
Group (1)	12±1.34			41±2.08		
Group (2)	67±6.91	S		89±4.99	S	
Group (3)	25±4.44	S	S	52±7.51	S	S
Group (4)	50±8.0	S	S	72±6.12	S	S
Group (5)	48±6.63	S	S	76±9.02	S	S

Data are given as

P: Statistically significantly different from the control group

P*: Statistically significantly different from the CCl_4 group

Table (2) represents MDA and Peroxynitrite in the five studied groups.

Parameters	MDA µmole/L Mean±SE	P	P*	Peroxynitrite µmole/L Mean±SE	P	P*
Group (1)	4.79±0.14			53.63±6.09		
Group (2)	12.49±2.1	S		71.59±14.22	S	
Group (3)	7.42±1.21	S	S	56.72±9.98	N.S	S
Group (4)	6.88±0.55	S	S	54.09±11.0	S	S
Group (5)	7.29±1.17	S	S	65.9±11.06	S	S

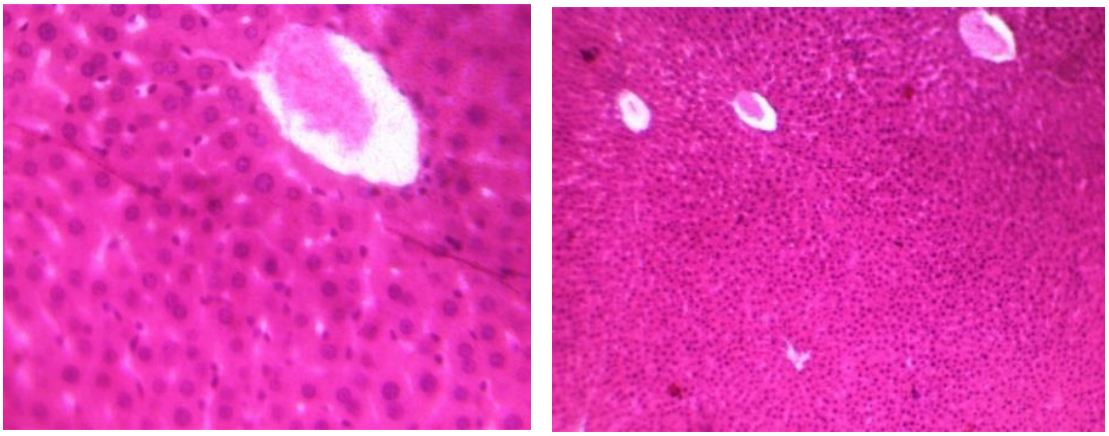

Figure 1. Section through the liver of the control group revealed the normal appearance of hepatic lobules with a normal hepatic plate and hepatocytes A: 100x, B: 400x respectively (H&E).

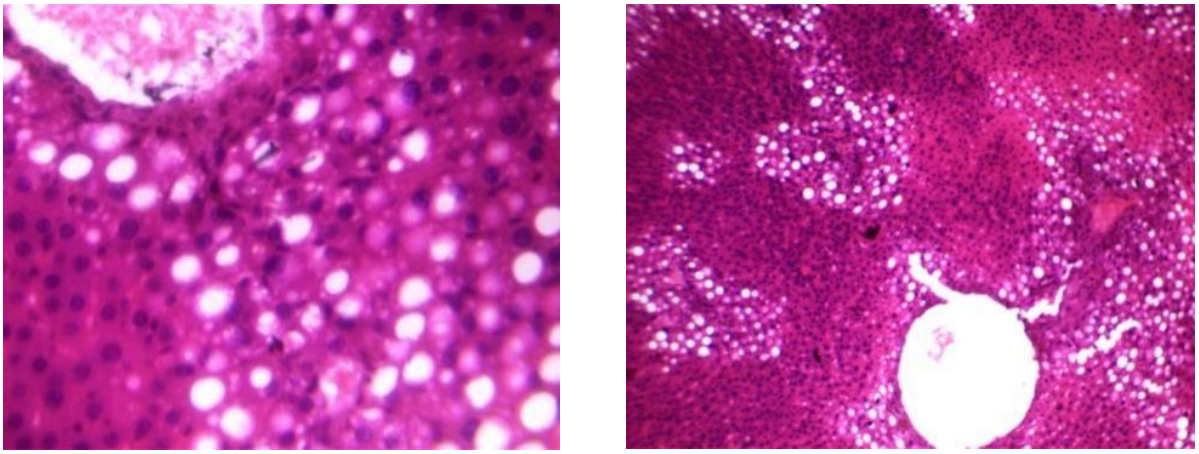

Figure 2. Section through the liver of CCl_4 group rats reveal the high number of fat droplets in the cytoplasm of the liver cell, disorganization of the hepatic lobules, and plates A: 100x, B: 400x respectively (H&E).

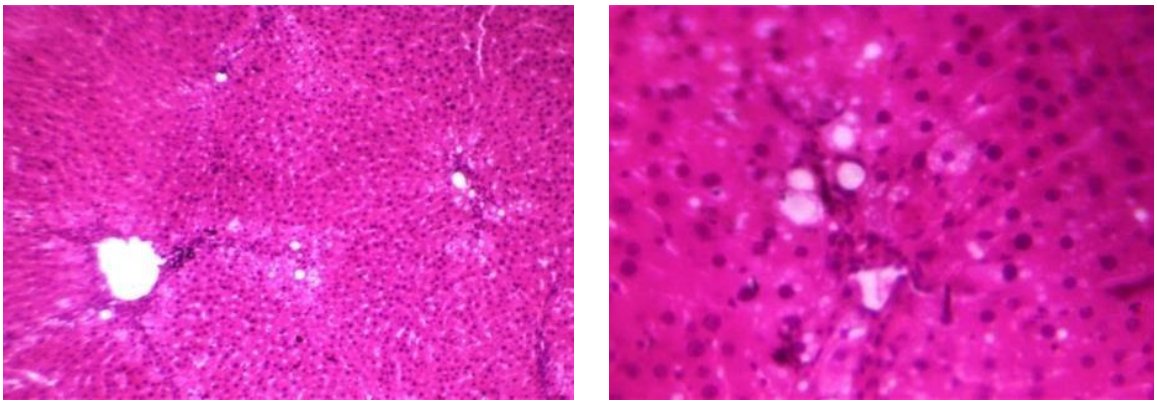

Figure 3. Section through the liver of Silymarin + CCl_4 group rats, showing clear decrease in the number of fat droplets, approximately normal appearance of the hepatic feature A: 100x, B: 400x respectively (H&E).

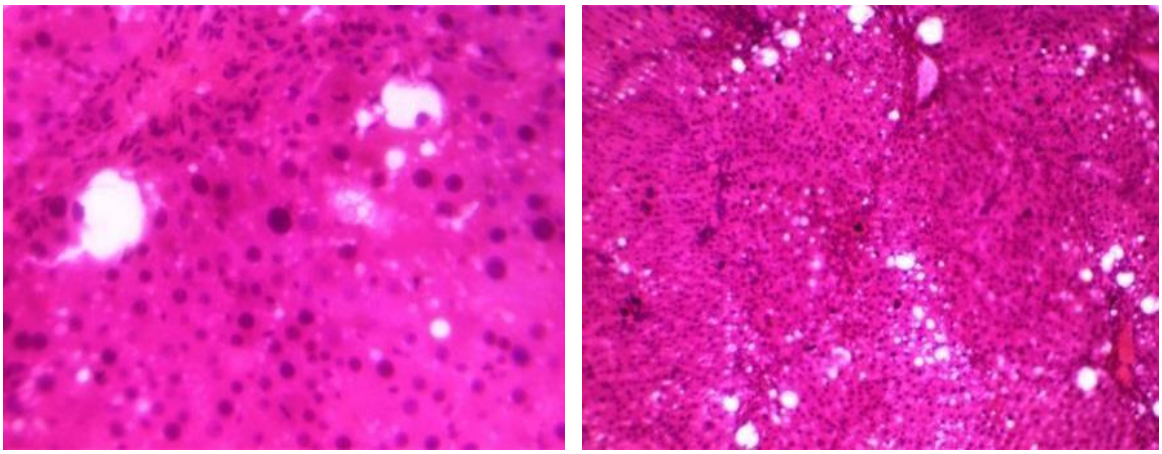

Figure 4. Section through the liver of Ursoflor + CCl_4 group rats, showed the better appearance of liver lobule compared with CCl_4 group alone but still obvious fat droplets found. A: 100x, B: 400x respectively (H&E).

5.CONCLUSION

This study demonstrated that CCl_4 when metabolized in the body is changed into very reactive free radicals that induce hepatic damage marked by alteration observed in enzyme activities and generation of reactive oxygen species indicated by elevated levels in MDA and Peroxynitrite. On the other hand, the treatment L-Cysteine, Ursodeoxycholic acid, and Silymarin minimized the hepatocellular damage induced by CCl_4 due to their antioxidant activity. For that, more studies are recommended on these active plant components with long duration and different doses.

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