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Hepatoprotective Effects of *Achillea millefolium* methanolic extract on carbon tetrachloride induced hepatotoxicity on albino male mice

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Abstract

Liver is a vital organ play a major role in metabolism and excretion of xenobiotics from the body. Liver injury is a major health problem that challenges not only for the health care professionals but also for the pharmaceutical industry and drug regulatory agencies. Liver cell injury caused by various toxic chemicals one of them is carbon tetrachloride (CCL4). Herbal medicines have been used in the treatment of liver diseases for a long time. The present review is aimed at compiling data on promising The present research was aimed to study the *in vivo* hepatoprotective activity of methanolic extract of *Achillea melifolium*in CCl4 induced hepatotoxicity in mice. The level of serum aspartate aminotransferase (SGOT), alanine aminotransferase (SGPT) and alkaline phosphatase (ALP) were determined to assay hepatotoxicity in comparison with non-treated mice and CCL4 infected. CCl4 administration caused severe hepatic damage in mice as evidenced by elevated serum SGOT, SGPT and ALP Levels. The extract of *Achillea melifolium.* administration prevented the toxic effect of CCl4 on the above serum parameters in both preventive and curative models. The present study concludes that, methanolic extract of *Achillea melifolium* has significant hepatoprotective activity against CCl4 induced hepatotoxicity, which support folkloric utilization and further confirmed by the histological investigation which present a significant activity at concentrations 100 and more significant at 200 mg/kg and play an important roles as treatment and protective effects to the liver. The observed activity may be associated with its high bioactive compounds including flavonoids and other compounds.

Keywords: Liver, hepatoprotective activity, *Achillea melifolium*in, flavonoids

Introduction

It is well known that the liver is considered as effective organ which play a substantial role in regulation of physiological activities because, It is involved in several biological activities such as; metabolism, secretion and storage. Even more, the detoxification of large number of drugs is performed by the liver (Ziech*et al*., 2010).Any defect in liver function which might result from the hepatotoxic chemicals, leads to produce certain damaged in liver cells thereby inducing lipid peroxidation and other oxidative damages, but it had been established that medicinal plants or their products may counteract such damages (Dhanabal*et al*., 2006).

Liver function is performed by different enzymes like: alkaline phosphatase (ALP), alanine aminotrsnferase

(GPT) and aspartate aminotransferase(GOT),…etc. ALP is responsible on the formation of an organic radicals and inorganic phosphate due to its ability to catalyze the hydrolysis of phosphate ester molecules (Anderson *et al*., 2005), while GPT and GOT activity were represented by acceleration of chemical reaction's rate by decreasing its activation the activating energy (Basketter *et al*., 2008).

Indeed, the function of liver may be affected by different factors, like exposure to viral infection which might lead to increase the level of liver enzymes at high rate or over dose of chemical drugs also lead to make disturbance in liver functions, all of those factors lead to produce hepatotrophy (Rahiman *et al*., 2011). So, it is very necessary to find a way to restrict this defect and that will be either by taking identified drugs or trying to find on some medicinal plants which has a good effect to restrict such phenomena.

It had been mentioned that importance of using those medical herbs goes back to 7000 years B.C. and, the first facts recorded for the use of medicinal plants in ancient Iran, Egypt, Middle East, Greece, India and China date back to around 3000 years B.C. (Omidbaigi *et al.*, 2012). The diversity in getting good results in the treatment of diseases came from the impressive pharmaceutical and biological activities of herbal plants

One of elected plants that were tested to check its effect on hepatotrophic effect is *Achillea millefolium*. This plant is related to the genus *Achillea* which is considered as fundamental genera in the *Asteraceae.* This family has more than 100 species distributed in different areas of the world, and it had been recorded that in Iran; there is more than 19 identified species of this plant (Zargari *et al.*, 1996 &Mozaffarian *et al.*, 1996).

So, this study aims to check the anti-hepatic effect of *Achillea millefolium* plant extract in laboratory mice that suffered from certain hepatic damage due to its treatment with CCL_4.

Materials and Methods

Plant collection

The plants were collected from Iraqi local market in Baghdad from the period between from Sep., 2016 till Oc., 2016. Then, this plant had been identified by Prof. Dr. Khulood WhayebAbbod/ College of Biotechnology / Al-Nahrain University.

Extraction of active compound

Dried plant material (10g) was extracted with 100 ml of ethanol/methanol kept on a rotary shaker for 24 h. Later on, it was filtered and centrifuged at 5000 g for 15 min. After evaporation of the solvent, the supernatant was collected to make the final volume one-fifth of the original volume. It was stored at 4° in bottles for further experiment (Nair andChanda, 2008).

Evaluation of Hepatoprotective Effects

Hepatoprotective effects were evaluated in albino male mice after stimulation of hepatic damage with carbon tetrachloride (CCl_4). The parameters of assessment were GPT, GOT and ALP enzymes in serum, as well as histopathological evaluation of liver tissue.

Two doses (100, 200 mg/kg) of plant extract were usedto assess thehepatoprotective effects in seven groups of mice (each group is composed of four mice).

- **Group I**: mice were administrated with a single dose 0.1 ml of D.W for 7 days.
- **Group II**: mice were administrated with a single dose 0.1 ml of 100 mg/Kg of *Achillea melifolium* for 7 days.
- **Group III**: mice were administrated with a single dose 0.1 ml of 200 mg/Kg of *Achillea melifolium* for 7 days.
- **Group IV**: Mice were administered with a single dose of 0.2% CCl_4 in olive oil (0.1ml) in day 1, and then received distilled water (0.1 ml) as a single daily dose for 7 days
- **Group V**: Mice were administered with a single dose of 0.2% CCl_4 in olive oil (0.1ml) in day 1, and then received 0.1 ml of the first dose (100 mg/kg) of *Achillea melifolium* extract once daily for 7 days.
- **Group VI**: Mice were administered with a single dose of 0.2% CCl_4 in olive oil (0.1ml) in day 1, and then received 0.1 ml of the second dose (200 mg/kg) of *Achillea melifolium* methanolic extract once daily for 7 days.

The tested materials were IP injected, and mice were killed then dissected in day 8. Blood was collected vid heart puncture, collected in Eppendorf tube and allowed to clot at room temperature for 15 minutes; the serum was separated by centrifugation at 3000 rpm for 10 minutes (Al-ezzy *et al*., 2016). Later on, it was

used for the evaluation of liver function enzymes (aspartate aminotransferase; GPT, alanine aminotransferase; GOT), in addition to alkaline phosphatase (ALP) as well as liver were obtained and stored in formalin (10%)for histological examinations(Fu *et al*., 2010)

Aspartate Amino-Transferase (GPT)

The enzyme activity of GPT was calculated in mouse serum according to evaluation method of Reitman and Frankel (1957). For this purpose a commercial kit (Randox Company) was used. The activity of GPT (Unit/L) was calculated from the kit standard curve.

Alanine Amino-Transferase (GOT)

The enzyme activity of ALT was determined in mouse serum according to Reitman and Frankel (1957), as in GOT determination using a commercial kit (Randox Company).The activity of GPT (Unit/L) was calculated from the kit standard curve.

Alkaline Phosphatase (ALP)

The enzyme ALP was assessed in mouse serum using a specific kit manufactured by Bio Merieux Company and the most traditional way used is that of Gometi *et al.* (2014)in which di-sodium phenyl phosphate is hydrolyzed with liberation of phenol and formation of sodium phosphate. The amount of phenol formed is determined colorimetrically.

C. Calculations

The following equation was employed to assess the activity of ALP.

$$\text{ALP Activity (Unit/ml)} = \left(\frac{\text{Sample Absorbance - Control Absorbance}}{\text{Standard Absorbance - Blank Absorbance}} \right)$$

Liver Tissue Preparation for Histology

The liver of each mouse is prepared for histopathological study as described by (Ibrahim *et al*., 2017). Samples were fixed in 10% formalin for 24h, followed by dehydration with a gradual series of alcohol (30-100%) for (5)min each. Then the samples cleared in two changes of xylene before embedded in paraffin wax for sectioning. Cross sections of (5)µm thickness are prepared and stained with hematoxylin (Harison) and eosin according to standard method. Histopathlogical changes are performed under light microscope as compared to control group (Alwachi *et al*., 2014)

Statistical Analysis

Graphpad 6 prims Omer *et al*., 2016.

Results

Here, we would like to refer that we injected the mice with CCL_4 before their treatment with our plant extract in order to produce certain damage in liver function.

GPT activity:

The results had shown that the GPT activity was 99±2.1 Unit/ L in control (Untreated mice) while, upon its injection with CCl_4; the GPT value increased to 176±5.2 Unit/L, suggesting that there was positive damage upon its treatment. Interestingly, we got a significant reduction in GPT value which was 95±2.5 Unit/L after its treatment with 100 mg/Kg of *Achilleamellifolium* plant extract compared with those group which were injected with CCl4 only, and this reduction increased slightly when we increases the dose of treatment with 200 mg/ kg of the plant extract which was 85.6±3 Unit/L, indicating that the plant extract supported a positive regulation of GPT activity in the group that suffered from such disturbance as in figure 1.

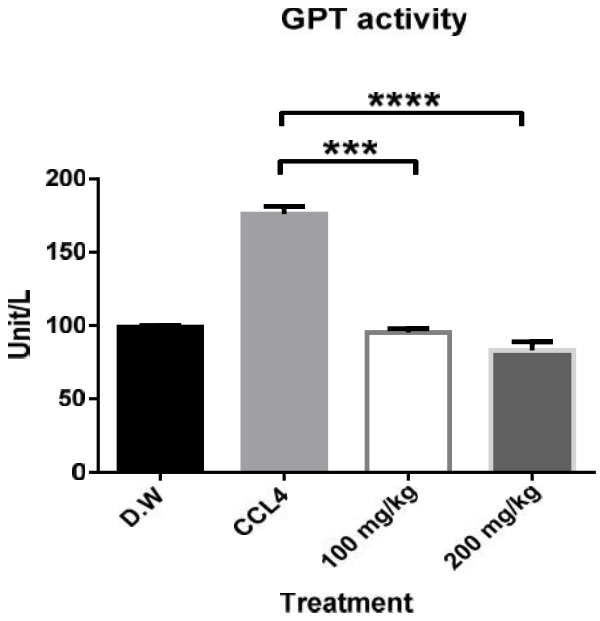


Figure1: represents GPT activity in mice upon treatment with *Achillea mellifolium* plant extract with 100 and 200 mg/Kg respectively. Data represents mean ± SD of four independently repeated experiments. One way Anova followed by Dunnett's multiple comparison test using CCL_4 as a control used for statistical analysis of each time point separately(***$p \leq 0.001$, ****$p \leq 0.0001$).

GOT activity:

GOT activity is another parameter checked in order to see the hepato-protective role of *Achillea mellifolium* plant extract. In figure 2, the GOT value of CCL_4 injected mice had shown high level 67.1±1.2 Unit/L compared with those untreated mice was 33±4.5 Unit/L, while; upon the treatment with *Achillea mellifolium* extract, we got a significant decrease in GPT value which was 38.3±3 and 23.9±2 Unit/L with a dose of 100 and 200 mg/Kg respectively. This means that the plant extract helped in reregulation of liver activity upon the reduction of GOT in CCL_4 mice.

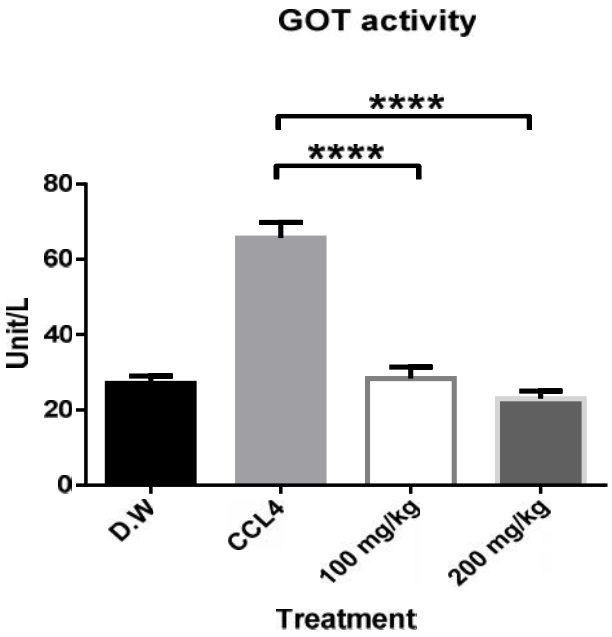


Figure 2: represents GOT activity in mice upon treatment with *Achillea mellifolium* plant extract with 100 and 200 mg/Kg respectively. Data represents mean ± SD of four independently repeated experiments. One way Anova followed by Dunnett's multiple comparison test using CCL_4 as a control used for statistical analysis of each time point separately (****$p \leq 0.0001$).

ALP activity

The mice that were injected with CCL4 also produced high levels of ALP 132.3±2.5 Unit/L compared with those untreated mice that was 93.6±4 Unit/L. But, when we treated the mice with *Achilleamellifolium* of 100, 200 mg/Kg; we found significant effect in reducing of ALP values 90.6±2.0 and 88.3±1.5 Unit/L respectively compared with those group that was treated with CCL_4 before plant extract treatment suggesting that the plant extract had a positive effect again in repairing ALP activity due to its ability to maintain the value near the normal range as in figure 3.

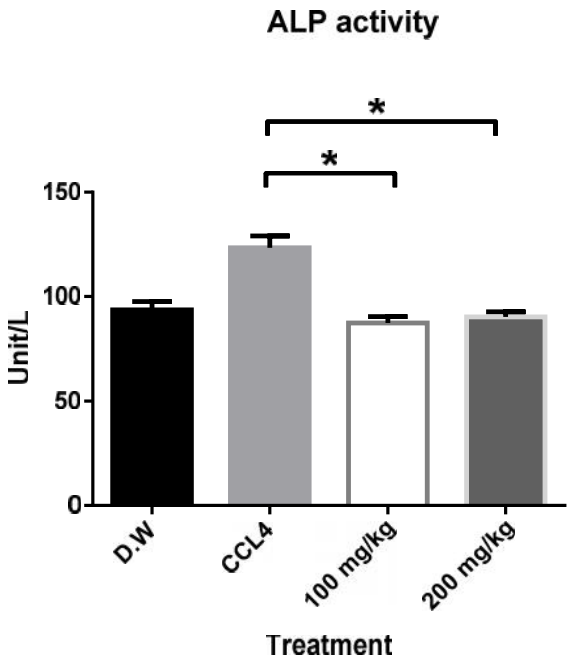


Figure 3: represents ALP activity in mice upon treatment with *Achillea mellifolium* plant extract with 100 and 200 mg/Kg respectively.Data represents mean ± SD of four independently repeated experiments. One way Anova followed by Dunnett's multiple comparison test using CCL_4 as a control used for statistical analysis of each time point separately($p \leq 0.05$).

From the results mentioned above which explain the important role of *Achillea mellifolium* plant extract in controlling the regulation of liver function that were represented by GPT, Got and ALP enzymes, we should look for the mechanism or any explanation behind that positive effect. To answer this question, we treated the mice with both of our plant extract and CCL4 together in order to check if this reduction in enzymatic activity that came from plant treatment is due to its ability to interact with the CCL4 or not.

Figures 4, 5 and 6 represent the results of dual action of plant extract and CCL_4 together on GPT, GOT & ALP respectively.

Regarding GPT activity, the dual treatment with CCL_4 and *Achillea mellifolium* plant extract had shown a significant, positive reduction in its values which were 101±3.6 and 93.3±3.5 Unit/L compared with the group which was treated with CCL4 alone. Indeed this result was confirmed by GOT values that produce a significant decrease in its activity upon dual treatment which were 27±2 and 27±2 Unit/L compared with CCL_4 treatment alone.

Again, ALP activity was down regulated significantly 91±3.1 and 89±4 Unit/ L if we compare it with single treatment of CCL_4.

So, we can conclude that the dual treatment of mice with CCL_4 and *Achillea mellifolium* extract produced a fundamental reduction in GPT, Got & ALP activity.

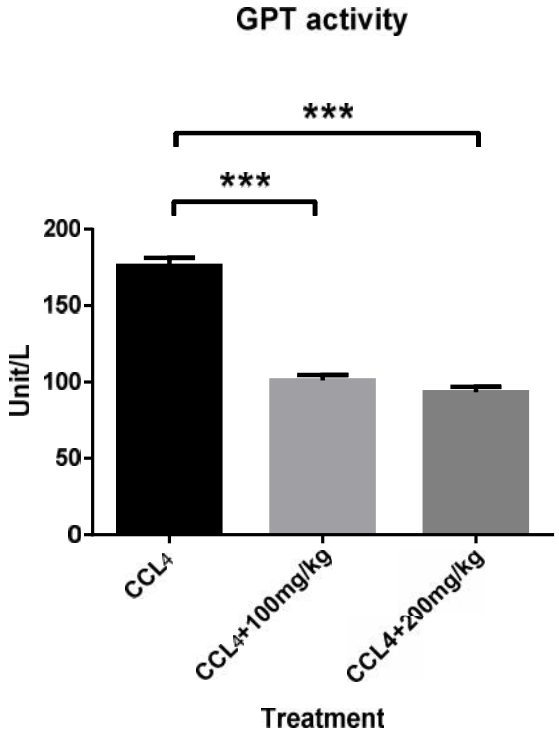
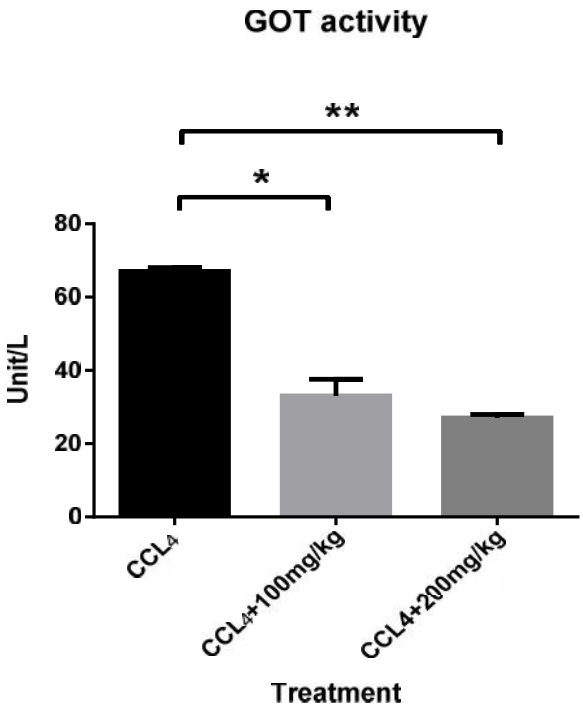


Figure 4: represents GPT activity in mice upon dual treatment with CCL_4 and *Achillea mellifolium* plant extract with 100 and 200 mg/Kg respectively. Data represents mean ± SD of four independently repeated experiments. One way Anova followed by Dunnett's multiple comparison test using CCL_4 as a control used for statistical analysis of each time point separately(***$p \leq 0.001$).



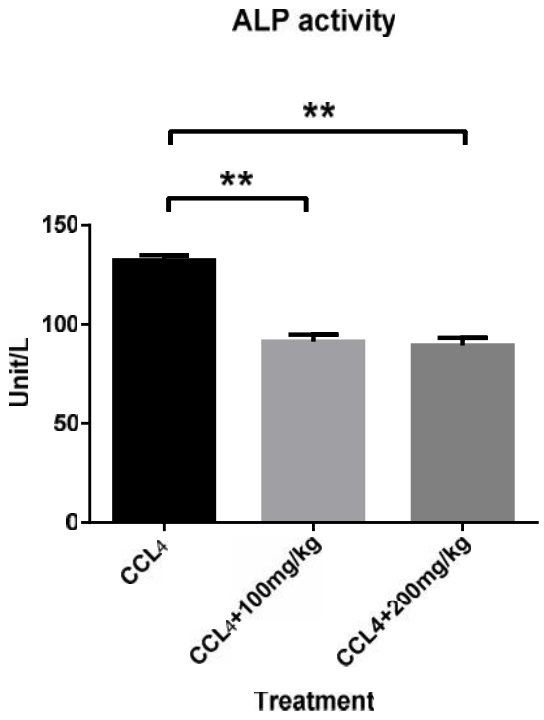


Figure 5: represents GOT activity in mice upon dual treatment with CCL_4 and *Achillea mellifolium* plant extract with 100 and 200 mg/Kg respectively. Data represents mean ± SD of four independently repeated experiments. One way Anova followed by Dunnett's multiple comparison test using CCL_4 as a control used for statistical analysis of each time point separately(*$p \leq 0.05$, **$p \leq 0.01$).

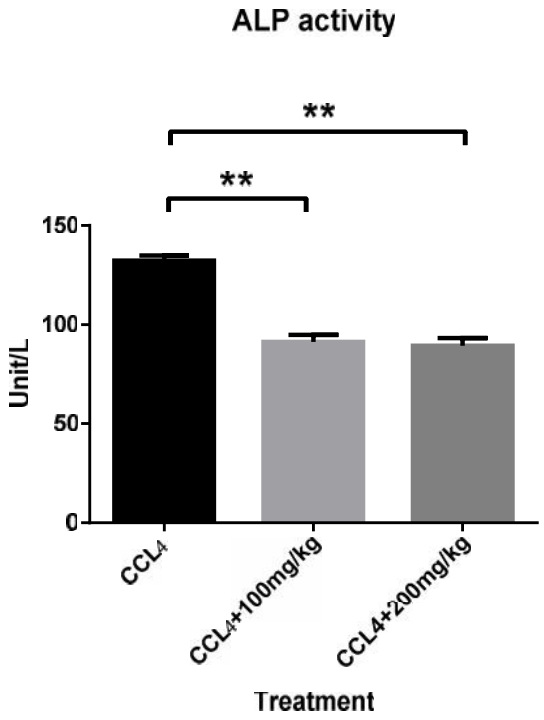


Figure 6: represents ALP activity in mice upon dual treatment with CCL_4 and *Achillea mellifolium* plant extract with 100 and 200 mg/Kg respectively.Data represents mean ± SD of four independently repeated experiments. One way Anova followed by Dunnett's multiple comparison test using CCL_4 as a control used for statistical analysis of each time point separately(**$p \leq 0.01$).

Histopathological activity of liver

The hisopathological studies on animal contain two groups of animals using plant extract only at tow concentrations 100 and 200 mg/ml, while other two groups infected with CCL_4 and treated with two concentrations 100 and 200 mg/ml and last two groups control and animal infected with CCL_4 only and compare results with each others.

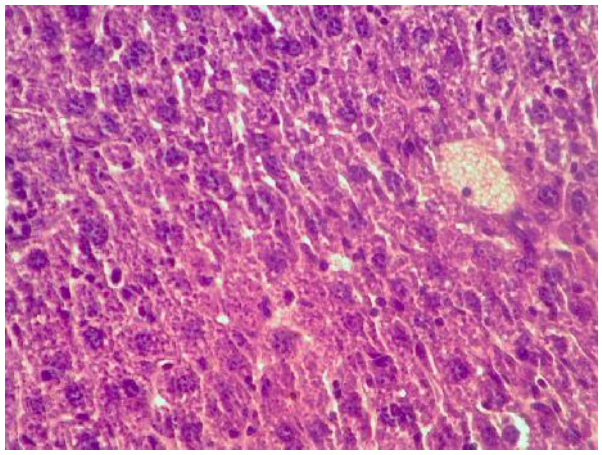


Figure 7: section of normal liver structure, which consists of central vein, surrounded by hepatocyte cells(H& E) X400

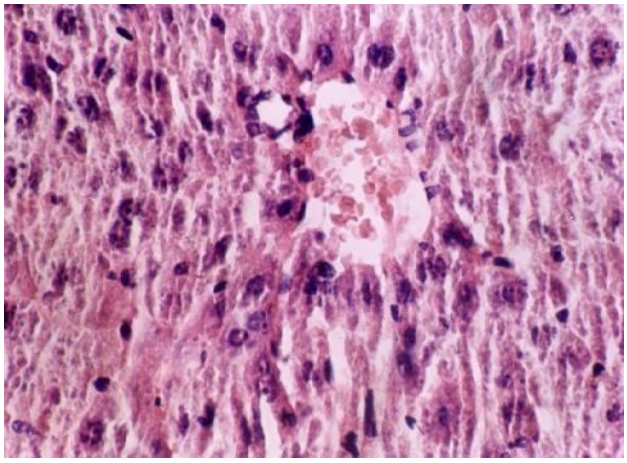


Figure 8: section of liver tissuetreated with CCL4 showing congestion, degenerative and necrosis of paranchymal tissue cells; with mild cells inflammation (H&E) x400

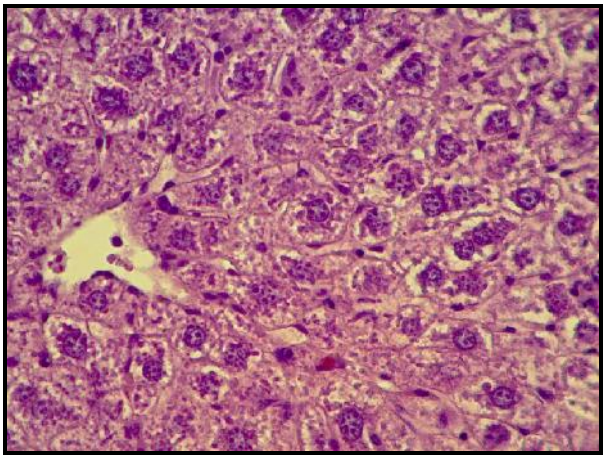


Figure 8: Mice treated with 100 mg/ml of *Achillia melifolium* for 7 days. Liver showing look like normal architecture with accumulation of glycoproteins granules and the cell become enlarged (H& E) X400.

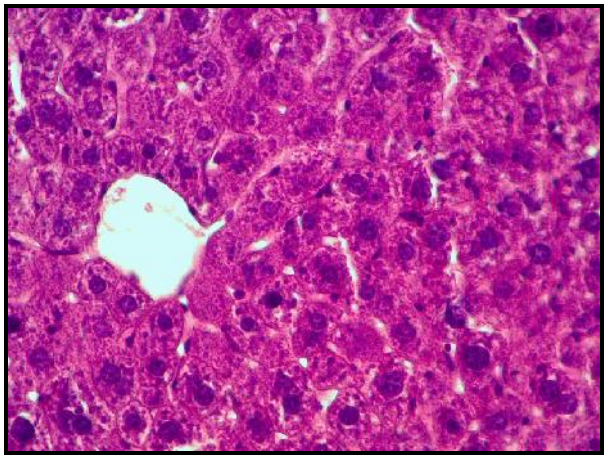


Figure 9: Mice treated with 200 mg/ml of *Achillia melifolium* for 7 days. Liver showing look like normal architecture with accumulation of glycoproteins this indicate the hepatocyte cells (H& E) X400.

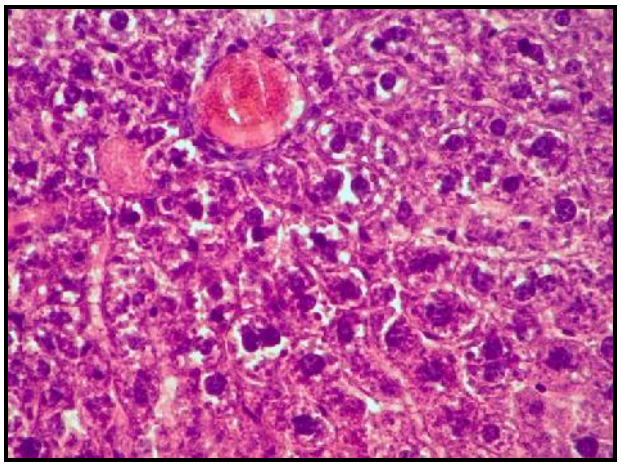


Figure 10: Mice infected with CCL_4 and treated with 100 mg/ml of *Achillia melifolium*. Liver showing congestion with accumulation of glycoproteins granules (H& E) X400.

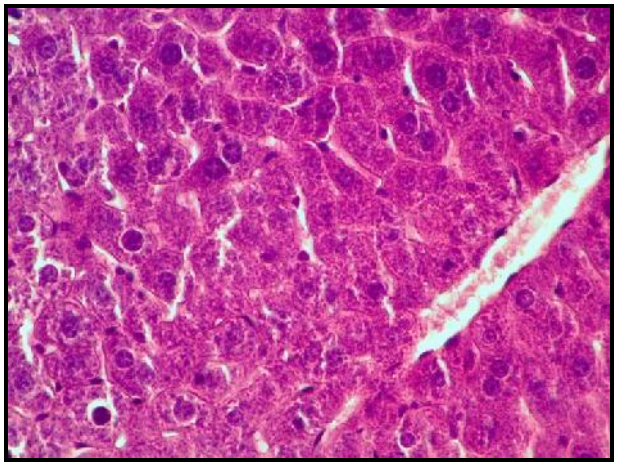


Figure 11: Mice infected with CCL_4 and treated with 200 mg/ml of *Achillia melifolium*. Liver showing normal appearance structure, consist of central vein and threads of hepatocyte cells (H& E) X400.

Discussion

It is well defined that the liver is the main target of detoxification and certain up take of the drugs lead to produce a substantial hepatic shut off due to the production of pro oxidant reactive oxygen species (ROS, that's in turn leads to activate cellular defect which effect on some biomolecules like DNA, Protein and (Ziech*et al*., 2010).

The main reason behind using of $CCL_4$is to induce this damage because Carbon tetrachloride-induced hepatotoxicity in mice resulting in a severe necrosis and damage in the structural integrity of liver which produce abnormal increase in liver enzyme levels (Jin *et al*., 2011). This compound is characterized by its ability to produce CCL_3 which is considered as a free radical which that alkylates cellular proteins, leading to liver damage, which is represented by cirrhosis and necrosis (Zeashan *et al*., 2008).

The imbalance situation between the production of ROS and the range of antioxidant level is called oxidative stress, the formation of those free radical is necessary for protection of cellular molecules from hepatotoxicity induced by CCL_4(Kim and Kim, 2011; Rahiman *et al*., 2011).

In this study, we detected significant increase in GPT, Got & ALP levels upon CCL4 treatment. So, to prevent such increase; it is necessary to inhibit the production of reactive metabolites (Wong *et al*., 2012).

Our study reveals that the methanolic extract of *Achillea mellifolium* possess a desirable effect on mice treated with CCL_4while there was no any defective effect on normal mice. That was clear in the reduction of liver enzyme levels when we treated the mice with our plant extract after their treatment with CCL_4.

Interestingly, we could detect a substantial effect which explained the ability of plant extract to interfere with the CCL_4 and prevents its harmful effect. Previous studies had shown that the active compounds found in *Achillea mellifoilum* species are considered as a strong anti-oxidant compound especially; the flavonoids (Popovici *et al.*, 2007; Benedec *et al.*, 2013,Serdar *et al.*, 2015 and Al-Anee *et al*.,2015).

In fact many species of *Achillea* plant exhibit a strong anti- oxidant activity, for example; the ethanolic extract of *Achillea schurii* had a strong anti- oxidant activity due to its ability to inhibit lipid peroxidation (Vlase *et al.,* 2014; Prior *et al.* 2005, and Benedec *et al.,* 2014). This plant had several poly-phenolic compounds which possess this anti-oxidant activity because; flavonoids of *Achillea* spp. have one catechol in one of aromatic rings and two hydroxyl groups which are necessary in such activity (Sestili et al., 2002).

It is very important to mention that, phenolic compounds may also participate directly to antioxidant action, because of their redox properties, that permit them to act as reducing factor, hydrogen donors and singlet oxygen quenchers (Rezende *et al*., 2014).

All of what mentioned above explains & supports the ability of the *Achillea mellifolium* plant extract to reduce the CCL_4 ability in production of certain damage which led to restrict the functional activity of liver in mice. But, the mechanism by which how this plant act against such defect still unclear so, its very necessary to find out an explanation about the way of action.

The results coincided with our investigations that mice infected with CCL_4 and treated with 200mg/ml showed the best protective effect against the carbon tetrachloride induced liver injuries, therefore the possible hepatoprotective mechanisms of extract of *Achillea mellifolium* may be due to preventing process of lipid per oxidation, inhabiting the cytochrome p-450 activity, stabilizing the hepatocellular membrane and enhancing the protein synthesis(Shama, 2004). Preliminary phytochemical studies have indicated the presence of flavonoids in *Achillea mellifolium*. Flavonoids consumed in large amounts in diet, are known to protect liver (Sobiya *et al*., 2009). Hence the anti hepatic toxicity of *Achillea mellifolium* may due to presence of flavonoids.

Liver sections in CCL_4 and treated with plant extract revealed a regeneration of hepatic cells (figure 10 and 11) in comparison with animal infected with CCL_4 and non-treated. Also a protective effect of this plant to liver may belong to flavonoids as active compounds of this plant.

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