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PROTECTIVE EFFECT OF *FICUS CARICA* FRUIT AGAINST CARBON TETRACHLORIDE INDUCED HEPATIC TOXICITY IN MICE

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ABSTRACT

Ficus carica (Fig) is considered to be first plant which is cultivated on earth. Fruit of this plant is delicious, medicinally important and is being consumed by human population since centuries. The present study was designed to assess the role of *Ficus carica* fruit as hepatoprotective agent in animal model. Crude methanolic extract of *Ficus carica* fruit and its derived *n*-hexane and aqueous fractions were used to study its curative effect on CCl_4 induced toxicity in mice. Animals were divided into seven groups (n=5). Group I animals were treated with normal saline only, Group II was treated with olive oil (vehicle control), Group III was treated with CCl_4 (toxic control) 0.2 ml/kg b.w, Group IV was treated with silymarin (standard drug) and Group V, VI and VII were treated with plant extract and fractions at different doses (200 mg/kg and 400 mg/kg b.w). Hepatoprotective effect was determined by investigating liver markers (Alanine transaminase, Aspartate aminotransferase, Alkaline phosphatase and bilirubin) using blood while *in vivo* antioxidant potential was found out by determining the activity of Glutathione peroxidase, Catalase and Superoxide dismutase. Histopathology of liver was also done. The aqueous fraction showed significant hepatoprotective effect against toxicity in mice as well as exhibited higher antioxidant value. The aqueous fraction of *Ficus carica* would be a good candidate for pharmaceutical industry as a hepatoprotective agent.

Key words: *Ficus carica,* Hepatoprotective, Antioxidant, Carbon tetrachloride.
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INTRODUCTION

Medicinal plant and their extracts are rich source of crude medication as they possessed therapeutic properties (Shahat *et al.,* 2018). From centuries people rely on medicinal plants instead of using synthetic drugs to get cure from ailments. They are used in both urban and rural area of country (Ouelbani *et al.,* 2016). Medicinal value of these plants is due to presence of some chemical substances known as secondary metabolites including phenols, terpenoids, flavonoids and tannins etc, used for treatment of different sicknesses (Tu *et al.,* 2019). Among secondary metabolites phenols have great importance because of their multiple biological effects that are found both in edible and non- edible part of plants (Li *et al.,* 2018).

Ficus carica is commonly known as fig and it belongs from Moraeceae family (mulberry tree). It is deciduous plant grow well in tropical and subtropical region. Its fruit have extraordinary nutritive and pharmacological value. It is richest source of calcium, fiber and amino acid. Among phytochemicals phenols are present extensively which are used for treatment of different ailments such as constipation, gout and other inflammatory diseases (Solomon *et al.,* 2006). Various activities of these polyphenolic compounds such as antispasmodic (Gilani *et al*., 2008), antipyretic (Patil *et al*., 2010), antidiabetic (Mopuri and Islam, 2016) and anti-inflammatory (Duke *et al*., 2002) have been reported.

Liver is the fundamental organ which carries out different functions such as storage and metabolism of foreign chemical (Latta and Mittal, 2017). Among liver diseases, liver toxicity is the most common risk factor which is caused by chemicals, air pollutants and alcohol consumption. In animal study, CCl_4 is most commonly used to induce liver toxicity, as mouse model is analogous to human while the hepatic microsomal cytochrome p450 convert CCl_4 into trichloromethyl free radical, as a result lipid peroxidation take place (Sahreen and Khan, 2017). Most commonly it results in the formation of free radical, which is the rate limiting process in tissue peroxidation damage. Oxidative stress is caused by reactive oxygen species which results in cellular metabolic changes like, elevation of the liver serum markers level, fragmentation of DNA and lipid peroxidation starts destroying the cells. As a result, hepatic cells loss their integrity and malfunctioning of liver takes place (Bahaduria *et al.,* 2008). In request to shield body from such malicious impact the natural antioxidants must be required which have no side effects. Medicinal plants contain such antioxidants which protect tissues from drug induced toxicity by scavenging them and act as a protective agent against disease (Fahmy *et al.,* 2016).

Various degenerative illnesses are caused by reactive oxygen species. These are exceedingly reactive species possess unpaired electron such as hydroxyl and nitric oxide radicals whereas hydrogen peroxide, peroxynitrite, singlet oxygen and hypocholous are non-radical oxidants (Vara *et al*., 2014). Environmental factors such as `UV, toxic chemical and sunlight are responsible for creation of these species and they may form as byproduct of oxygen metabolism in living organism. Human body contains antioxidative enzymes which protect from Reactive Oxygen Species. Now humans rely on plant either as sustenance source as restorative or as a medication source because of their ability to cope with free radicals. Plants contain antioxidant compounds among which phenols and flavonoids are of great importance because of their specialized structure by which they reduce free radical by donating hydrogen and extinguish singlet oxygen (Arika *et al.,* 2019). The phenolic content of plant and antioxidative potential is correlated to each other (Aryal *et al.,* 2019).

Based on the nutritive value of *Ficus carica*, the study was designed to study phytochemistry, *in vivo* hepatoprotective and antioxidant activity. To the best of our knowledge, data regarding the hepatoprotective effects of *Ficus carica* fruit is scarce and this is detailed study on protective potential of fig fruit. The study aimed to elucidate hepatoprotective effect and possible mechanism of *F. carica* fruit in carbon tetrachloride induced toxicity in experimental animal.

MATERIALS AND METHODS

Plant sample: Fresh fruit of *Ficus carica* was collected from Islamabad, Pakistan in the month of June and July 2017. Identification was done by expert taxonomist of Botany Department, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi and was submitted as specimen (voucher no124) for future reference.

Extraction and fractionation: The fruit sample was cleaned and dried in shade. After drying, it was chunked into small pieces and pulverized into fine powder. Maceration was done with methanol and kept on shaking for 72 hours. After 3 days filtration was done and solvent was evaporated in rotary evaporator at 40 ºC under reduced pressure. The solid residue obtained was suspended into water and further fractionated with solvents such as *n*-hexane and aqueous fraction. The extract and fractions were also concentrated under reduced pressure by using rotary evaporator (Heidolph, Germany) and for further uses, extract and fractions were stored in refrigerator at 4 ºC.

Chemicals and reagents: All chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (USA).

Total phenolic content estimation: It was estimated by colorimetric method. Plant sample of 200 µl was mixed with 1.5 ml of folin reagent. After 5 minutes, Sodium bicarbonate (1.5 ml) was added to the reaction mixture and incubated for 90 mins and optical density was measured at 725 nm by using spectrophotometer (Shimadzu, Japan 1900 UV/Vis) and gallic acid as a standard (Kim *et al.,* 2003).

Flavonoid content estimation: To determine the flavonoid content, plant sample (0.5 ml) was mixed with 0.3 ml of sodium bicarbonate (5 %). After 5 minutes 0.3 ml of aluminum chloride (10 %) and 10% NaOH was added. Absorbance was evaluated at 510 nm, using quercetin standard. Values were expressed as milligram Quercetin Equivalent per gram (mg QE/g). The test was done in triplicates (Marinova *et al.,* 2005).

***In vivo* hepatoprotective study**

Experimental animal: Total fifty Swiss albino male mice of 30 to 40 gram bodyweight with age of six to eight weeks were procured from National veterinary laboratory, Islamabad, Pakistan. They were kept in stainless steel cages at room temperature 25 ± 3 ºC, with 12:12 hours of light/dark cycles. They had free access to laboratory diet and water *ad libitum*. This research activity was approved from ethical committee of university before preceding the experiment on animals with issued reference no PMAS_AAUR/BCH/326.

Experimental design: Five animals were randomly assigned to each group.

Groups I: (normal control) remain untreated for 14 days.

Group II: (vehicle control) received 1 ml of olive oil post orally for 14 days.

Group III: (toxic control) animals were served with CCl_4 0.2 ml/kg body weight intra peritoneally twice a week.

Group IV: received CCl_4 twice a week and silymarin drug for remaining days (positive control).

Group VA & VB: received CCl_4 twice a week and crude methanolic extract 200 and 400 mg/kg bw for remaining days.

Group VI A & VI B: received CCl_4 twice a week and *n*-hexane fraction 200 & 400 mg/kg bw for remaining days.

Group VII A & VII B: received CCl_4 twice a week and aqueous fraction 200 & 400 mg/kg bw for remaining days.

Blood sampling and tissue collection: After 24 hours of treatment blood was collected from anesthetized animals by cardiac puncture. Centrifugation (Labnet, USA) was done to separate the serum for liver biochemical parameters determination. After collection of blood animals were then weighed and sacrificed and liver were dissected out, they were weighed and cleared to remove

debris and divided into two parts one part was directly transferred to 10 % formalin solution for histopathological studies and second part was stored on ice to study oxidative stress.

Analysis of biochemical parameters: The separated serum was further used to estimate the biochemical markers of liver such as Alanine transaminase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), and total bilirubin using standard AMP diagnostic kits purchased from Pioneer diagnostic company (Qureshi *et al.,* 2010) and total protein was determined with method described by Lowry *et al.,* 1951.

Assessment of oxidative enzymes: Homogenization of hepatic tissues was carried out by adding 100 mg of tissue in KH_2PO_4 buffer (100 mM, PH 7.4) containing 1 mmol of EDTA. Centrifugation was done at 12000 X g for 30 minutes and supernatant used for further assays.

Superoxide Dismutase (SOD) activity: In order to determine this activity, first of all reaction mixture was prepared containing 50 µl of phenazine methosulphate (186 mM) and 600 µl of 0.052 mM sodium pyrophosphate buffer of PH 7.0. About 150 µl of tissue homogenate was added to the reaction mixture. Reaction was initiated by adding 100 µl NADH (186 mM). To stop reaction, 500 µl of glacial acetic acid was added after 1 min. Absorbance was measured at 560 nm to enumerate the color intensity (Kakkar *et al.,* 1984).

Catalase (CAT) activity: To determine the catalase activity about 35 µl of supernatant was mixed with reaction mixture, containing 625 µl of KH_2PO_4 (50 mM) of pH 5 and 100 µl of H_2O_2 (5.9 mM). Change in the absorbance was measured after one minute at 240 nm (Chance and Maehly, 1955).

Glutathione peroxidase (Gpx) activity: To find out the glutathione peroxidase activity, reaction mixture involved 2 ml of phosphate buffer (75 mM, pH 7.0), 0.1 ml of glutathione reductase (30 units/ml), 0.1 ml of EDTA (15 mM), 50m of glutathione (60 mM) and 0.1 ml of NADPH (3 mM). Toward the end tissue supernatant was added to this mixture by making total volume 3 ml. Hydrogen peroxide (0.1ml) was added to initiate reaction. Absorbance was evaluated at 340 nm by rate of progress of transformation of NADPH to NADP for 3 minutes. Glutathione peroxidase activity was determined as oxidation of µmoles of NADPH to NADP + min-1mg-1protein (Lawrence and Burk, 1976).

Histopathological Studies: The liver tissues were fixed in formalin (10 %) and embedded into paraffin. About 5 µm of tissue section was obtained and finally staining was done by haematoxylin and eosin stains. Light microscope (AmScope, USA) was used to observe tissue slides.

Statistical analysis: The values were expressed as mean of triplicate along with ± standard error of mean. Animal study was performed under completely randomized design. To find out the consequences of different treatments, one-way analysis of variance was used by computer software graph pad prism 5.0. Multiple comparisons among treatments were made by Tukey– post hoc test. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Phytochemical analysis: Total phenolic and flavonoid content of *Ficus carica* fruit was determined colorimetrically, shown in Table 1. The total phenolic content was expressed in milligram of gallic acid equivalent per gram of dry weight. Crude methanolic extract showed highest phenolic content (107.3 ± 0.88 mg GAE/g), followed by aqueous fraction (101.8 ± 1.38 mg GAE/g) and *n-hexane* fraction (44 ± 2.64 mg GAE/g). Crude methanolic extract also exhibited high flavonoid content (24.6 ± 1.44 mg QE/g), aqueous fraction showed less than methanolic extract (16.9 ± 0.24 mg QE/g), while *n*-hexane fraction (13.9 ± 0.30 mg QE/g) contained less amount. Secondary metabolites present in fruit and vegetables are of great importance due to their medicinal values (Wadood *et al.,* 2013). Phenolic compounds attain special attention due to their scavenging activity which is due to their ideal structure by which they reduce free radical and prevent oxidation. Flavonoids perform their function by inhibiting various enzymes also prevent cell proliferation and apoptosis. These compounds perform therapeutic role against different diseases (Al-Said *et al.,* 2016). In the present study polar solvents were found best for extraction of phenols and flavonoid from *Ficus carica* fruit. While *n. hexane* is non-polar solvent, it was found less effective for extraction of phenols and flavonoid from *Ficus carica* fruit.

Hepatoprotective study

Effect on liver markers: Liver is the vital organ; perform multiple function**s**, which also involves detoxification of drugs and xenobiotic. The pre exposure of liver to the chemicals results in liver injury. CCl_4, a potent hepatotoxic agent, is mostly used to study hepatoprotective activity of medicinal plants (Wang *et al*., 2019).The protective effect of fig fruit on liver enzymes and protein were shown in Figure 1. Induction of CCl_4 significantly increased ($P < 0.05$) the level of liver markers such as alanine transaminase, aspartate aminotransferase, alkaline phosphatase and bilirubin as compared to the normal group. Treatment with positive control silymarin and plant extract both at high dose 400 mg/kg and low dose 200 mg/kg significantly reduced ($P < 0.05$) the elevated enzymes level. Among the fractions the maximum hepatoprotective effect was shown by aqueous fraction as compared to *n*-hexane fraction. There was significant

decreased ($P < 0.05$) in level of protein by treatment with CCl_4 and there was a remarkable increased on treatment with plant extract and silymarin. Hepatotoxicity induced by CCl_4 in the liver of mice was confirmed by parameters such as liver marker enzymes and histopathological. The rise in level of liver enzymes such as ALT, AST and ALP is due to hepatocellular damage and this is one of the ways to estimate the level of damage caused to the liver (Balakrishnan *et al*., 2019). The oxidative damage in liver is initiated by conversion of CCl_4 into CCl_3 by enzyme cytochrome P_{450}, this free radical degenerate the membrane of hepatocytes, as a result, the membrane permeability increases and cause cellular death. This results increase in ALT, AST, and ALP that leaks into the serum as a result their level is high in blood (Lu *et al.,* 2018). Treatment with *Ficus carica* fruit extract and its fraction showed significant decrease in enzymes level among which aqueous fraction showed more activity than other fractions. This curative effect of plant extract and fraction was due to presence of bioactive compounds which scavenged the free radicals. These compounds are antioxidant in nature and prevented lipid peroxidation, restored function of liver cells. Antioxidant effect of many phenols has been reported (Ngo *et al.,* 2017). They are correlated with each other. Phenols possessed specialized structure, a phenol (OH) ring which transfer electron to free radical and scavenged the free radical by neutralizing them. As a result chain of production of reactive oxygen species terminated and hepatocytes cell membrane re-stabilized and liver tissues get repaired. Hepatoprotective activities of phenols have been already reported (Saha *et al.,* 2019). Liver damage may also block the bile excretion as result of which serum bilirubin get increase. CCl_4 intoxication exhibited significant rise in bilirubin whereas on treatment with extract and its fractions the level declined as shown in Figure 1. This recovery may result from healing of parenchyma and hepatocytes regeneration by curative effect of phytochemicals present in plant (De *et al.,* 2017). CCl_4 toxicity also decreased the level of serum total protein as shown in Table 2; this is another indicator of liver toxification. It results from dissociation of polyribosomes which are present on endoplasmic reticulum (Kumar *et al.,* 2009). Aqueous fraction of *Ficus carica* fruit exhibited highest hepatoprotective potential which was due to presence of high phenolic content. Because free radicals are responsible for hepatic injury and phenols act as antioxidant compounds by scavenging them and reversed the activity of liver enzymes. While lower activity was exhibited by *n.hexane* fraction which possessed low phenolic content. This study suggested that bioactive compounds responsible for hepatoprotective activity of *Ficus carica* fruit were polar in nature and better fractionated by aqueous solvent.

Table 1. Total phenolic and total flavonoid content of *Ficus carica* fruit.

Plant extract/ Fraction	TPC (mg GAE/g dw)	TF (mg QE/g dw)
Crude methanolic extract	107.3 ± 0.88	24.6 ± 1.44
n-hexane fraction	44 ± 2.64	13.9 ± 0.30
aqueous fraction	101.8 ± 1.38	16.9 ± 0.24

All values are expressed as mean of triplicate ± S.E.M (where n=3)

***In vivo* antioxidant activity:** The redox homeostasis is controlled in body by liver**.** It controlled the production and scavenging of reactive oxygen species. Excess reactive oxygen species are scavenged by antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase (He *et al.,* 2017). Table 2 showed the curative effect of fig fruit on liver's antioxidant enzymes. The level of these oxidative enzymes was significantly ($P < 0.05$) lower in CCl_4 treated animals and was remarkably elevated in the group administered with silymarin and plant extract. When compared the plant extract and its fractions, higher dose 400 mg/kg was much effective than lower dose 200 mg/kg so the curative effect was dose dependent. In this study, the activity level of antioxidant enzymes of liver tissue was decreased which was indicator of oxidative stress generated after administration of CCl_4 in mice. Decrease in the level of Glutathione peroxidase, catalase and superoxide dismutase is also indicator of lipid peroxidation (Pallerla *et al.,* 2019). These enzymes scavenged the free radicals in coordination with each other. When the free radical generates excessively, the activity of these antioxidant enzymes suppressed. The role of SOD is to convert free radical in H_2O_2 which is decomposed by CAT and glutathione peroxidase in case of normal metabolism and they eliminate the hazardous effect of hydroxyl radical (Zhoa *et al.,* 2019). However, when liver get toxicated as in case of CCl_4 in toxicity, the concentration of H_2O_2 elevated in hepatic tissues and as a result severe injury take place. The result of this study was also similar to other studies where CCl_4 intoxication increased level of H_2O_2 and suppress the antioxidant enzymes in hepatic tissue (Sahreen *et al.,* 2013). Whereas, the group treated with *Ficus carica* fruit showed significant increase ($P < 0.05$) in activity level of SOD, CAT and glutathione peroxidase which was due to presence of antioxidant compounds in plant extract and fraction. Which stopped the production of free radical by termination of chain reaction. So, the results supported the evidence that scavenging of free radicals restored the antioxidant enzyme level. The results suggested significant ($P < 0.05$) increased in the activity of antioxidant enzymes by aqueous fraction which was due to high phenolic and flavonoid content which prevented lipid peroxidation as reported earlier (Kondeva-Burdina *et al .,* 2017).

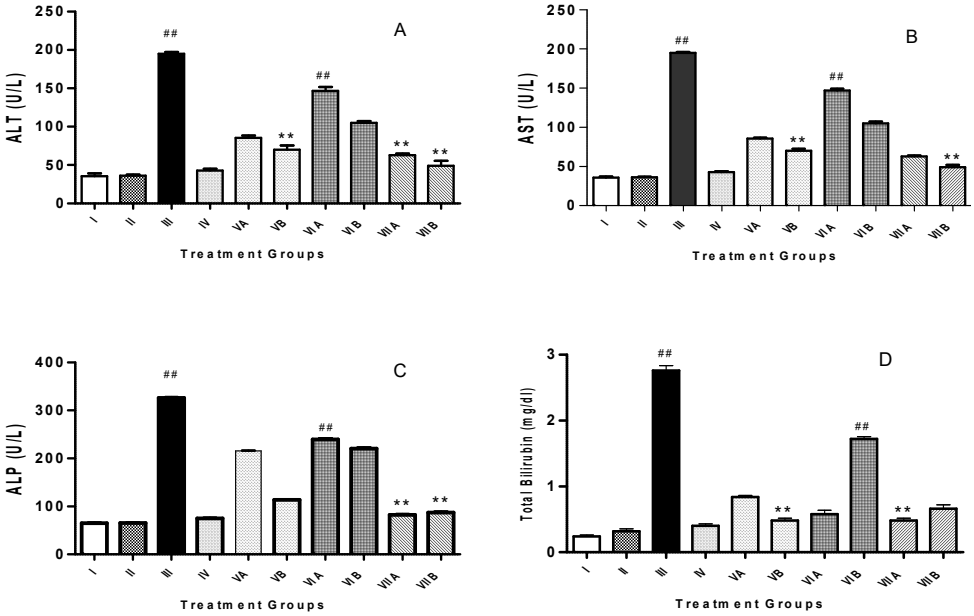

Figure1. *Ficus carica* fruit extract/fractions effect on level of A. Alanine aminotransferase, B. Aspartate aminotransferase, **C**. Alkaline phosphatase, and **D**. Bilirubin. Cumulative values are reported as mean ± S.E.M for five mice in each group$^{\#\#}$ significantly different from normal group ($P < 0.05$) **significantly different from CCl_4-treated group ($P < 0.05$).

Table 2. Effect of *Ficus carica* fruit extract and its fractions on antioxidant enzymes.

Groups	GPx(mol/min/mg protein)	SOD (U/mg protein)	CAT(U/mg protein)	Total protein(g/dL)
I	37.8 ± 1.04	11.8 ± 0.24	9.2 ± 0.12	4.4 ± 0.24
II	36.1 ± 1.34	9.8 ± 0.11	8.2 ± 0.39	4.4 ± 0.39
III	24.2 ± 0.23$^{\#\#}$	5.4 ± 0.13$^{\#\#}$	4.8 ± 0.40$^{\#\#}$	5.1 ± 0.27$^{\#\#}$
IV	34.6 ± 0.17	9.1 ± 0.32	8.3 ± 0.37	3.3 ± 0.17
VA	32.7 ± 1.20**	8.1 ± 0.11	7.3 ± 0.18	4.5 ± 0.14
VB	34.6 ± 0.43**	8.5 ± 0.23**	8.1 ± 0.51**	4.7 ± 0.11
VI A	26.8 ± 1.07	7.7 ± 0.17	54 ± 0.44	3.9 ± 0.09
VI B	27.8 ± 1.11	7.8 ± 0.13	5.6 ± 0.19	4.0 ± 0.28
VII A	32.7 ± 1.03**	8.6 ± 0.18**	7.8 ± 0.21	4.2 ± 0.30
VII B	32.9 ± 0.77**	9.0 ± 0.21**	8.3 ± 0.19**	3.6 ± 0.13**

Cumulative values are reported as mean ± S.E.M for five mice in each group**significantly different from CCl_4-treated group at ($P < 0.05$), $^{\#\#}$significantly different from normal group at ($P < 0.05$).

Histopathological studies: The histopathological studies of liver section supported the evidence of biochemical study. In case of normal control group the hepatic cells had well preserved cytoplasm; nucleus was prominent as compared to CCl_4 administered group which showed complete loss of structural integrity of liver cells. However the administration of *Ficus carica* extract reversed the gross disturbances which were observed in hepatic cells architecture as shown in Figure 2. Histopathological study like biochemical findings also confirmed the toxic effect of CCl_4, which completely lost the architecture of liver cells, whereas the plant sample retrieved the cell architecture, this capability might be due to the presence of various phytochemical especially polyphenols (Sadeghi *et al .,* 2014).

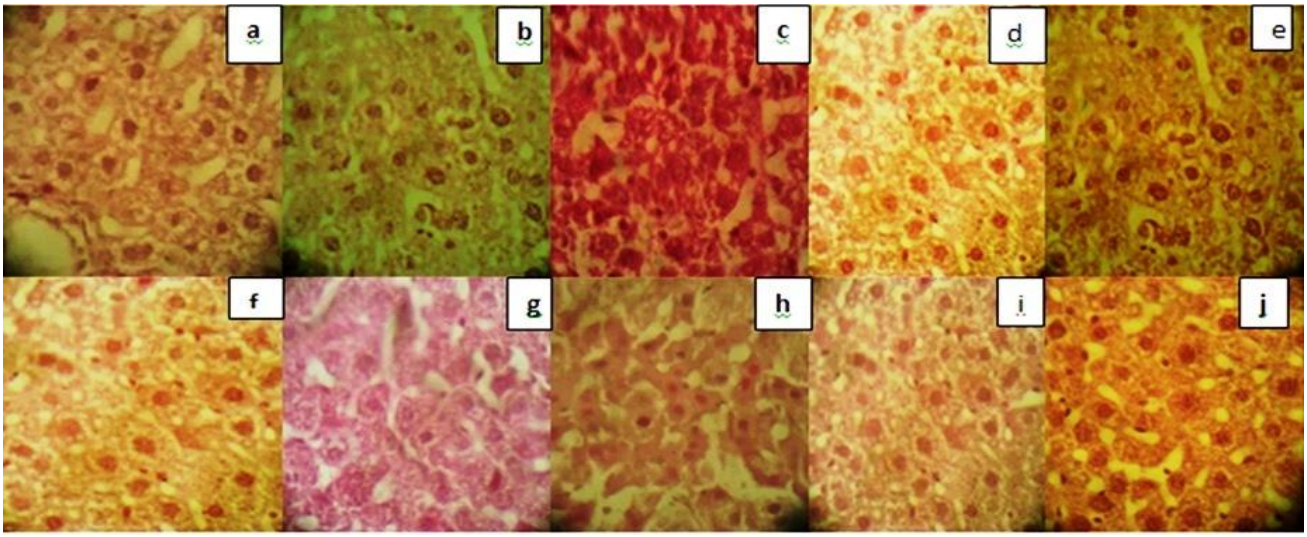

Figure 2: Histopathology of liver sections of mice a. normal group b. olive oil group c. CCl_4 intoxicated d. silymarin treated e. crude methanolic extract 200mg/kg treated f. crude methanolic extract 400mg/kg treated g. *n*. hexane fraction 200mg/kg treated h. *n. hexane* fraction 400mg/kg treated i. aqueous fraction 200mg/kg treated j. aqueous fraction 400mg/kg treated.

Conclusion: Fruit of *Ficus carica* had potential to treat injuries and biochemical changes caused by carbon tetrachloride and reversed the activity of antioxidant enzymes, which could be due to presence of phytochemicals such as phenols and flavonoids. So, it could be suggested from these findings that methanolic extract of *Ficus carica* had hepatoprotective effect against CCl_4 induced toxicity due to presence of antioxidant compounds. Aqueous fraction was found most effective.

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Conflict of interest: Authors are fully responsible for all research and have not any conflicts of interest.

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