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ORIGNAL ARTICLE

Hepatoprotective effects of black grapes (*Vitis vinifera*) seed extract against CCl_4 induced liver injury in rats

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Abstract

Black grape (*Vitis vinifera*), seeds extract is a rich source of flavonoids, proanthocyanidins and oligomer which have antibacterial, anti-inflammatory and antioxidant activity. Silymarin is a flavonolignan complex derived from the seeds and fruits of Silybum marianum, a milk thistle. It is originally used to treat a variety of liver illnesses and dysfunctions, including as hepatitis, alcoholic cirrhosis (due to drug or viral infection), and hepatic issues associated with diabetes. Carbon tetrachloride (CCl4) is a xenobiotic that is extensively used to cause oxidative stress and is one of the most commonly utilized hepatic toxins in the laboratory to induce liver injury. This study was designed to investigate the protective effect of grape seed extract (GSE) and silymarin against carbon tetrachloride induced liver injury in rats. Mature wistar rats were separated into five equal groups (each with six rats) and given the following treatments: Group 1 was retained as a control group and given normal saline orally; group 2 was kept as a control positive and administered CCL4 (1.0 mg/ kg b.w.) orally the day before to produce liver toxicity. Silymarin (50 mg/kg) was given to group 3 on a daily basis. GSE daily oral dose (100 mg/kg) was given in Group 4. Group 5 received a combination of silymarin (50 mg/kg) and GSE (100 mg/kg) treatments. A considerable increase in serum levels of aminotransferases (AST), alanine transaminase (ALT), and alkaline phosphatase was generated by CCl4 (ALP). Results shows that there is decrease in level of ALT (25.00±3.464) and AST (114±6.489) in Group 5 as compared to groups G2, G3 and G5, while ALP was decrease in Group 5 (118.67±10.68) as compare to G2, G3 and G4. Sample of blood were collected at 0 day and after administration of recommended doses treatment according to experimental protocol. The hematology results shown that there was increased level of RBCs 5.51±0.081 in G5 compare to other groups, WBCs was increased in G4 compare to other groups, Hb was increase in G5 compared to other groups. Histopathological analysis was accompanied by taking sample of liver. The data was statistically analyzed by using ANOVA.

Keywords

Black grape (*Vitis vinifera*)
Flavonoids
Xenobiotic
Silymarin and
Carbon tetrachloride (CCl4).

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Introduction

Many edible and non-edible plants (such as ginger, pomegranate, grape and banana) which are cited in the Holy Quran are is use as remedies for various ailments such as cough, intestinal bleeding and diarrhea. Therefore plants mentioned in the Holy Quran have been attracted by the botanists, biochemist and pharmacognosist for research proposes (Ahmad Wani et al., 2011).

The health benefits of black grapes (*Vitis vinifera*), one of the world's most widely consumed fruits, are enormous. It contains many active ingredients, including polyphenols, flavonoids, anthocyanins, procyanidins, proanthocyanidins, and components of stilbene. Anticarcinogenic, immunomodulatory, anti-diabetes, anti-obesity and anti-aging are the main medical characteristics of resveratrol and its constituent. (Nassiri-Asl and Hosseinzadeh, 2009) Although there are mainly bioactive components of grapes in grapes skin and grapes seed grape skin and seeds are typically discarded during daily feed consumption and grapes juice industries and winery. These wastes have bioactive constituents with a free radical scavenging ability, strong antioxidant and oxidative stress reduction, which is an effective stimulant for hepatocytes stellate cell activation (Li and Friedman, 1999; Xia et al., 2010) Consumption of *V. Vinifera* seed was observed to delay the incidence and progression of cataracts in diabetic rats. (Satyam et al., 2014).

Grape Proanthocyanidins were better antioxidants and free radical scavengers than vitamin C. (Katiyar, 2008). Grape proanthocyanidin are strong antioxidant that have been manifest in scientific study to upgrade cardio vascular functions (Karthikeyan et al., 2009).

The preventive effect of *V. vinifera* aqueous seeds extract against pancreatic dysfunction in diabetes is currently unknown. The purpose of this study was to see how Vitis vinifera seeds extract affected pancreatic function as well as levels of, inflammation, and oxidative stress, apoptosis in diabetic pancreas (Badavi et al., 2013). The combination of V. vinifera seeds, S. chinensis seeds, and Ttaraxacum officinale extract protected against hepatic harm caused by persistent alcohol exposure. (Kim et al., 2006).

Grapes seeds extract (GSE) is a by-product of the juice business that contains phenols, catechins, epicatechins, procyanidins, and proanthocyanidins, among other active components. antiaging, anti-inflammatory, anticancer, antibacterial, anti-diabetic and anti-viral properties of GSE as a dietary supplement have been demonstrated in animal models and people (Nowshehri et al., 2015).

Grapes, as well as grape leftovers (pomace or marc), including seeds, are noteworthy items in the wine making industry because of the health advantages of their components. Natural sources of bioactive components have been reported in by-products such as oil seeds and pomace yeasts. (Paradelo et al., 2010; M and H, 2016).

Grape berry phenolic contribute to wine's organoleptic qualities, albeit their composition varies depending on grape varietal, harvest conditions, and even grape age. Tannins and non-flavonoid stilbenes (resveratrol) are numerous in skin, while non-flavonoid hydroxycinnamic acids are prevalent in flesh. Seeds, on the other hand, include primarily flavan-3-ols as well as a variety of non-flavonoids, including those listed in the skin and flesh, and have a total phenol concentration 10 times that of the peel.(Tang et al., 2018).

Grape seed oil's hydrophilic and lipophilic components both help the body recuperate from the inflammation that occurs in many chronic conditions (Santangelo et al., 2007; Shinagawa et al., 2015).

Silymarin is an herbal substance that is derived from the dried seeds of the milk thistle plant. It is more concentrated in this portion of the plant than in other parts. It is widely utilized for its hepatoprotective properties in the treatment of liver illnesses all over the world. Other favorable effects of Silymarin supplementation have been emphasized by laboratory investigations and clinical trials in the last decade, including antioxidant, anti-inflammatory, immunomodulatory, and recently, antifibrotic and liver regenerative abilities. Silymarin has hepatoprotective as well as regenerating properties. Silymarin produces a compound that prevents toxins from entering the inside of the liver cells (AbouZid, 2012).

Silymarin has been proven to be a safe herbal substance in pharmacological investigations. Silymarin has been shown to be harmless in animal experiments. Silymarin has been found to be non-teratogenic and to have no post-mortem harmful effects (Nijris et al., 2020).

Silymarin has been shown to be a potent hepatoprotective in liver fibrosis caused by CCl_4 in new in vivo experiments (CCl4) by reducing the liver's damaging alterations once hepatic fibrosis has been induced (Jung et al., 2013). Silymarin anti-inflammatory properties prevent intrahepatic nuclear factor kappa B. activation, lowering levels of interelukin-2 (IL-2), tumor necrosis factor alpha interferon gamma and inducible nitric oxide synthase. (Colturato et al., 2012)

Materials and Methods

Drugs and chemicals: Silymarin and resveratrol were purchase from sigma-Aldrich. All reagents were analytical grade.

Preparation of ethanolic exctract: Black grapes (*Vitis vinefera*) were purchased from local market of

Faisalabad. Seeds were separated from the pulp of grapes and then the dried in hygienic conditions under a shade. After drying the required amount of seeds were powdered and extracted by using 95% ethanol than the extract was concentrated by use a rotary evaporator at 40±5°C and stored for further use in research (Giribabu et al. 2018).

Total flavonoids contents: Plant extracts' total flavonoid content was assessed used the method described by (Chang et al., 2006). In a 6-minute incubation period, 0.5ml of extract was combined with 2mL of D, water and 0.15mL of a 5% NaNO2 solution. After that, 0.15mL of 10% AlCl3 solutions was add to the mixture and it was incubated for another 6 minutes before adding 4 percent NaOH solution. The volume of the reaction mixture was increased to 5mL by adding methanol and mixing thoroughly. After fifteen minutes of incubation, the reaction mixture's absorbance was measured at 510nm. The catechin linear regression curve was used to compute the total flavonoids contents of the extract, which was then reported as catechin equivalents.

Total phenolic contents: The total phenolic content of sample extracts was determined using the Folin Ciocalteu method. For this 1 mL of material was mixed with 5ml Folin Ciocalteu (10 percent) and 4ml Na_2CO_3 (20%) for 1 hour of incubation. The absorbance of the blue color complex that resulted was measure at 765nm. The measurement was carried out in compliance with the standard (gallic acid). Different amounts of gallic acid were used to create the calibration curve. 1 ml aliquots of 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 0.10mg/mL gallic acid solution in methanol were combined with 5ml Folin Ciocalteu reagent (diluted ten times) and 4 ml Na_2CO_3 (20 percent). The calibration curve was built using absorbance as a function of concentration after 1 hour of measurement at 765nm. Using the following procedure, the total concentrations of phenolic compounds in plant extracts was quantified in gallic acid equivalents.

Quantitative analysis of grapes seeds extract (HPLC analysis): For quantitative and qualitative analysis, high performance liquid chromatography (HPLC) test, total flavonoids test, anti-oxidant activity test and total phenolic contents tests were done (Giribabu et al., 2018).

***In vitro* anti-oxidant activity**

DPPH assay: The approach of Yen and Chen was used to measure DPPH radical scavenging activity with slight modifications. 3 mL plant extracts were combined with 1 mL newly produced 0.004% DPPH in methanol solution and stored in the black dark for 30 minutes. The absorbance was then measured at 517 nano-meters. A reaction combination with a low absorbance indicates a significant free radical scavengings activity. The antioxidant activity of BHT was compared to that of Vitamin-C a control substance. As a control, a solution without plant extract was employed. Each test was repeated three times. The formula below was used to compute the percentage inhibition of DPPH radical samples.

Inhibition of DPPH (percentage) = Absorbance of Blank, Absorbance of Sample/ Absorbance of Blank 100.

Experimental animals: Healthy adult male Wister rats (230 to 250 gram), were used in experimental procedure according to the ethical guidelines for laboratory animals (Giribabu et al 2018).

Blood collection: Blood was collected directly from the heart during the treatment protocol for serum liver enzymes inspection (Giribabu et al., 2018).

Table 1: Experimental design

Groups	Treatments
Control negative	Normal control.
control positive	CCl_4
Standard	CCl_4; silymarine.
Treatment	CCl_4; grape seed extract.
Combine treatment	CCl_4; grape seed extract plus silymarin.

Biochemical parameters

Estimation of alanine aminotransferase or alanine transaminase (ALAT or ALT): The ALT was determined using a Merck Company commercially available kit (Bergmeyer et al., (1986).

Procedure: Mix 100 liters of reagents 2 with 400 liters of reagent 1 then add 50 liters of serum sample. Examine and record the absorbance.

Calculation: The computation was measured for the first two minutes inside Hg 340 nm and then multiplied by the 1745 factor. A 0.1 mL serum sample dilutes into 0.9 mL physiological saline when the absorbance limit is exceeded. The reading was taken and the outcome was ten folded. Enzymatic activity (U/L) = (Δ A/ min) × F

Estimation of Aspartate aminotransferase AST: A commercial kit (Merck, Germany) L-Aspartate + 2-Oxoglutarate ASAT was used to assess the level of AST in the blood.

Procedure: Combine 100 ul of serum with 1000 ul of mono reagent at 37 °C for one minute then read absorbance A1, A2, and A3. $\Delta A = A1 - A2$

Calculation: ASAT, activity (U/L) $= \triangle A/\text{min} \times$ factor (U/L)

Factor =1745 at 340 nm wavelength.

Hematology and histopathology: Hematological parameters including red blood cell counts (RBCs), white blood cell counts (WBCs), platelet count and hemoglobin concentration were calculated. At end of this study liver samples were collected for histopathology.

Statistical analysis: Data were analyzed by appropriate statistical method.

Results

Polyphenol contents and DPPH %inhibition: The TPC in GSE determined by Folin Ciocalteau method was 270 mg GAE/g of sample and TFC was 58.55 mg CE/g of sample. GSE exhibited 39.29% DPPH* free radical scavenging activity as shown in Figure 1.

Hematological parameter

Red blood cells (RBCs): The mean (± SE) RBCs level of rats in control negative was 4.78±0.139. In control positive 5.31±0.081, in standard 5.28±0.040, in treatment 5.30±0.081 and in combine treatment 5.51±0.081. Statistical analysis showed significant (P<0.0023) value of RBCs level of rats as shown in Figure 4.

White blood cell (WBCs): The mean (± SE) WBC level of rats in control negative was 2.70±0.058, in control positive 5.40±0.100, in standard 8.10±0.115, in treatment 13.30±0.35 and in combine treatment 8.60±0.289. Statistical analysis showed significant (P<0.0000) value of WBC level of rats as shown in Figure 4.

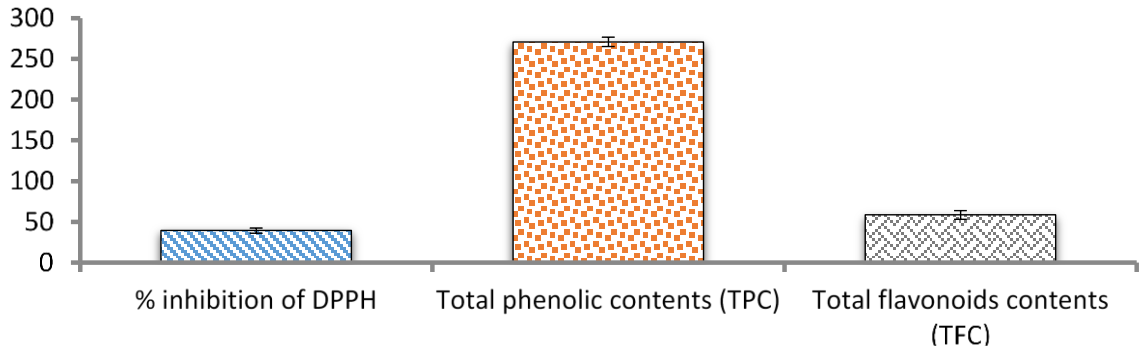

Figure 1: Total phenolic and flavonoid contents of grape seeds extract.

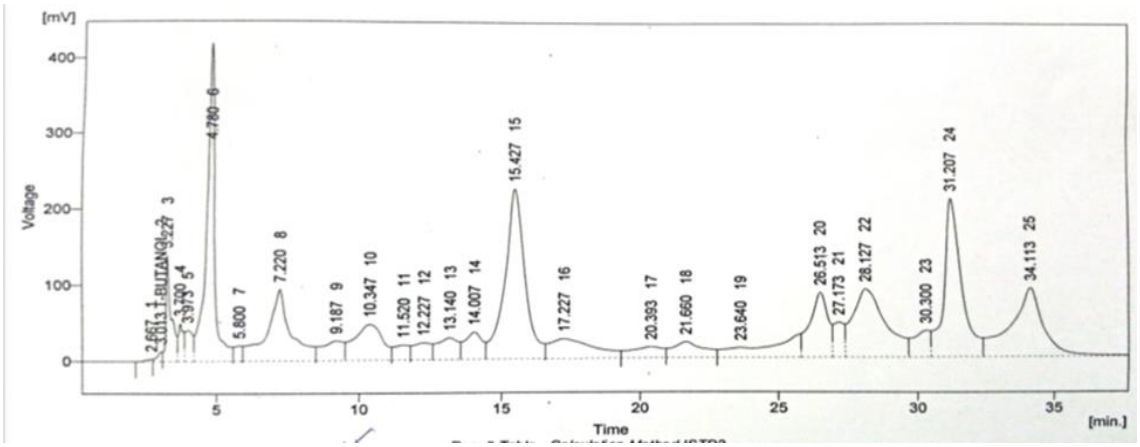

Figure 2: Preperation of grape seeds extract for gradiant mode HPLC analysis.

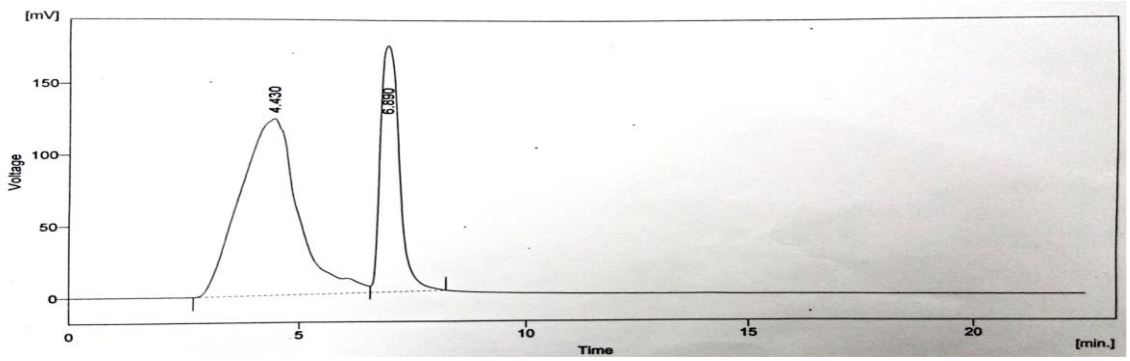

Figure 3: Preparation of drug sample for isocratic mode HPLC analysis.

Hemoglobin levels estimation: The mean (± SE) hemoglobin (Hb) level of rats in control negative 10.20±0.289 in control positive 12.50±0.173 in standard 12.40±0.231 in treatment 12.60±0.058 and in combine treatment 13.10±0.404. Statistical analysis showed significant (P<0.0001) value of Hb level of rats as shown in Figure 4.

Platelets count: The mean (± SE) PLT level of rats in control negative was 194.00±5.196, in control positive 513.00±16.92, in standard 473.00±14.01, in treatment 453.00±9.238 and in combine treatment 196.00±8.083. Statistical analysis showed significant (P<0.0000) value of PLT level of rats as shown in Figure 4.

Biochemical parameter

a) ALT: The mean (± SE) ALT level of rats in control negative was 27.00±1.155, in control positive 42.67±2.028, in standard 40.00±1.155, in treatment 33.67±2.028 and in combine treatment 25.00±3.464. Statistical analysis showed significant (P<0.0035) value of ALT level of rats as shown in Figure 5.

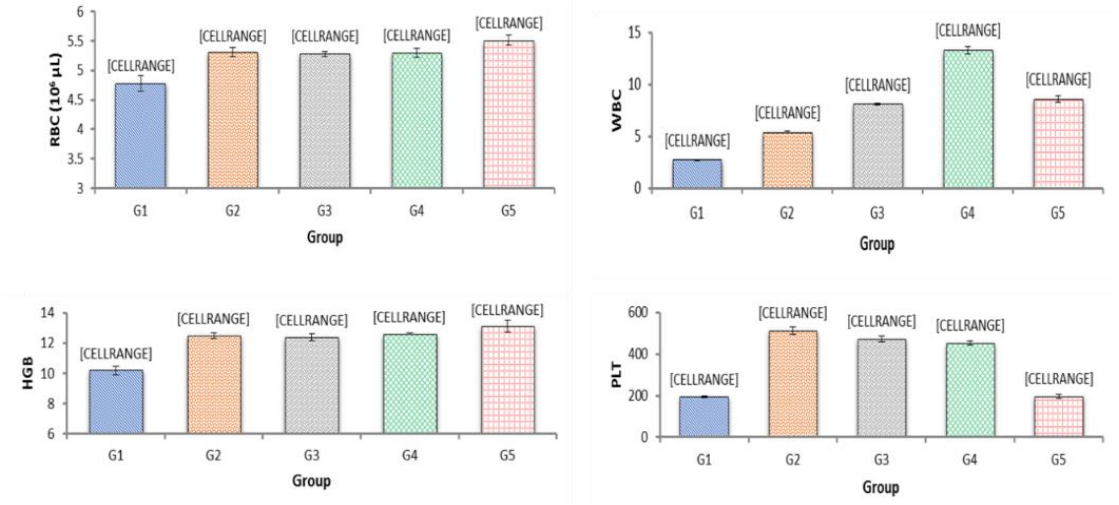

Figure 4: The mean (± SE) levels of RBCs, WBCs, hemoglobin (Hb) and platelets (PLT) of rats in different groups of experiment.

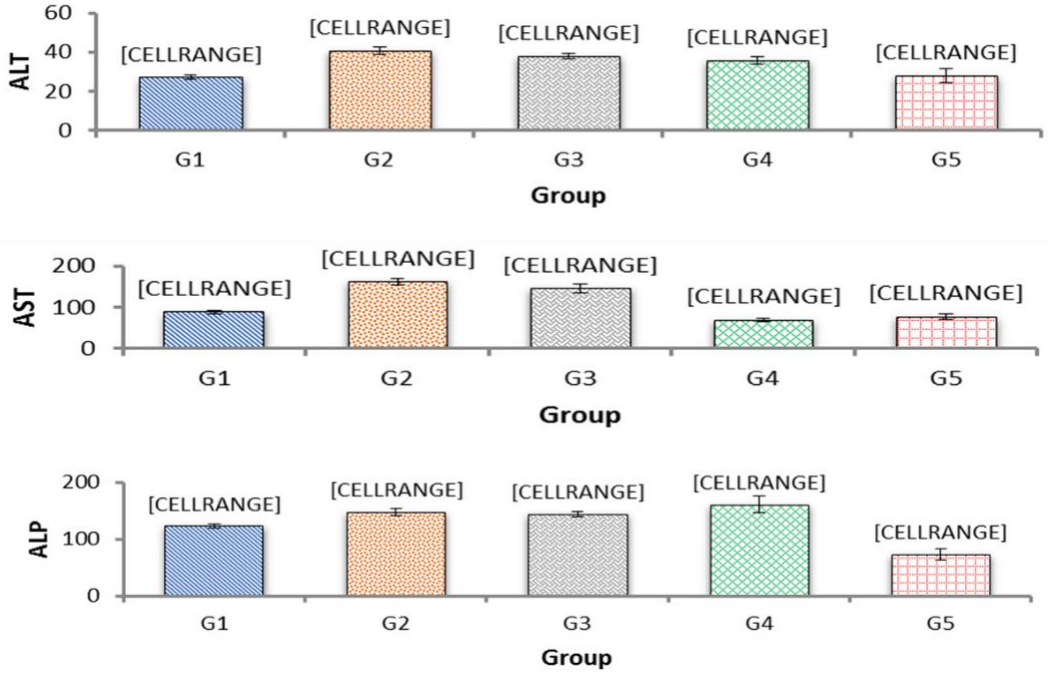

Figure 5: The mean (± SE) levels of ALT, AST and ALP of rats in different groups of experiment.

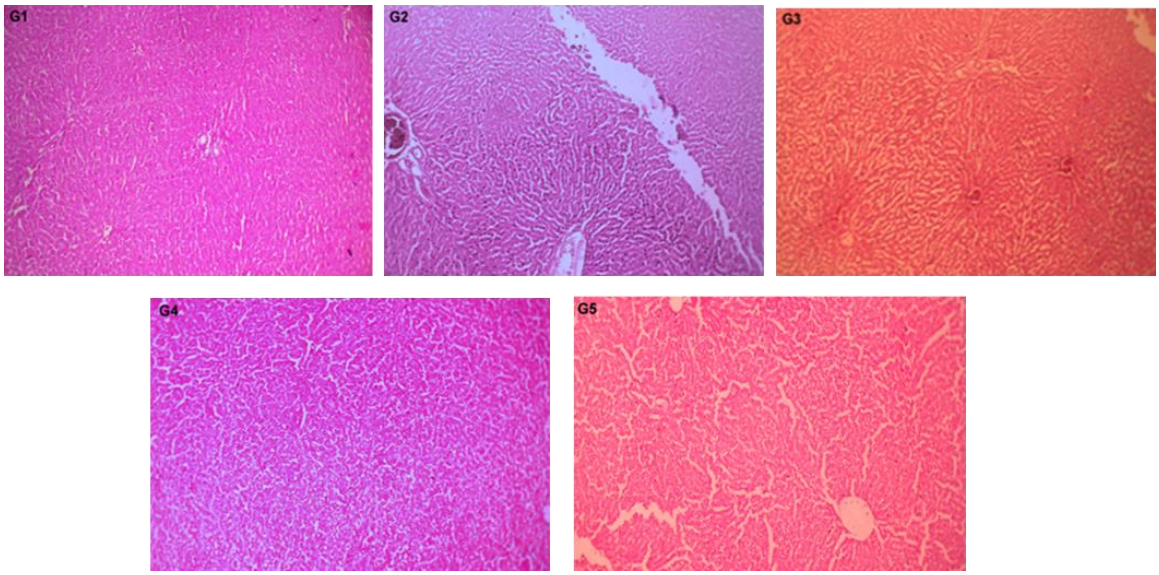

Figure 6: Photomicrographs of liver of rats in different groups of experiment.

b) AST: The mean (± SE) AST level of rats in control negative group was 88.00±4.933, in control positive 158.67±8.007, in standard 137.00±10.39, in treatment 123.00±4.163 and in combine treatment 114±6.489. Statistical analysis showed significant (P<0.0035) value of AST level of rats as shown in Figure 5.

c) ALP: The mean (± SE) ALP level of rats in control negative was 123.00±3.000, in control positive 155.67±5.783, in standard 143.00±5.196, in treatment 130.00±14.64 and in combine treatment 118S.67±10.68. Statistical analysis showed significant (P<0.0003) value of ALP level of rats as shown in Figure 5.

Histopathological examination: Photomicrograph of liver of rat in group 1 normal, showed the normal structure of sinusoidal space, hepatocytes and no infiltration of inflammatory cells. The group 2 treated with CCL_4 showed mononuclear cellular infiltration and vacuolation in between the hepatocytes. The group 3 treated with Silymarin showed the normal structure of hepatocytes and has no cellular infiltration around the portal area or central vein, in between the hepatocytes. The group 4 treated with GSE, showed the normal structure of hepatocytes and has no cellular infiltration around the portal area or central vein, in between the hepatocytes. The group 5 treated with GSE + Silymarin, showing the prominent improve in hepatocytes as shown in Figure 6.

Discussion

In the current study, the results indicated that CCl_4 induced a severe toxicity to the animals and were in agreement with the previous reports. Significant alterations in serum biochemical markers, similar to those reported in the literature, were caused by CCl4. The aim of this research was induced acute hepatitis by ccl_4 in rats and then treated with grapes (*vitis vinifera*) ethanolic seeds extract alone and synergistic treatment with silymarin standard and *vitis vinifera* seeds extract. Hepatic DNA (strand breaks) and RNA were damaged by CCl4. This shows that the oxidation products of hepatic nucleic acids assessed in this study are effective indicators of oxidative liver damage caused by CCl4. Different types of oxidative stress produce different oxidation products, and biomarkers sensitive to other insults may not be the same as those that were obviously raised in this CCl4 model (Abdel-Aziem et al., 2011). The increased ALT, AST, and ALP levels found in this study suggested significant hepatic parenchymal cell damage (Abdel-Wahhab et al., 1999; Barton et al., 2001). These findings confirmed that CCl4 has a detrimental and stressful effect on hepatic tissue, which is in line with previous findings (Abdel-Wahhab et al., 2010; Bhattacharjee and Sil 2007). In experimental hepatic fibrosis, combined administration of grape seeds extract and silymarin effectively attenuated thioacetamide induced liver fibrosis and biochemical parameters were greatly improved, hepatic content was reduced, oxidative stress was averted, and growth stimulating hormone levels were restored. Our findings revealed that GSE and silymarin combinations have the potential to protect animals from oxidative stress-related harm by boosting antioxidant defense mechanisms and increasing antioxidant state (Nada et al., 2015). Silymarin (50 mg/kg) treatment dramatically reduced blood LFT, tests and total bilirubin levels while considerably increasing serum total proteins level. These effects could be related to direct protection of liver cells by maintaining the cell membrane and

limiting lipid peroxidation by preventing hepatic glutathione depletion. (Li et al., 2012). Regulate cell membrane permeability and suppressing leukotriene, and scavenging reactive oxygen species are all pharmacological activities of silymarin. (22 mg/kg) silymarin lowered plasma discharge of hepatic enzyme ALT and AST, lowered serum level of ALP and slowed the progression of carbon tetrachloride-induced liver damage and liver fibrosis (CCl4) (Abdel-Salam et al., 2007; Basit, 2014). Transaminase activity is also a sensitive predictor of acute hepatic necrosis because the liver is assumed to be the primary target organ for CCl4 (Sarhan et al., 2012; MM, 1987).

In current study, serum AST, ALT and ALP level were influenced as a response to ccl4 indicated in liver injury at species of rats which utilized. In group of control positive AST level is 161.67±8.007. Treatment of GSE the AST level is 70.00±4.163. The silymarin standard group AST level indicated 146.00±10.39 and synergistic treatment of GSE and silymarin standard AST level is 77.67±6.489. The level of AST was increased in control positive group. ALT level in control positive group is 27.00±1.155. Level of ALT in GSE treatment group is 22.67±2.028. In group of silymarin standard ALT level is 38.00±1.155. In combine treatment group level of ALT is 28.00±3.464. So, the level of ALT decreased in the GSE treatment. ALP level in control positive group shows 147.67±5.783. ALP level in silymarin standard group of treatment is 144.00±5.196. In group of GSE is 161.00±14.64. In combine treatment group the level of ALP is 72.67±10.68. In combine treatment group ALP level is significantly decreased.

The delivery of apitherapy formulations to groups with experimentally produced hepatopathy by CCl4 causes an increase in WBC, an improvement in RBC, Hb, and RDW values, and an increase in thrombocytes, as measured by blood parameters. In case of CCl4-induced hepatopathy, the blood count values are like WBC 4.86±0.69 (103/mcL), RBC (103/mcL) 7.22±1.21, Hgb 13.27±1.47 (g/dL) and HCT (%) 39.9±4.3. The blood count values of rats with CCl4-induced hepatopathy are normalized when they are fed an apitherapy diet are WBC 10.78±3.12 (103/mcL), RBC 7.53±0.61 (103/mcL), Hb 14.48±1.49 (g/dL) and HCT 44.25±3.24 (%).

In current study CBC result shown that, WBC in control positive group show that 2.70±0.058. In control negative show that 5.40±0.100. In silymarin standard group are WBC counted 8.10±0.115. In GSE treatment group WBC was counted 13.30±0.351. In combined treatment group WBC level is 8.60±0.289. In the GSE treatment WBC counted significantly increased. The level of RBC in normal control group is 4.78±0.139. In control positive group the level of RBC is counted 5.31±0.081. In silymarin standard group level of RBC is 5.28±0.040. In GSE treatment group level of RBC is 5.30±0.081. In combine treatment group level of RBC counted 5.51±0.081. Level of RBC is significantly increased in combined treatment. Hb in normal control group is 10.20±0.289. In control negative level of Hb recorded 12.50±0.173. In silymarin standard treatment group Hb level is 12.40±0.231 and in GSE treatment group recorded 12.60±0.058. In combine treatment group the Hgb level is 13.10±0.404. Hgb level is significantly increased in combined treatment group.

Bioflavonoids have been widely studied in terms of their biological, pharmacological, and therapeutic effects. Proanthocyanidins and other polyphenolic chemicals in GSE are gaining popularity due to their pharmacological, medical, and therapeutic potential. Polyphenolic chemicals found in GSE, such as proanthocyanidin and procyanidin have a strong free radical scavenging's activity (Bagchi et al., 1998). In rats, GSE was shown to reduce liver fibrosis and dysfunction caused by prolonged arsenic injection in a previous study (Xu et al., 2010).

In present excremental study The TPC in GSE determined by Folin Ciocalteau method was 270 mg GAE/g of sample and TFC was 58.55 mg CE/g of sample. GSE exhibited 39.29% DPPH* free radical scavenging activity as shown in Figure 1.

Conclusion: It is concluded that grape seeds extract and silymarin combination have synergistic effect as anti-hepatocytic therapy than single treatment with silymarin or grapes seeds extract alone and combination with silymarin has a good resultant effect in protecting the CCl_4 induced liver injury in rats.

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