Hepatoprotective and toxicological studies of *Salvia bucharica* methanolic extract in rabbits

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Abstract: Most of the species of genus Salvia are famous for having medicinal properties due to their chemical constituents. *Salvia bucharica* (Lamiacea) is found in Balochistan near Quetta in Hannaurak and Kalat. It is used in traditional system of medicine and claims to cure liver ailments. In current study crude methanolic extract (CME) of *Salvia bucharica* was obtained from the leaves and tested for hepatoprotective activity and possible toxicity in rabbits. Liver toxicity was induced in rabbits by administration of carbon tetra chloride (CCl_4) and evaluated by biochemical tests and histopathology of tissues. In this study rabbits were divided in to 3 groups (5 rabbit in each group). Rabbits of group I (control) were administered only vehicle (0.9% sodium chloride) orally. Rabbits of group II were given CCl_4 and group III were treated with CCl_4 and *S. bucharica* CME orally. For hepatoprotective effect serum enzyme level and total protein level were calculated. Histopathology of liver sections of rabbits was also carried out to observe protective effect. Biochemical, hematological and histoptahological parameters were studied on rabbits for toxicological studies. *S. bucharica* CME showed significant liver protection with reduction in total bilirubin, direct bilirubin, Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), gamma glutamyl transpeptidase (γ-GT). And decrease in Albumin and globulin. In toxicological studies, biochemical and histoptahological parameters showed no significant toxicity in liver, heart and kidneys. It is concluded that *S. bucharica* CME showed hepatoprotective effects with nontoxic profile.

Keywords: Hepatoprotective, *Salvia bucharica,* Crude methanolic extract (CME), Balochistan. Carbon tetra chloride (CCl4)

INTRODUCTION

Salvia is one of the main genera of Lamiaceae family, there are about 1000 species of salvia, (Fernandez *et al.,* 2003, Garcia *et al.,* 2006). The word Salvia is Latin word "salvare" means "to be unharmed and safe or to heal", which accounts for folkloric faith of its "enchanted" healing properties for many types of disorders and its acceptance in folk medicine (Ulubelen 2002). Out of 1000 species of Salvia, only 134 have been studied. For example, dried roots of *S. miltiorrhiza* is the most popular traditional herbal medicine in Asia and other countries and used extensively for various forms of hepatitis, cerebrovascular diseases, chronic renal failure, dysmenorrhea, for the cure of coronary artery diseases, myocardial infarction and angina pectoris (Watzke *et al.,* 2006, Wang *et al.,* 2010). *S. caveleriei* is used for the cure of boils, dysentery and fall injuries, *S. desoleria* for treatment of CNS, digestive and menstrual disorders, *S. bucharica* for liver disorder (Ibrahim *et al.,* 2007).

Salvia bucharica (Lamiaceae) is a perennial plant found in Pakistan, Afghanistan and Central Asia (Ahmad *et al.,* 1999). In Pakistan it is found in Balochistan at herboi (Kalat) and near Quetta at Hannaurak. Local name is `sur-sunda. Locally used for the cure of liver ailments (Ali *et al.,* 2007, Tareen *et al.,* 2010). In Balochistan the decoction of the entire plant is prepared by soaking in water and then it is used for the colic pain, kidney pain, fever, jaundice and hypertension (Tareen *et al.,* 2010). Current study was carried out on *S. bucharica* crude methanolic extract (CME) of leaves to find out its hepatoprotective effect and toxicity.

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MATERIAL AND METHODS

Plant material

Plant material was collected from Hanna Urak near Quetta. Plant was identified by Dr. Atta Muhammad Sarangzai, Associate Professor, Department of Botany, University of Balochistan, Quetta. A voucher specimen was deposited in the herbarium of the Department of Botany, University of Balochistan, Quetta (Voucher No. S-912).

Animals

For chronic toxicity test New Zealand white rabbits (male) were used. Animals were purchased from Animal house of Dow university of Health Sciences. Standard protocol in-accordance with GLP (Good Laboratory

Practice) Regulations of WHO was followed (Aniagu *et al.,* 2005). Rabbits were acclimatized seven day before study. Rabbits were housed in standard condition of humidity, temperature and 12 hour light/ darkness cycle.

CCL_4 induced hepatotoxicity

White healthy rabbits (1000-1400gm) were used in the study. Rabbits were divided into three groups (5 rabbits in each group). In Control (Group I) 0.9% NaCl (1ml/Kg) was given orally. CCl_4 treated group (Group II) received distill water (1ml/Kg orally) and administered CCl_4, liquid paraffin (1:1, 1.5ml/Kg) after 36 hour of last dose of vehicle. CME *S. bucharica* treated group (Group III) was administered aqueous suspension of *S. bucharica* CME 300mg/kg for 15 days. The group III was received CCl_4: liquid paraffin (1:1, 1.5ml/kg) after 36 hour of last dose of extract (Yasmin *et al.,* 2009, Prochezian *et al.,* 2005).

Sample collection

Blood samples were collected after 36 hours of last dose. Blood (approximately 5cc in tubes) was collected through cardiac puncture technique (Feroz *et al.,* 2011b, Etang *et al.,* 2013). It was centrifuged at 4000 rpm for 8 minutes, to separate the serum (using Eppendorf 5810R centrifuge).

Assessment of hepatic parameters

Within 3 hours of sample collection serum was analyzed for Total Bilirubin, Direct Bilirubin, Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Gamma glutamyl transpeptidase (γ-GT), Albumin and Globulin on automatic analyzer (Merck) at 37^{o}C by using standard reagent kits obtained from Merck (Feroze *et al.,* 2013, Etang *et al.,* 2013).

Histopathological examination of liver tissues

Animals were sacrificed and their livers were collected. Randomly selected liver tissues were selected for hsitopathological examination. It was carried out by microscopy of liver tissue to observe the histological changes. 3- 4micron thick sections were taken from wax blocks by rotary manual microtome. The sections were fixed on the slides and were dried gently by pressing with the filter paper. Standard staining procedure was followed for histopathological analysis (Feroze *et al.,* 2013).

Chronic toxicity studies

Rabbits were divided into 2 groups (5 animals each group). Group A (Control group) received distilled water 1ml/Kg orally and Group B, *S. bucharica* CME treated (300 mg/ kg body weight) group, given the drug orally for 90 days.

Mortality and clinical signs

During the dosing period (90 days), all animals were observed daily for any clinical signs and mortality, once before the dosing, immediately after the dosing and up to 4 hours after dosing.

Blood collection

On day 91 blood was collected by cardiac puncture technique, by using sterile 10ml syringes, in anti-coagulant free serum separator tubes, allowed to stand for one hour to clot, then centrifuged at 4000 rpm for 8 minutes (using Eppendorf 5810R centrifuge). Serum was transferred in tubes, and stored at temp of -20^{o}C till further use (Etang *et al.,* 2013, Feroz *et al.,* 2011b).

Biochemical tests

The blood for serum biochemistry was collected into tubes containing anti-coagulant. These tests were carried out to determine the Liver function Tests i.e. Total Bilirubin, Direct Bilirubin, Alkaline Phosphatase, Gamma GT and SGPT, Total proteins i.e. Albumin, Globulin, Lipid profile i.e. Cholesterol, Cholesterol HDL ratio, Triglycerides, LDL, HDL and VLDL, on automatic analyzer (Merck) at 37^{o}C by using standard reagent kits obtained from Merck (Germany).

Haematology

On day 91 blood samples were taken and drawn into a test tube contains EDTA (an anticoagulant) to prevent clotting. Sample was analyzed in automatic analyzer (using Beckman Coulter HMX analyzer, USA). Parameters hemoglobin, Red blood cells count, Hematocrit (HCT/PCV), MCV, MCH, MCH, Total white blood cells Count and Platelets Count were carried out (Etang *et al.,* 2013).

Organ collection

After collection of blood, rabbits were sacrificed and their organs i.e. Livers, Hearts and Kidneys were removed and stored in formalin for histopathological examination.

Gross pathology and microscopic examination

Tissue biopsies from the Liver, heart and kidneys were taken. Biopsies were fixed in 10% formalin. After dehydration tissues were embedding in paraffin, sections were cut at 3-4μ with the microtome, stained with hematoxylin and eosin and examined with light microscope (Aniagu *et al.,* 2005, Feroze *et al.,* 2013).

STATISTICAL ANALYSIS

All values were compared with control by taking mean and standard error of the mean using t-test and value of $p<0.05$ were considered as significant (Feroze *et al.,* 2013).

RESULTS

Hepatoprotective activity

Liver function test

In group I (Control) Total Bilirubin level was 0.26±0.015, Direct Bilirubin (mg/dL) 0.01±0.008, Serum ALT (U/L) level was 52.05±0.745, Alkaline Phosphatase (ALP U/L) was 97.63±0.518, Gamma GT(U/L) level was 2.12±0.382 and AST (U/L) level was13.19±0.724 (table 1).

Table 1: Hepatoprotective activity: Effects of *S. bucharica* CME in liver function test of rabbit.

S No.	Test	Control (Mean ±SEM)	CCl_4 treated (Mean ±SEM)	Drug treated (Mean ±SEM)
1	Total Bilirubin (mg/dL)	0.26±0.015	1.356±0.65**	0.20±0.003*
2	Direct Bilirubin (mg/dL)	0.01±0.008	0.914±0.005**	0.07±0.004*
3	SGPT (ALT) (U/L)	52.05±0.745	94.80±1.070**	65.80±1.598*
4	Alkaline Phospatase (ALP)(U/L)	97.63±0.518	148.60±1.032**	63.00±0.709*
5	Gamma GT (U/L)	2.12±0.382	7.01±0.134**	4.30±0.089*
6	AST (U/L)	13.19±0.724	24.80±0.584**	21.52±0.454*

Table 2: Hepatoprotective activity: Effects of *S. bucharica* CME in total protein test of rabbits.

S No.	Test	Control (Mean± SEM)	CCl_4 treated (Mean ±SEM)	Drug treated (Mean± SEM)
1	Total proteins (g/dL)	8.08±0.319	4.72±0.073**	9.34±0.092*
2	Albumin (g/dL)	4.62±0.149	2.38±0.049**	4.938±0.139*
3	Globulin (g/dL)	3.58±0.196	6.32±0.080**	4.58±0.037*

All values are mean ± SEM; n=5; * = Significant results (p<0.05), ** = highly significant results (p<0.01).

Table 3: Chronic toxicity test: Effect of *S. bucharica* CME on kidney function test on rabbits.

S No.	Test	Control (Mean± SEM)	Drug treated (Mean ±SEM)
1	Urea (mg/dL)	54.02±1.42	69.02±1.160**
2	Creatinine	0.67±0.012	1.47±0.005

Table 4: Chronic toxicity test: Effect of *S. bucharica* CME on blood glucose (random) and Hb A1c % of rabbits.

S No.	Test	Control (Mean± SEM)	Drug treated (Mean± SEM)
1	Blood Glucose Random	112.10±2.199	78.10±0.680**
2	Hb A1c (%)	3.496±0.015	3.02±0.037*

Table 5: Chronic toxicity test: Effect of *S. bucharica* CME on cardiac enzymes of rabbits.

S No.	Test	Control (Mean± SEM)	Drug treated (Mean ±SEM)
1	LDH (U/L)	289.02±1.807	34.7±0.083**
2	CPK (U/L)	1261.2±0.584	1980.00±2.746**
3	CK-MB (U/L)	27.02±0.694	26.98±0.723
4	SGOT (U/L)	13.01±0.724	24.04±0.790**

Table 6: Chronic toxicity test: Effect of *S. bucharica* CME on Lipid profile of rabbit.

S No.	Test	Control (Mean± SEM)	Drug treated (Mean±SEM)
1	Cholesterol HDL ratio	3.10±0.031	4.21±0.039*
2	Cholesterol (mg/dL)	54.01±0.838	26.20±1.396**
3	Triglycerides (mg/dL)	135.03±1.116	104.80±1.022**
4	HDL (mg/dL)	17.00±0.709	7.03±0.576**
5	LDL (mg/dL)	26.00±0.549	2.20±0.20**
6	VLDL (mg/dL)	27.04±0.709	28.20±1.160**

Table7: Chronic toxicity test: Effect of *S. bucharica* CME on blood of rabbit.

S No.	Test	Control (Mean± SEM)	Drug treated (Mean± SEM)
1	Hb (g/dl)	13.20±0.070	12.54±0.103
2	RBC Count (million/ul)	6.10±0.031	6.048±0.139
3	Hematocrit (HCT/PCV) %	42.06±0.322	38.42±0.244
4	MCV (fl)	63.2±1.070	63.08±0.229
5	MCH (pg)	22.10±0.783	20.90±0.031
6	MCHC (g/l)	32.10±0.332	32.80±0.070
7	Total WBC Count (×10^9/L)	11.40±0.176	10.58±0.037
8	Platelet Count (×10^9/L)	490.02±2.155	556±5.747**

All values are mean ± SEM; n=5; *=Significant (p<0.05), **=highly significant (p<0.01).

In group II (CCl4 treated group), highly significant ($p<0.01$) increase was observed in levels of Serum Total Bilirubin, Direct Bilirubin, ALT, Alkaline Phosphatase (ALP), Gamma GT and AST (table 1) as compare to group I. In group III (*S. bucharica* CME treated group), significant ($p<0.05$) decrease was observed in levels of serum Total Bilirubin, Direct Bilirubin, ALT, Alkaline Phosphatase (ALP), Gamma GT and AST (table 1) as compare to group II.

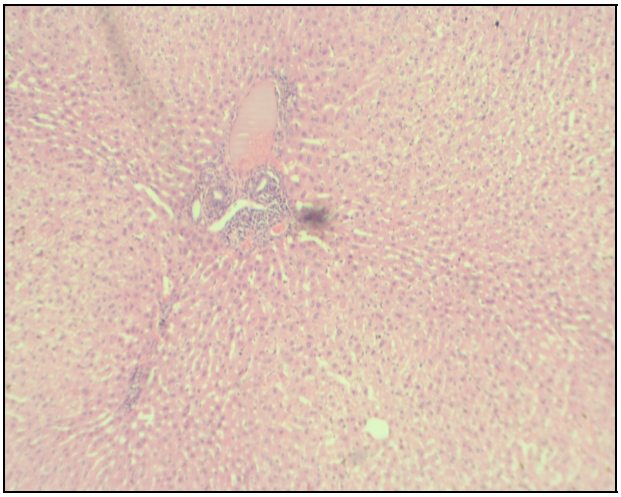

Fig. 1: Photomicrograph (Lens10x) of control group rabbit liver revealing parenchyma with normal looking hepatocyte and portal tract.

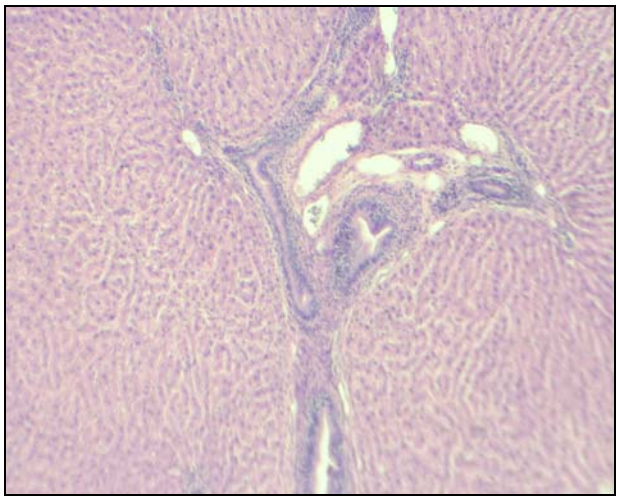

Fig. 2: Photomicrograph (Lens 10x) of CCl_4 treated group rabbit revealing mild inflammation.

Total protein test

In-group I (Control) Total Proteins (g/dL) was 8.08±0.319, Albumin (g/dL) was 4.62±0.149 and Globulin (g/dL) was 3.58±0.196 (table 2). In group II (CCl4 treated group) highly significant ($p<0.01$) decrease was observed in levels of serum Total protein and Albumin and highly significant ($p<0.01$) increase was observed in levels of serum Globulin (table 2) as compare to group I. In group III (*S. bucharica* CME treated group) significant ($p<0.05$) increase was observed in levels of serum Total protein and Albumin and significant ($p<0.05$) decrease was observed in levels of serum Globulin as compared to group II (table 2).

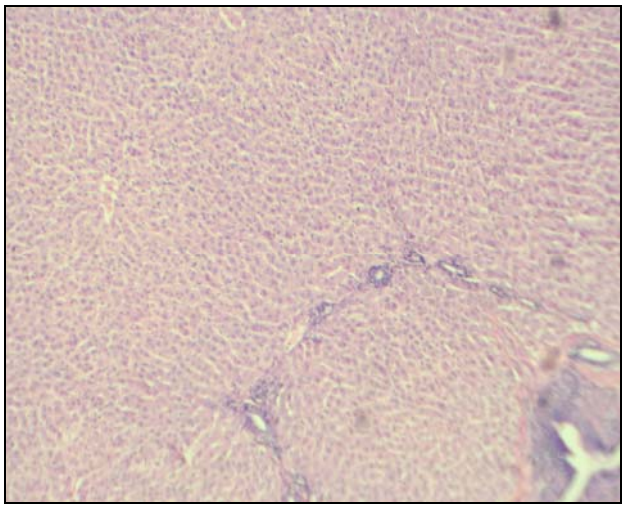

Fig. 3: Photomicrograph (lens 10x) of Liver. Rabbit treated with *S. bucharica* shows normal looking hepatocyte and portal tract.

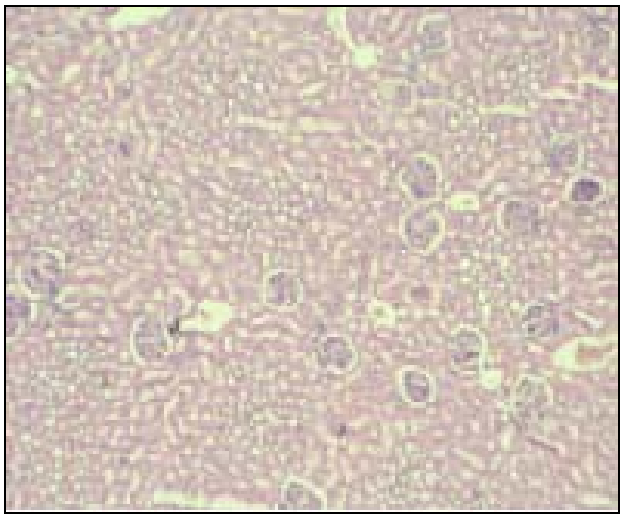

Fig. 4: Photomicrograph (Lens10x) of *S. bucharica* treated group reveals renal parenchyma with normal looking glomeruli.

Histopathological observations

Histoarchitecture of liver of control group showed normal cells with distinctive hepatic cells (fig. 1). Histoarchitecture of CCl_4 treated group showed mild inflammation (fig. 2). *S. bucharica* CME treated group did not reveal any microscopic changes as compared with control group.

Chronic toxicity test

Biochemical test

In-group I Urea (mg/dL) concentration was 54.02±1.4 and Creatinine level was 03.67±0.012. In-group II (*S.*

bucharica CME treated group) there was a significant ($p<0.05$) increase in concentration of urea and creatinine (table 3).

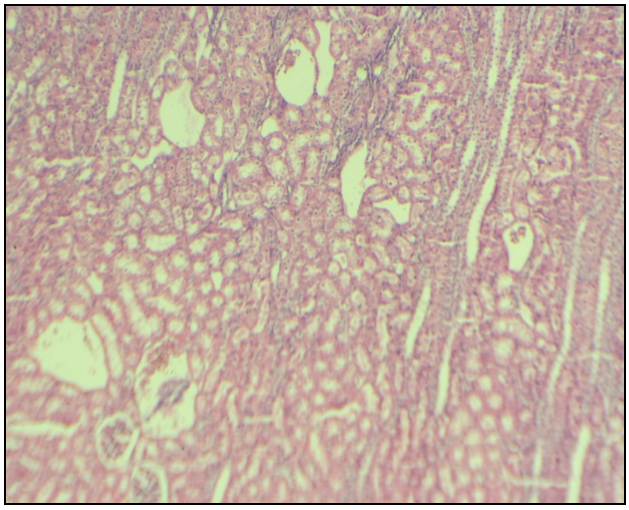

Fig. 5: Photomicrograph (Lens10x) of *S. bucharica* treated group shows renal parenchyma with normal looking glomeruli.

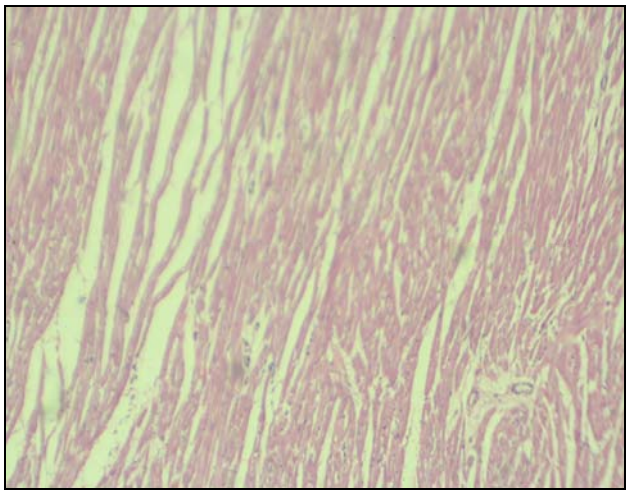

Fig. 6: Photomicrograph (Lens 10x) of *S. bucharica* treated group heart shows normal myocardium

Blood glucose test of rabbit shows that Blood glucose level (random) in-group I was 112.10±2.199. Highly significant ($p<0.01$) decrease in Blood glucose level of group II observed (table 4). In cardiac enzyme assay values for group I were, LDH (U/L) 289.02±1.807, CPK (U/L) 1261.2±0.584, CK-MB (U/L) 27.02±0.694 and SGOT (U/L) 13.01±0.724. In-group II (*S. bucharica* CME treated group) there was a significant decrease ($p<0.01$) in LDH, and there was significant increase ($p<0.01$) in CPK and SGOT. There was no significant change in concentration of CK-MB (table 5).

In Lipid profile test, In-group I (control) the Cholesterol HDL ratio was 3.10±0.031, Cholesterol (mg/dL) level was 54.01±0.838, HDL (mg/dL) level was 17.00±0.709, LDL (mg/dL) level was 26.00±0.549 and VLDL (mg/dL) level was 27.04±0.709. In-group II (*S. bucharica* CME treated group) there was a significant ($p<0.05$) increase in Cholesterol HDL ratio, and significant ($p<0.01$) decrease in Cholesterol, HDL and LDL was observed. In VLDL increased was non-significant (table 6).

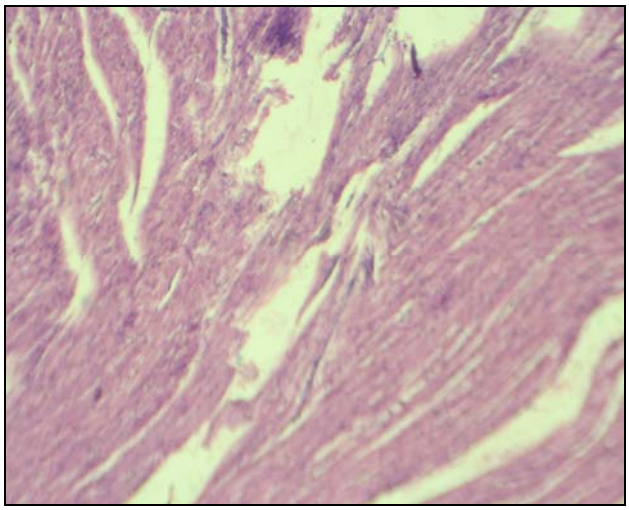

Fig. 7: Photomicrograph (Lens 10x) of *S. bucharica* treated group heart reveals mild myocardial edema.

Hematological profile

In Hematological profile, group I (control) Hb (g/dl) was 13.20±0.070, RBC Count was (million/ul) 6.10±0.031, Hematocrit (HCT/PCV) % was 42.06±0.322, MCV (fl) was 63.2±1.070, MCH (pg) was 22.10±0.783, MCHC (g/l) was 32.10±0.332 and Total WBC Count (×10^9/L) was 11.40±0.176 (table 7).

In-group II (*S. bucharica* CME treated group) there was non-significant decrease in Hb, RBC count, MCV. Significant ($p<0.05$) decrease in Hematocrit (HCT/PCV) % and MCH was observed. There was non-significant increase in WBC. And significant ($p<0.05$) increase in Platelets count was observed (table7).

Histopathological studies

In chronic toxicity test histopathology of rabbits kidney and heart was done to evaluate possible toxic effects on these organs. Adminstartion of *S. bucharica* CME 300mg/kg showed no change in Histoarchitecture of kidney (fig. 5) as compared with control (fig. 4) and mild inflammation was observed in heart (fig. 7) as compared with Control group (fig. 6).

DISCUSSION

Many drugs, viral infections and industrial toxic chemicals have been known to cause hepatic injuries, which are difficult sometime to treat by medical remedies. It is significant to evaluate the plant based extracts that can be utilized to improve the treatment of hepatic failure due to necrosis and severe oxidative stress (Toklu *et al.*,

2008). Carbon tetrachloride is a hepatotoxin, was used for hepatic damage in current study, as it has been previously reported to exert toxic effects on liver (Javdan *et al.,* 2011). For the type and extent of hepatocellular damage serum enzymes estimation is a suitable quantitative marker. Increased level of ALP, AST and ALT in serum may be due to damaged structural integrity of liver, since these markers are cytoplasmic and are released in the circulation upon autolytic cellular necrosis or breakdown (Recknagel *et al.,* 1989).

Consequently, increased release of ALP, AST, ALT (Lin *et al.,* 2008) SGOT and SGPT (Kus *et al.,* 2004) in the circulation is indication of severe hepatic tissue membrane damage during Carbon tetrachloride intoxication (Lin *et al.,* 2008).

Administration of *S. bucharica* CME significantly ($p<0.05$) decreased the Serum Total Bilirubin, Direct Bilirubin, ALT, Alkaline Phosphatase (ALP), Gamma GT, AST and Globulin level and significant ($p<0.05$) increase was observed in levels of serum Total protein and Albumin as compare to group CCL4 treated group. Reversal of elevated serum enzymes in Carbon tetrachloride induced liver injury by the *S. bucharica* CME may be due to prevention of leakage of the intracellular enzymes by its membrane stabilizing activity. This is in covenant with commonly adopted view that hepatocytes regeneration and healing of hepatic parenchyma returns the serum level of transaminases to normal (Thabrew *et al.,* 1987). This was supported by histo-pathological results in the current study. Similar hepatoprotective effects have been reported for *S. hypoleuca* (Javdan *et al.,* 2011).

For possible toxic effects on kidney, heart and blood, *S. bucharica* CME was studied for toxic effects. Current study showed that *S. bucharica* CME at the dose of 300 mg/kg did not produced significant toxicity. There was significant increase in concentration of urea and creatinine and there were no significant changes in the histopathologic architecture of the kidney. According to Harita *et al*., (2008) lower serum creatinine level there is an increased risk of the type 2 diabetes. Therefore, the increased in level of serum creatinine of extract treated rabbits suggest that the *S. bucharica* CME possess anti-diabetic effects. This finding is also supported by significant ($p<0.05$) decrease in serum glucose level of extract treated rabbits.

Cardiac injury can be detected by measuring the serum levels of cardiac enzymes marker, such as CK-MB (creatine kinase-MB fraction), LDH, AST and serum lipid profile (Komolaf *et al.,* 2013). Administration of *S. bucharica* CME at the dose of 300 mg/kg showed a significant decrease ($p<0.01$) in LDH and there was significant increase ($p<0.01$) in CPK and SGOT. There was no significant change in concentration of CK-MB. However repetitive sampling may induce mild increase in creatine kinase in plasma, which then returned towards normal when animals were allowed to rest (Lefebvre *et al.,* 1992). Histopathological observations for heart showed mild inflammation of cardiac muscles.

An important parameter for the prognosis and management of diabetes is HbA1c, as it increases proportionally to FBG (fasting blood glucose) levels (Narendhirakannan *et al.,* 2006). The significant decrease in HbA1c levels by *S. bucharica* CME at 300mg/kg indicates the glycemic control induced by the plant extract.

Abnormal lipid level manifested the severe health problem and cardiovascular disease. There are numerous pharmacological and dietary treatments for reducing the elevated cholesterol levels in blood but still problem is there. In this perspective specific attention is given on LDL: HDL ratio, a resilient conjecturer of cardiac events (Hermansen *et al.,* 2003). In *S. bucharica* CME treated group, there was significant ($p<0.05$) increase in Cholesterol HDL ratio and significant ($p<0.01$) decrease in Cholesterol, HDL and LDL and significant increase VLDL was observed.

In Hematological profile administration of *S. bucharica* CME at 300mg/kg showed no significant sign of toxicity. A significant ($p<0.05$) increase in Platelets was observed. Active constituents may act on bone marrow, may prevent its damage and increase the ability to produce platelets. Moreover, it may also prevent platelet destruction in blood and in that way may increase the life of platelets in the circulation.

CONCLUSION

In conclusion, administration of *S. bucharica* CME at 300mg/kg showed hepatoprotective effect, significantly decreased in the serum Total Bilirubin, Direct Bilirubin, ALT, Alkaline Phosphatase (ALP), Gamma GT, AST and Globulin level and significantly ($p<0.05$) increases levels of serum Total protein and Albumin as compare to group CCL_4 treated group. In histopathological studies *S. bucharica* CME showed protection against CCl_4 damaged liver. It had significantly decreased the blood glucose level and significantly increased platelet count. In toxicological studies *S. bucharica* CME showed no significant toxicity. Although current study has established the hepatoprotective effect and safety of *S. bucharica* CME, further studies are required to identify active chemical constituents and relate their specific hepatoprotective activity.

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