Toxicity studies on herbal formulation used in diabetes mellitus

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Abstract: In current study herbal formulation was prepared for Diabetes mellitus (type 2). It consists of the extracts of *Salacia reticulate*, *Cinnamomum zeylanicum*, *Lagerstroemia speciosa*, *Camellia sinensis* and *Gymnema sylvester*. Toxicity studies were carried out on heart, liver, kidney and blood of both male and female rabbits. Drug was administered in a dose of 15mg/kg body weight daily for 90 days. On 91th day, blood was drawn from animals and investigated for changes in biochemical and hematological levels. After that animals were sacrificed and their organs (liver, heart and kidney) were analyzed for histo-pathological changes. In biochemical tests for lipid profile, significant decreased (male-70.64±0.321; female-69.80±0.365) in triglycerides level were observed, no significant change was recorded in Cholesterol HDL ratio, LDL, VLDL level. A significant increase (male-16.00±1.418; female-10.00±0.709) was observed in HDL level. In liver function test significant decrease was observed in Gamma GT (male-10.08±0.862; female-7.00±0.709). Alkaline phosphatase (male-79±0.838; female-51.1±1.810), SGPT (male-54±0.709; female-43.04±2.060), direct bilirubin (male-0.024±0.005; female-0.014±0.002) and total bilirubin (male-0.109±0.003; 0.106±0.049) were observed. Non-significant changes were observed in serum total protein, globulins, albumin and A/G ratio. No significant changes were noticed in urea level and serum electrolytes. In cardiac enzymes significant decrease was observed in LDH (male-443±5.61; female-360±1.848) and SGOT (male-27±0.709; female-28±1.418) level and highly significant rise in CPK (male- 3128±8.478; female-1598±7.483) and CK-MB (male-446±2.308; female-438±2.819). In hematological profile, significant decrease was observed in Hb (male-12.3±0.392; female-12.4±0.1), RBC count (male-6.60±0.167; female-5.74±0.25) and Hematocrit (HCT/PCV) % in both male and female rabbits (male-45.70±0.255; female-43.50±0.448) and significant ($p<0.5$) increased in WBC count (male-8.40±0.401; female-9.10±0.054). Significant ($p<0.5$) decrease in blood glucose level and HbA1c (male-3.36±0.113; female-3.16±0.076) was observed. In histopathological studies mild edema was observed in heart and there was no change in histo-architecture of liver and kidneys. It is concluded that formulation does not showed any chronic toxicity in adult dose.

Keywords: Toxicity, biochemical tests, poly-herbal formulation, diabetes type 2.

INTRODUCTION

Diabetes mellitus is a condition in which there is increased blood glucose level that results from decreased insulin production in pancreas or resistance of insulin in the peripheral tissues. Diabetes causes abnormalities in the metabolism of lipid and protein, in addition to deficiency of glucose and sugar metabolism. Diabetes is a severe chronic metabolic disorder, which now affects 3% of worldwide population (Liu *et al.,* 2013). Oral hypoglycemic drugs are beneficial in the cure of diabetes, but their usage is limited due to pharmacokinetic properties, secondary failure values, and associated side effects. Herbal drugs are often considered to have lesser toxic and side effects as compare to synthetic drugs (Bangar *et al*., 2009). Keeping above data in view, the herbal formulation was made, containing extracts of *Salacia reticulate* (100mg), *Cinnamomum zeylanicum* (50mg), *Lagerstroemia speciosa* (150mg), *Camellia sinensis* (50mg) and *Gymnema Sylvester* (100mg). This formulation was selected to evaluate its anti-diabetic and toxic properties.

MATERIALS AND METHODS

Animals

Both male and female rabbits weighing about 1000 -1400 g were used for chronic toxicity studies. Rabbits were obtained from Animal house of DUHS (Dow University of Health Sciences). Standard conditions of temperature, humidity and 12 hours light/darkness cycle were maintained. Seven days before of the study animals were familiarized. Standard protocol in-accordance with GLP (Good Laboratory Practice) Regulations of WHO were followed (Aniagu *et al.,* 2005)

Chronic toxicity studies

Dosage design

Rabbits were divided into 2 groups (5 Rabbits in each group), First group (Group A) Control group and 2nd group (Group B) Drug treated (15mg/kg body weight) group. Drug was administered orally for 90 days.

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Monitoring of body weight
During the familiarization period, body weight of each rabbit was assessed, by using electrical balance. Body weight was noted before commencement of drug dosing, once weekly during dosing period of the drug and on the day of sacrifice.

Clinical signs and mortality
During the dosing period (90 days), animals were monitored daily, for clinical signs and mortality patterns, before dosing, after the dosing and up to 4 hour after the dose.

Blood collection
Blood of the animal was drawn from marginal ear vein by using sterile needle (Uko *et al*., 2000), into tubes (anti-coagulant free serum separator), allowed for 1 hour to clot and after that centrifuged for 10 minutes at 3000 rpm (using Eppendorf 5810R centrifuge). Serum was shifted into tubes, and kept at -20^{o}C till further utilization (Ajagbonna *et al.*, 1999).

Biochemical tests
Method described by Ahamefule *et al.*, (2008) was used for biochemical tests. These tests were carried out to determine the Total proteins i.e. Albumin, Globulin, Electrolytes, Bicarbonate, Potassium, Sodium and Chloride, Lipid Profile i.e., Cholesterol, Cholesterol HDL ratio, Triglycerides, HDL, VLDL and LDL, Liver function test i.e. Direct Bilirubin, Total Bilirubin, Alkaline Phospatase, SGPT and Gamma GT (Roche Diagnostics, Germany).

Analysis of hematological profile
Blood sample was drawn by using sterile needle of 22gauge from marginal ear vein. The Sample was drawn in test tube containing EDTA (anticoagulant) for preventing clot. Sample was analyzed by using automatic analyzer. Parameters Hemoglobin (Hb), Red Blood Cells (RBC) Count, Total White blood cells count (WBC), Platelets count, HCT/PCV (hematocrit/packed cell volume) count, mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) were determined (Ozkan *et al.*, 2012).

Organ collection
Rabbits were sacrificed and kidney, liver and heart were removed, and stored in formalin for histo-ptahological examination.

Gross pathology and microscopic examination
Tissue biopsy from the liver, heart and kidneys was taken. Biopsies were fixed in 10% formalin. After dehydration tissues were embedding in paraffin, sections were cut at 5µ with the microtone, stained with hematoxylin and eosin and examined with light microscope (Ullah *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).

Table 1: Effect of formulation on lipid profile

Test Sample	Cholesterol (mg/dL)	Cholesterol HDL ratio	Triglycerides (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)	HDL (mg/dL)
Control	20.00±0.448	3.30±0.083	74.00±1.051	7.00±0.137	14.00±0.709	6.00±0.634
Drug treated male rabbit	21.08±0.862	3.12±0.058	70.64±0.321*	7.80±0.491	11.96±0.319*	16.00±1.418*
Drug treated female rabbit	22.2±0.862	3.80±0.155	69.80±0.365*	6.00±0.709	11.50±0.709*	10.00±0.709*

Table 2: Effect of formulation on liver function test

Test Sample	Gamma GT (U/L)	Alkaline Phospatase (U/L	SGPT (U/L)	Direct Bilirubin (mg/dL)	Total Bilirubin (mg/dL)
Control	13.0±0.448	91.0±0.951	70.0±1.585	0.012±0.076	0.162±0.015
Drug treated male rabbit	10.08±0.862	79±0.838	54.00±0.709	0.024±0.005	0.109+0.003
Drug treated female rabbit	7.00±0.709	51.1±1.810	43.04±2.060	0.014±0.002	0.106±0.049

Table 3: Effect of formulation on total protein test

Test Sample	Total proteins (g/dL)	Globulin (g/dL)	Albumin (g/dL)	A/G ratio
Control	11.13±0.95	4.93±0.016	6.21±0.263	0.80±0.031
Drug treated male rabbit	10.14±0.256	4.39±0.141	5.76±0.293	0.76±0.024
Drug treated female rabbit	10.08±0.491	4.71+0.087	6.09±0.031	0.77±0.048

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

RESULTS

Biochemical tests

Lipid profile

Level of Cholesterol was 20.00±0.448 for control, 21.08±0.8 and 22.2±0.862 for drug treated male and female rabbit respectively. Level of Cholesterol HDL ratio was 3.30±0.083 for control, 3.12±0.058 and 3.12±0.058 for drug treated male and female rabbit respectively. Level of triglycerides was 74.00±1.051 for control, 70.64±0.321 and 69.80±0.365 for drug treated male and female rabbit respectively. Level of LDL was 7.00±0.137 for control, 7.80±0.491 and 6.00±0.709 for drug treated male and female rabbit respectively. Level of VLDL was 14.00±0.709 for control, 11.96±0.319 and 11.50±0.709 for drug treated male and female rabbit respectively. HDL level was 6.00±0.634 for control, 16.00±1.418 and 10.00±0.709 for drug treated male and female rabbit respectively (table 1).

Table 4: Effect of Formulation on Kidney function test

Test Sample	Urea (mg/dL)	Creatinine
Control	32.00±0.709	0.84±0.039
Drug treated male rabbit	33.00±0.709	0.96±0.006
Drug treated female rabbit	34.00±0.634	0.95±0.050

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

Liver function test

Level of Gamma GT (U/L) was 13.0±0.448 for control, 10.08±0.862 and 7.00±0.709 for drug treated male and female rabbit respectively. Alkaline phospatase (U/L) level was 91.0±0.951 for control, 79±0.838 and 51.1±1.810 for drug treated male and female rabbit respectively. SGPT (U/L) level was 70.0±1.585 for contro1, 54.00±0.709 and 43.04±2.060 for drug treated male and female rabbit respectively. Direct Bilirubin (mg/dL) level was 0.012±0.076 for contro1, 0.024±0.005 and 0.014±0.002 for drug treated male and female rabbit respectively. Total Bilirubin (mg/dL) level was 0.012±0.076 for control, 0.109±0.003 and 0.106±0.049 for drug treated male and female rabbit respectively (table 2).

Total protein

Level of Total proteins (g/dL) was 11.13±0.95 for control, 10.14±0.256 and 10.08±0.491 for drug treated male and female rabbit respectively. Globulin (g/dL) level was 4.93±0.016 for control, 4.39±0.141and 4.71±0.087 for drug treated male and female rabbit respectively. Albumin (g/dL) level was 6.21±0.263 for control, 5.76±0.293 and 6.09±0.031 for drug treated male and female rabbit respectively. A/G ratio was 0.80±0.031 for control, 0.76±0.024 and 0.77±0.048 for drug treated male and female rabbit respectively (table 3).

Kidney function test

Level of Urea (mg/dL) was 32.00±0.709 for control, 33.00±0.709 and 34.00±0.634 for drug treated male and female rabbit respectively. Level of Creatinine was 0.84±0.039 for control, 0.96±0.006 and 0.95±0.050 for drug treated male and female rabbit respectively. Result of the test shows that there was non-significant increase in the level of urea and significant increase in level of creatinine (table 4).

Serum electrolytes test

Level of Sodium (mEq/L) was 145.10±0.874 for control, 144.00±1.05 and 149.00±0.317 for drug treated male and female rabbit respectively. Potassium (mEq/L) level was 4.93±0.016 for control, 6.70±0.184 and 5.3±0.083 for drug treated male and female rabbit respectively. Chloride (mEq/L) level was 112.03±1.650 for control, 107.00±0.448 and 111.00±0.709 for drug treated male and female rabbit respectively. Bicarbonate (mEq/L) level was 19.00±0.549 for control, 23.00±0.709 and 22.00±0.709 for drug treated male and female rabbit respectively (table 5).

Cardiac enzymes

Level of LDH (U/L) was 461.2±2.913 for control, 443±5.61 and 360.00±1.848 for drug treated male and female rabbit respectively. CPK (U/L) level was 1324.8±7.43 for control, 3128±8.478 and 1598.00±7.483 for drug treated male and female rabbit respectively. CK-MB (U/L) level was 349.8±3.550 for control, 446±2.308 and 438±2.819 for drug treated male and female rabbit respectively. SGOT (U/L) level was 70.0±2.435 for control, 27±0.709 and 28.00±1.418for drug treated male and female rabbit respectively (table 6)

Hematological profile

In control group Hb (g/dl) level was 13.20±0.054, RBC Count was 6.81±0.085 (million/µl), Hematocrit (HCT/PCV) was 45.50±0.158 (%), MCV was 66.62±0.660 (fl), MCH was 19.32±0.183 (pg), MCHC was 29.0±0.317 (g/l), Total WBC Count was 10.60±0.219 (×10^9/L) and Platelet Count (×10^9/L) was 381±1.02.

In formulation treated male rabbits Hb (g/dl) level was 12.3 0±0.392, RBC Count was 6.60±0.167 (million/µl), Hematocrit (HCT/PCV) was 45.70±0.255 (%), MCV was 69.9±0.375 (fl), MCH was 18.70±0.184 (pg), MCHC was 27.00±0.634 (g/l), Total WBC Count was 8.40±0.401 (×10^9/L) and Platelet Count (×10^9/L) was 403.00±3.75.

In formulation treated female rabbits Hb (g/dl) was 12.40±0.100, RBC Count was 5.74±0.250 (million/µl), Hematocrit (HCT/PCV) was 43.50±0.448 (%), MCV was 75.96±0.427 (fl), MCH was 21.90±0.976 (pg), MCHC was 28.40±0.680 (g/l), Total WBC Count was 9.10±0.054 (×10^9/L) and Platelet Count (×10^9/L) was 394.20±2.423 (table 7).

Table 5: Effect of formulation on serum electrolytes

Test Sample	Sodium (mEq/L)	Potassium (mEq/L)	Chloride (mEq/L)	Bicarbonate (mEq/L)
Control	145.10±0.874	4.93±0.016	112.03±1.650	19.00±0.549
Drug treated male rabbit	144.00±1.05	6.70±0.184*	107.00±0.448	23.00±0.709*
Drug treated female rabbit	149.00±0.317	5.3±0.083	111.00±0.709	22.00±0.709

Table 6: Effect of formulation on cardiac enzymes

Test Sample	LDH (U/L)	CPK (U/L)	CK-MB (U/L)	SGOT (U/L)
Control	461.2±2.913	1324.8±7.43	349.8±3.550	70.0±2.435
Drug treated male rabbit	443±5.618*	3128.00±8.478**	446±2.308	27.00±0.709**
Drug treated female rabbit	360.00±1.848*	1598.00±7.483**	438±2.819	28.00±1.418**

Table 7: Effect of Formulation on hematological profile

S No.	Test	Control (Mean± SEM)	Drug treated male rabbit (Mean± SEM)	Drug treated female rabbit (Mean± SEM)
1	Hb (g/dl)	13.20±0.054	12.30±0.392*	12.40±0.100*
2	RBC Count (million/µl)	6.81±0.085	6.60±0.167	5.74±0.250*
3	Hematocrit (HCT/PCV) %	45.50±0.158	45.70±0.255	43.50±0.448*
4	MCV (fl)	66.62±0.660	69.9±0.375*	75.96±0.427*
5	MCH (pg)	19.32±0.183	18.70±0.184	21.90±0.976
6	MCHC (g/l)	29.0±0.317	27.00±0.634	28.40±0.680
7	Total WBC Count (×10^9/L)	10.60±0.219	8.40±0.401*	9.10±0.054*
8	Platelet Count (×10^9/L)	381±1.02	403.00±3.751*	394.20±2.423*

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

Blood glucose and HbA1c (%)

Level of Blood Glucose was 122.00±0.951 for control, 108.00±1.382 and 111±0.709 for drug treated male and female rabbit respectively. HbA1c (%) was 4.17±0.096 for control, 3.36±0.113 and 3.16±0.076 for drug treated male and female rabbit respectively (table 8).

Table 8: Effect of formulation on blood glucose

Test Sample	Blood Glucose Random	HbA1c (%)
Control	122.00±0.951	4.17±0.096
Drug treated male rabbit	108.00+1.382	3.36±0.113
Drug treated female rabbit	111+0.709	3.16±0.076

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

Histopathological studies

For chronic toxicity test microscopic studies (histopathology) of rabbit's heart, liver and kidney was done to observe probable toxic effects on these organs. Mild inflammation was observed in heart (fig. 2, 3) as compared with Control group (fig. 1). No significant change was observed in tissues of Liver (figs. 4-6) and kidney (figs. 7-9).

DISCUSSION

The use of CAM (complementary and alternative medicine) is increasing among general people (Frass *et al.*, 2012). The use of herbal drugs has advantage that they have lesser side effects, as compared with synthetic drugs. For examples, metformin may increase the risk of lactic acidosis and gastro-intestinal side effects (Ali *et al.*, 2012). Chinese medicinal plants are used in clinics for treatment of diabetes since years. *Aloe vera, Gymnema sylvestre* and *Momordica charantia* showed clinical effectiveness in the patients in short-term metabolic trials or non-randomized trials (Leatherdale *et al.*, 1981, Ghannam *et al.*, 1986, Shanmugasundaram *et al.*, 1990). In current study we studied the toxicological profile of poly-herbal anti-diabetic formulation.

Administration of formulation results in significant (p<0.5) decrease in the level of triglycerides no significant change in cholesterol HDL ratio, LDL, VLDL and significant (p<0.5) increase in HDL level. Amongst the lipids, elevated level of blood triglycerides, low density lipids (LDL) and very low density lipids (VLDL) as well as decreased level of high density lipids (HDL) has been recognized as cause of hyperlipidemia. In majority of the cases it leads to diabetes mellitus (Viswanath *et al.*, 2014). Due to active chemical constituents of the herb (Lee *et al.*, 2000) formulation decreased the triglycerides level and increased the HDL

level. It might be proposed that formulation increased the lipase activity, which is responsible for hydrolysis of triglycerides.

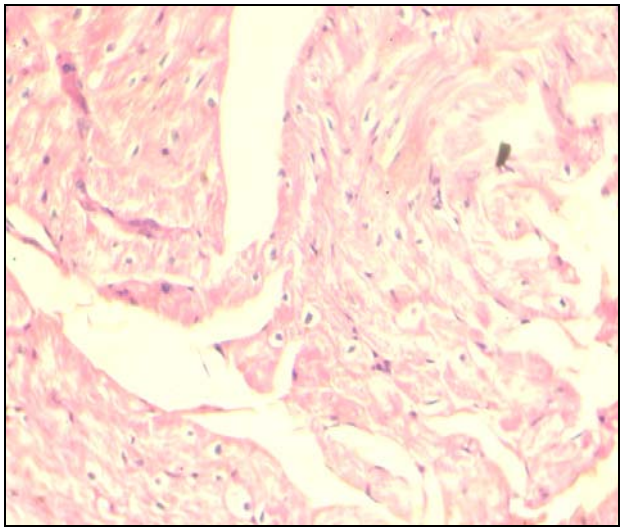

Fig. 1: Photomicrograph (40x) of control (non-treated) rabbit heart revealing normal myocardium

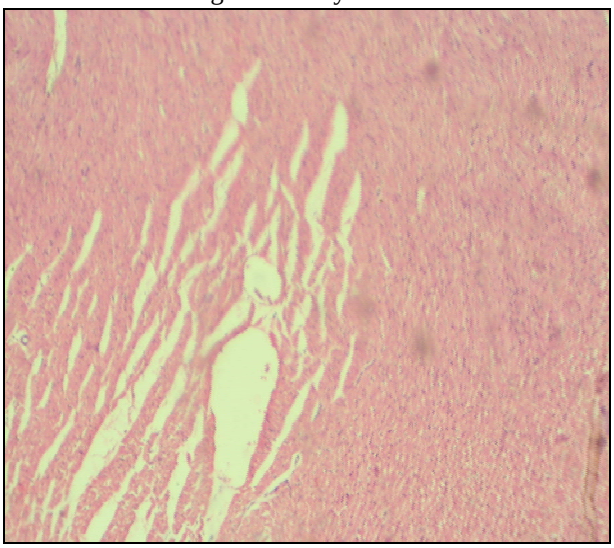

Fig. 2: Photomicrograph (10x) of drug treated male rabbit heart revealing edematous myocardium

Serum level of enzymes such as ALP, SGOT and SGPT revealed the dysfunctioning of liver. These enzymes level changed due to distortion of the liver, as a result of cellular injury of organ due to diseases and toxic metabolites (Ngaha *et al.*, 1989). Administration of formulation results in significant ($p<0.5$) decrease in gamma GT, alkaline phospatase (U/L), SGPT, direct bilirubin and total bilirubin. Level of serum liver function enzymes like AST, ALT, alkaline phosphatase and bilirubin were reported to be elevated in hepatic damage by Alshawsh *et al.* (2011). In current study serum enzymes level was decreased which revealed that formulation does not showed toxic effects on liver function of the animals.

Omonkhua *et al.* (1999) reported that different liver disorder may cause increase in serum bilirubin level. The results of total protein test showed non-significant change in serum total protein, globulin, albumin and A/G ratio as compared with control. This suggests that administration of formulation did not reduce the protein synthesis capacity of liver.

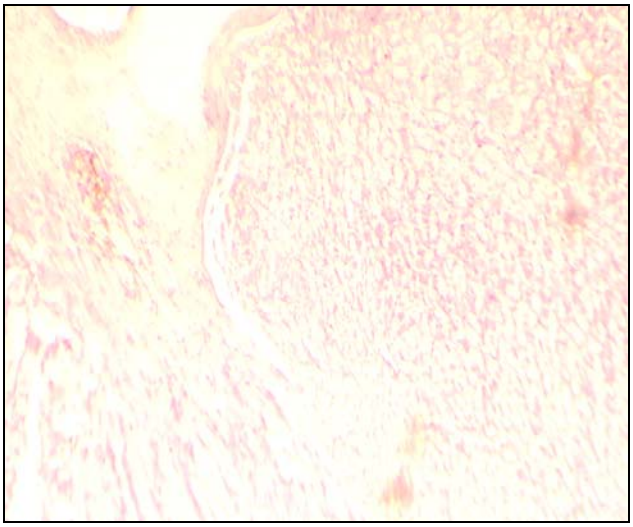

Fig. 3: Photomicrograph (10x) of drug treated female rabbit heart revealing edematous myocardium

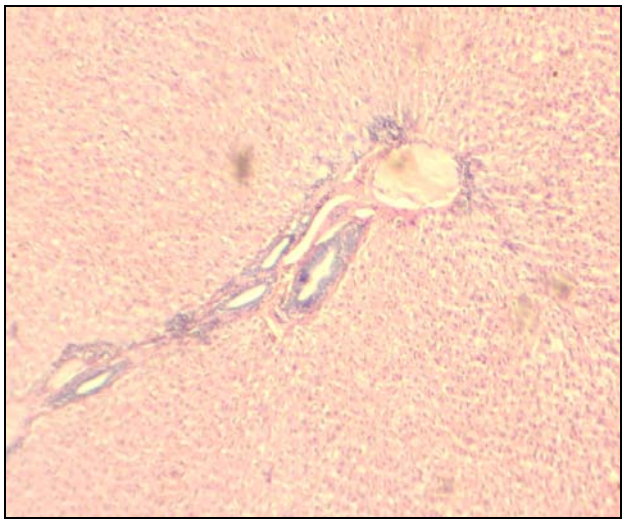

Fig. 4: Photomicrograph (10x) of control control rabbit liver showing normal hepatic parenchyma with portal tracts.

Urea, creatinine and electrolytes are mostly used parameters to measure the state of kidney. The increased levels of urea are reported to be due to the dysfunction of kidney (Eteng *et al.*, 2009). Administration of formulation results in no significant change in urea level. In serum electrolyte, no significant changes were observed in serum sodium, potassium, chloride and bicarbonate concentration in both male and female rabbits except significant ($p<0.05$) increase in potassium level in male

rabbit and marked increase in bicarbonate level ($p<0.05$) was observed in both male and female rabbits.

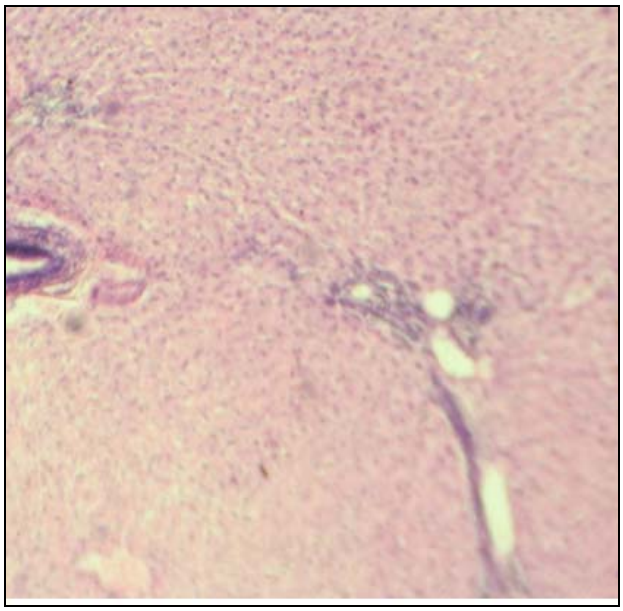

Fig. 5: Photomicrograph (10x) of drug treated male rabbit liver showing normal hepatic parenchyma with portal tracts

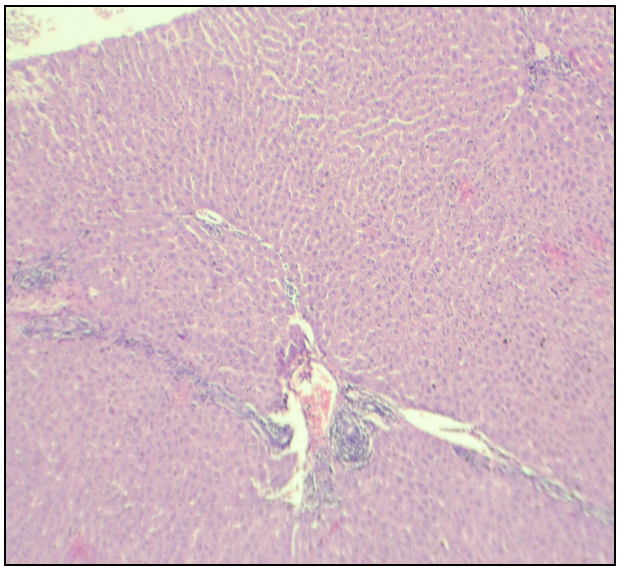

Fig. 6: Photomicrograph (10x) of drug treated female rabbit liver showing normal hepatic parenchyma with portal tracts.

In cardiac enzymes, there was significant ($p<0.5$) decrease in LDH and SGOT level and highly significant raise in CPK and CK-MB. The metabolic injury of the myocardium results in rise in concentration of serum marker enzymes such as LDH and CK-MB (Changes in enzymatic activity of SGPT and SGOT is clinically important in the patient of diabetes. The rise in SGOT level with only slight rise in SGPT level suggest the cardiac damage (Sundaram *et al.,* 2009). Decrease level of SGOT in drug treated rabbits shows the protective effect on heart.

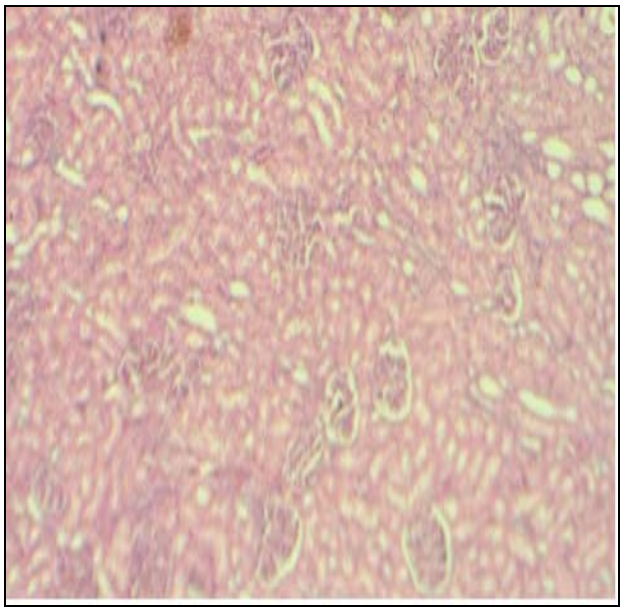

Fig. 7: Photomicrograph (10x) of control (non-treated) rabbit kidney showing renal cortex with normal glomeruli.

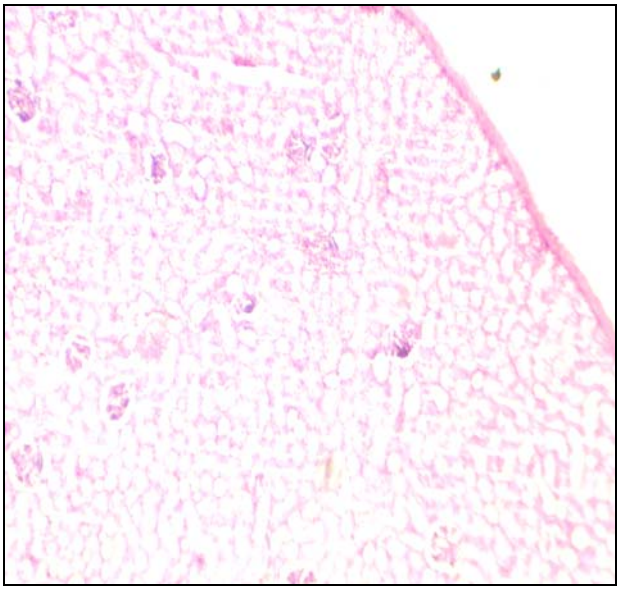

Fig. 8: Photomicrograph (10x) of drug treated male rabbit kidney showing renal cortex with normal glomeruli.

In hematological profile there was significant ($p<0.5$) decrease in Hb in both male and female rabbits. RBC count and Hematocrit (HCT/PCV) % was decreased in female rabbit. WBC count was significantly ($p<0.5$) decreased and platelet count was significantly increased.

Usually WBC count is increased in reaction to noxious environment (Ogbonnia *et al.,* 2014). Administration of formulation results in increase in WBC may be due to

noxious effects of formulation. No significant changes in MCV and MCHC signifying that, there is no regenerative anemia (Ogbonnia *et al.,* 2014). Hematological profile of formulation treated male and female rabbits showed no significant toxicity.

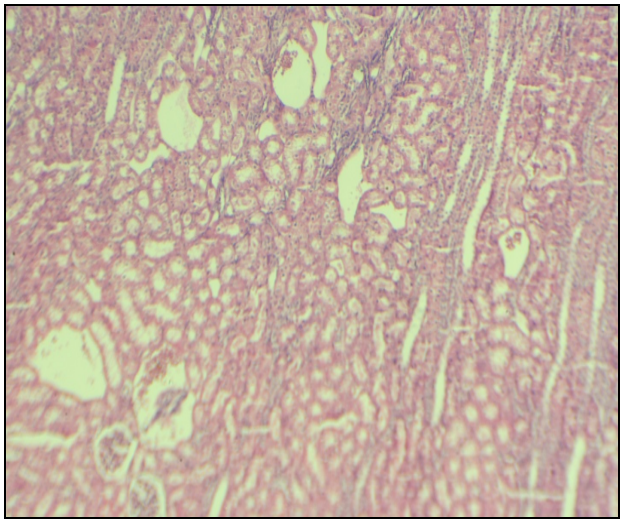

Fig. 9: Photomicrograph (10x) of drug treated male rabbit kidney showing renal cortex with normal glomeruli**.**

The formulation significantly decreased ($p<0.5$) blood glucose level and HbA1c in both male and female rabbits. The decrease in HbA1c is reported to be associated with enhancement of diabetic complications and glycemic control (Lo *et al.,* 2014).

CONCLUSION

Present study was carried out to study the toxicity of herbal formulation for Diabetes mellitus. Chronic Toxicity studies were carried out on heart, liver, kidney and blood of both male and female rabbits. Administration of formulation at the dose of 15mg/kg body weight daily for 90 days does not showed any significant chronic toxicity in bio-chemical, hematological and histopathological studies in adult dose. However histopathology of rabbit heart showed mild inflammation. However studies on large number of animals and humans are required to reach any conclusion.

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