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Antidiabetic and anti-apoptotic profile assessment of the seed of Holarrhena antidysenterica in streptozotocin-induced diabetic rat: An approach through proteomic and genomic study.

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Introduction:

Diabetes mellitus (DM) is a metabolic dysfunction characterized by unstable or persistent hyperglycaemia due to reduction of insulin action. When the body's insulin supply is diminished from the normal level, carbohydrate processing in body is not in normal range and it compensates by over production of fats and protein. Diabetes is a disease highly prevalent in developing countries as well as in developed countries (McAnuff et al., 2003). This is characterized by chronic elevation of blood glucose level and occurrence of glucose in urine. Overtime complications can results like nerve injury, blindness and kidney failure (Feldman, 1988). The severe hyperglycaemia was found to high amount of production of free radicals which is related to long term damage, malfunction and collapse of various organs, especially the eyes, kidnies, nerves, heart and blood vessels (Baynes, 1991; Mohamed et al., 1999). The elevated production of and/or ineffective scavenging of reactive oxygen species (ROS), result tissue damage (Maxwell et al., 1997). Severe hyperglycaemia induced oxidative stress has been related with dysfunction and apoptosis of several cell types, including pancreatic β cells (Wu et al., 2004). From the literature review, more than 800 plant species have been noted antihyperglycemic activity (Sellamuthu et al., 2009). Most of the plants content glycosides, alkaloids, terpenoids, flavonoids, carotenoids, etc., those are frequently used as having antihyperglycemic efficacy (Loew and Kaszkin, 2002). The medicinal values of *Holarrhena antidysenterica* have been published previously by us (Ali et al., 2009). We already explore the therapeutic efficacy of the hydro-methanol :: 40:60 extract of seed of *Holarrhena antidysenterica* against hyperglycaemia and oxidative stress condition in STZ induced diabetic model animals (Ali et al., 2011).

Aims and objectives of the project:

Present work was conducted to explore the scientific basis regarding the antidiabetic effect of seed of *H. antidysenterica*. In this context, all the experiments were carried out on experimentally induced diabetic rat to fulfil the following aims –

- **To search out the most active fraction of most active hydro-methanol :: 40:60 extract of *Holarrhena antidysenterica* for its antidiabetic activity in STZ induced diabetic rat.**
- **To find out the diabetes induced carbohydrate metabolic disorders, oxidative stress and apoptosis in pancreatic tissue at the level of genomics and proteomics and their recovery by most active fraction followed by toxicity searching of it if any.**

<u>Long term</u>

- **To enlighten the possible characterization of the most effective part of active sub-fraction in this concern and to develop a herbal drug for the management of diabetes.**

EXPERIMENT NO. – 01

Protective effect of hydro-methanol :: 40:60 extracts of seeds of *Holarrhena antidysenterica* in streptozotocin-induced diabetic rat: A dose dependent analysis

Animals and their maintenance

Healthy, normoglycemic Wistar strain adult male albino rats having 3 months of age and weighing 150±10 g were selected for the entire experimental schedules in this project work. During pre-experimental check-up and also during the period of experiments, the animals were maintained under standard laboratory condition i.e. 12 hrs light: 12 hrs dark and 25±2ºC temperature with free access of foods and water *ad libitum*.

In each set of experiments, the animals were acclimatized to this laboratory condition for 15 days prior to experimentation. The principal of laboratory animal care [NIH Publication No. 85-23, revised 1985] was followed throughout the all experimental schedules. Experimental protocol has been designed as per the instruction of our Institutional animal ethical committee (IAEC). In each cage, six rats were caged maximally. Composition of standard laboratory diet which was provided to the animals.

Plant materials

Holarrhena antidysenterica seeds were collected from local market in the Midnapore town, District Paschim Medinipur, West Bengal, India and were recognised by taxonomists of Botany and Forestry department, Vidyasagar University, Midnapore where voucher specimen was preserved having the No. Bio-Med/V.U/H.A/16/08. The hydro-methanol :: 40:60 extract of seed of *H. antidysenterica* has been prepared as per our standard method reported earlier (Maiti et al., 2004). In brief, by using electric griender the fresh seeds were crushed and then pulverized. Out of this powder, 50 g was suspended in

250 ml of hydro-methanol :: 40:60 and kept in room temperature. The slurry was stirred intermittently for 2 hrs. After completion of 48 hours at room temperature, the mixture was then filtered through Whatman grade No. 3 filter paper.

Induction of diabetes mellitus in rats

As per our earlier publication, the standard dose of STZ was chosen which generates diabetes in adult rat (Mallick et al., 2006). In brief, to the overnight fasted rats a single intramuscular injection of STZ were subjected at the dose of 4 mg/ 0.1 ml of citrate buffer/ 100 g body weight/ rat that produce diabetes, after 48 hrs of STZ injection having fasting blood glucose level more than 300 mg/dl. After 7^{th} day of STZ injection, these hyperglycaemic rats were used to fulfil the study. In this manner, 30 rats were made diabetic.

Animal treatment

Six equal groups were made by using 36 rats. Hydro-methanol :: 40:60 extract treatment of seeds of *H. antidysenterica* was started from the 7^{th} day of post injection period of STZ and was considered as 1^{st} day of treatment. The duration of treatment was 28 days.

Group I (Vehicle treated control group) Normoglycemic animals of this group was treated with citrate buffer (0.1 ml/100 g body weight/ rat) by a single intramuscular injection.

Group II (Vehicle treated diabetic group) Animals were made diabetic by a single intramuscular injection of STZ at a dose of 4 mg/ 0.1 ml citrate buffer/ 100 g body weight/ rat having high level of fasting blood sugar.

Group III (10 mg extract treated diabetic group) From the 7^{th} day of STZ injection, the diabetic rats of this group were forcefully feed by gavage with hydro-methanol :: 40:60 extract of seeds of *H.*

antidysenterica at the dose of 10 mg/ 0.5 ml of 3% tween 80/ 100 g body weight/ day and the drug treatment was continued for 28 days at fasting state.

Group IV (20 mg extract treated diabetic group) Animals belongs to this group were orally administered with hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 20 mg/ 0.5 ml of 3% tween 80/ 100 g body weight/ day at fasting state for 28 days.

Group V (40 mg extract treated diabetic group) STZ induced diabetic animals of this group were treated with oral administration of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 40 mg/ 0.5 ml of 3% tween 80/ 100 g body weight/ day and the drug treatment was continued for 28 days at fasting state.

The duration of entire experiment was 28 days. Different fraction of above mentioned extract was given orally at 11:00 A.M. to animals (overnight fasting state).

Treatment was started from the 7^{th} day of STZ injection to the diabetic rats. Level of FBG was measured by using single touch glucometer in all the groups on every 7^{th} day (Chatterjee et al., 2009). After completion of 28 days of drug treatment, the animals were maintained in fasting condition for overnight and finally taking the body weight on 29^{th} day of post drug treatment period, than all the animals were sacrificed by decapitation using ether anaesthesia. From the dorsal aorta, blood was collected by a syringe and the serum was separated by centrifugation at 3000g for 5 minutes for the estimation of different parameters. Liver, kidnies, pancreas and skeletal muscular tissues were dissected out from each animal, washed in normal saline, soaked in blotting paper and stored in deep freezer (-20ºC) for the measurement of different relevant biomarkers and genomic sensors in this concern.

Measurement of fasting blood glucose (FBG) level

At the time of the grouping of the animals, FBG level was measured. FBG level was further recorded from all the animals of all groups throughout the experiment at a specific days of intervals. Blood was collected from the tail vein by syringe following warm-up the tail or by the retro orbital puncture in alternative manner. FBG level was measured by single touch glucometer (Chatterjee et al., 2009).

Biochemical assay of hexokinase activity in hepatic tissue and skeletal muscle

By spectrophotometrically, hepatic tissue and skeletal muscular hexokinase activity was assessed. Assay mixture consists of 7.5 mM M_gCl_2, 3.7mM glucose, 11 mM thioglycerol and 45 mM HEPES buffer. By using ice-cold 0.1 M phosphate buffer saline (pH 7.4) all the tissues were homogenised at 50 mg/ml concentration. After that 0.9 ml of assay mixture was added with 0.03 ml of 0.22 M ATP in a spectrophotometer cuvette and mixed properly. Then into this spectrophotometer cuvette 0.1 ml of the tissue supernatant was added and 340 nm the absorbance was noted. One unit of this enzyme was expressed as µg/g of tissue (Chou and Wilson, 1975).

Biochemical assay of glucose-6-phosphatase activity in hepatic tissue and skeletal muscle

Hepatic and skeletal muscular glucose-6-phosphatase activity was assessed according to the standard procedure. At the concentration of 50 mg/ ml both the tissues were homogenized in 0.1 M ice-cold phosphate buffered saline (pH 7.4). after that in to a calibrated centrifuge tube glucose-6-phosphate solution at the volume of 0.1 ml of 0.1 M and 0.3 ml of 0.5 M of malic acid buffer (pH 6.5) were taken and incubated for 15 minutes at 37ºC in water bath. By the addition of 1 ml of 10% trichloroacetic acid (TCA) the entire reaction was stopped, followed by ice chilling and centrifugation at 3000 × g for 10 minutes. The OD was noted in 340 nm and the activity of the enzyme was expressed as mg of inorganic phosphate liberated/g of tissue (Swanson, 1955).

Biochemical assay of catalase (CAT) activities of hepatic and renal tissues.

Hepatic and renal catalase activity was measured according to the standard protocol. Both that tissues were homogenized separately by using 0.05 M Tris-HCL buffer solution (pH 7.0) at a concentration of 50 mg/ ml. centrifugation of these homogenized samples were performed at 10000×g for 10 minutes at 4ºC. 0.5 ml of 0.00035 M H_2O_2 and 2.5 ml of distilled water were added in a spectrophotometer cuvette and mixed properly. Optical density was noted at 240 nm before the addition of supernatant. At a volume of 40 µl supernatant from the sample was added to the cuvette and 30 second interval subsequent six reading was noted (Beers and Sizer, 1952).

Biochemical assay of peroxidase activities of hepatic and renal tissues.

The peroxidase activities were measured in target tissues according to the standard method (Sadasivam and Manickam, 2008). The tissue samples were homogenized in ice-cold of 0.1 M phosphate buffer saline (pH 7.0) at the tissue concentration of 50 mg/ml. Next, 20 mM guiacol was mixed with 0.1 ml supernatant collected from the homogenate. In presence of 0.3 ml of 12.3 mM H_2O_2, the time was recorded for an increase in the absorbance by 0.1 at 436 nm.

Estimation of lipid peroxidation from the concentration of thiobarbituric acid reactive substances (TBARS) level

Tissues was homogenized at the concentration of 50 mg/ ml in 0.1 M of ice-cold phosphate buffer (pH 7.4) and the homogenates were centrifuged at 10000×g at 4ºC for 5 minutes. Each supernatant was used for the estimation of TBARS. For the measurement of TBARS, the homogenate mixture of 0.5 ml was mixed with 0.5 ml of normal saline (0.9 g % NaCl) and 2 ml of TBA-TCA mixture (0.392 g thiobarbituric acid in 75 ml of 0.25 (N) HCL with 15 g trichloroacetic acid). The volume of the mixture was made up to 100 ml by 95% ethanol and boil at 100ºC for 10 minutes. This mixture was then cooled

at room temperature and centrifuge at 4000×g for 10 minutes. The whole supernatant was transfer in spectrophotometer cuvette and read at 535 nm (Okhawa et al., 1979).

Bio-chemical assay of serum urea, uric acid and creatinine

Quantitative determination of serum urea was carried out on Olympus Auto Analyzer at 340 nm using infinity urea reagent. The enzyme methodology employed in the said reagent is based on the reactions first described by Talke and Schubert. Here, urea concentration is proportional to the absorbance change over a fixed time interval (Tifanny et al., 1972).

In vitro quantitative determination of serum uric acid was done by Olympus Auto Analyzer at 520 nm using infinity uric acid reagent. This reagent is based upon the method of Trivedi and Kabasakalian with a modified trider peroxidase assay using 2, 4, 6-Tribromo-3-hydroxy benzoic acid (TBHB). Uric acid is oxidized to allantoin by uricase with the production of H_2O_2. The peroxide reacts with 4-amino antipyrine (4-AAP) and TBHB in the presence of peroxidase to yield a quinoneimine dye. The resulting change in absorbance at 520 nm is proportional to uric acid concentration in the sample (Kabasakalian et al., 1973).

Serum creatinine was measured spectrophotometrically on Olympus Auto analyser. The method is based on the Jeffe reaction. In alkaline (Sodium hydroxide) solution, creatinine formed a yellow-orange complex with picrate. The colour intensity is directly proportional with the creatinine concentration and can be measured photometrically (Junge et al., 2021).

Histological study

For histological study, pancreas of all the animals from all the groups of respective experiment were dissected out and then fixed in Bouin's fluid. After fixation pancratic tissues were embedded in paraffin wax. Sections were prepared in semi auto microtome (LEICA) at 5 µm thickness. All the sections were finally stained with haematoxylin-eosin as per standard protocol and then examined under light

microscope at 40X magnification for detection any pathological and morphological changes (Mallick et al., 2010).

Statistical analysis

For statistical analysis of collected data, 'Analysis of Variance (ANOVA) followed by multiple comparison student's two tail t test' was performed (Sokal and Rohle, 1997). All the value was indicated by Mean±SEM, (n=6). Significant differences were considered at the level of $p<0.05$ for analysis of data.

Results

Fasting blood glucose (FBG) level

FBG level was significantly elevated ($p<0.05$) in STZ-induced diabetic rats in respect to the vehicle treated control group. After the treatment of hydro-methanol :: 40:60 extract of seed of *H. antidysenterica* at different doses for 28 days to the diabetic animals resulted a significant diminution ($p<0.05$) in fasting blood glucose level when comparison was made with vehicle treated diabetic group. But when comparison was made among different doses prepared from of hydro-methanol :: 40:60 extract treated to the diabetic animals, there was a significant variation was noted. In this concern 40 mg dose/ 100 g body weight/ day of hydro-methanol :: 40:60 extract of seed of *H. antidysenterica* was the most potent dose (Table. 1).

Assessment of hexokinase activity

Activities of hexokinase in liver and skeletal muscular tissues were diminished significantly ($p<0.05$) in STZ induced diabetic group in compare with the vehicle treated control group. After the administration of hydro-methanol :: 40:60 extract of seed of *H. antidysenterica* at different dose for 28 days to the STZ-induced diabetic animals resulted a significant elevation ($p<0.05$) in the activities of the enzymes

in respect to the diabetic group. Results focused that 40 mg/ 100 g body weight/ day of hydro-methanol :: 40:60 extract of seed of *H. antidysenterica* was significantly effective ($p<0.05$) (Fig. 1).

Assessment of glucose-6-phosphatase activity

Activities of glucose-6-phosphatase in hepatic and skeletal muscular tissues were increased significantly ($p<0.05$) in the diabetic animals in compared with the vehicle treated control animals. Vehicle treated diabetic animals treated with different dose of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* resulted a significant diminution ($p<0.05$) in the activities of glucose-6-phosphatase in respect to the vehicle treated diabetic group. Comparative analysis of the data in the activities of the enzyme indicated that 40 mg dose treated group showed significant effective result ($p<0.05$) in compare with another treated group (Fig. 1).

Assay of catalase

Catalase activities in hepatic and renal tissues were significantly reduced ($p<0.05$) in STZ treated diabetic rats in compared with the vehicle treated control rats. Administration of several doses of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* for 28 days to the diabetic rats resulted a significant elevation ($p<0.05$) in the activities of catalase in liver and kidney tissues in compare with vehicle treated diabetic group. In contrast, after 28 days treatment of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 40 mg/ 100 g body weight to diabetic rats, a significant recovery ($p<0.05$) was noted in the activities of the concerned parameters in respect to other dose treated groups (Fig. 2).

Assay of peroxidase

Activities of peroxidase in liver and kidney tissues were decreased significantly ($p<0.05$) in vehicle treated diabetic group in respect to vehicle treated control group. Treatment of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at different dose to the diabetic rats for 28 days resulted a

significant increase ($p<0.05$) in the activities of these enzymes in respect to the vehicle treated diabetic group. When the comparison was made among the different dose of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* extract treated groups, 40 mg/ 100 g body weight showed most effective outcome (Fig. 2).

Thiobarbituric acid reactive substances (TBARS) levels

Level of TBARS in liver and kidney tissues were significantly increased ($p<0.05$) in the vehicle treated diabetic group when comparison was made with the vehicle treated control group. TBARS level in liver and kidney tissues showed a significant correction ($p<0.05$) towards the control level in different dose of hydro-methanol :: 40:60 extract treated diabetic groups in respect to vehicle treated diabetic group. From the comparative analysis of the different doses of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* extract treated diabetic groups it was observed that 40 mg/ 100 g body weight was significantly effective ($p<0.05$) in this concern in compare with other doses of extract treated diabetic groups (Fig. 3).

Serum uric acid, creatinine and urea level

Experimental results showed that there is a significant elevation ($p<0.05$) in serum uric acid, creatinine and urea levels in vehicle treated diabetic rats in respect to vehicle treated control which were corrected significantly ($p<0.05$) towards the control after the treatment with hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* extract at different doses to the vehicle treated diabetic animals. Levels of serum uric acid, creatinine and urea showed maximum recovery in 40 mg/ 100 g body weight treated group in respect to other dose treated groups (Table. 2).

Histo-architectural assessment of pancreas

Pancreatic histological analysis showed that the diameter and the cell population density of pancreatic islets of Langerhans were significantly diminished in the vehicle treated diabetic group in compare with

the control group. The size and cell population density of pancreatic islets were resettled significantly after the treatment of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* to the diabetic animals at the doses of 10 mg, 20 mg and 40 mg/ 100 g of body weight. From the comparison with other different dose treated group, it was assessed that highest recovery was observed in 40 mg/ 100 g body weight of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* which enlighten that this dose is the threshold dose (Fig. 4).

Discussion

In the present study, 40 mg dose/ 100 g of body weight of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* administered to the STZ induced diabetic rats showed effective diminution of fasting blood glucose level in respect of STZ induced diabetic rats. So, it may be state that the extract stimulates the β-cells of pancreas for the secretion of insulin or it has the capacity for restoration of pancreatic β-cells which has been projected by other, using other plant products (Chauhan et al., 2008). The view for the regenerative activities of this extract for pancreatic β-cells in STZ induced diabetes has been reflected here from the recovery in the activities of glucose-6-phosphatase and hexokinase in liver and skeletal muscle as the enzyme hexokinase is regulated by insulin in positive way and glucose-6-phosphatase in negative way (Mitra et al., 1996; Prience et al., 1998). Activities of catalase and peroxidase were decreased in hepatic and renal tissues in diabetic condition and recovered significantly by extract treatment. The said recovery may be due to the antioxidant activity of the phytomolecule(s) present in said extract which protects the catalytic protein molecules in cells from diabetes induced reactive oxygen species biomolecule injury (Chatterjee et al., 2007). This antioxidative activity has been strengthen here from the quantification of thiobarbituric acid reactive substances (TBARS) level in above said tissues as there is an inverse relationship between the activities of antioxidant enzymes and quantity of free radical by-products which is consists with other report (Pari and Sarvanan, 2002) as well as by our previous work (Mallick et al., 2007; Ali et al., 2009). The increased level of serum

urea in diabetic animals may be due to high rate of protein catabolism and utilization of excess amino acids for energy production which results increased serum uric acid level as it is the product of purine metabolism produced due to excess muscle wastage in diabetic condition (Ghosh et al., 1991). Serum creatine level was also increased in diabetic condition which also due to muscle wastage in diabetic state as creatinine produced from dephosphorylation of creatinine phosphate of muscle (Ghosh and Suryawanshi, 2001). All these deformities were corrected by the extract treatment which may be due to recovery in serum insulin (Mallick et al., 2010).

Here, histological observation of pancreas strengthen the prediction about the β-cell regenerative activity of 40 mg dose/ 100 g of body weight of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* where haematoxylin-eosin staining of pancreas reflected the maximum recovery in the relative size of pancreatic islets, population density of islet's cells in compare with the vehicle treated diabetic group.

Conclusion

From the results of this experiment it may be concluded that 40 mg dose of prepared from hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* is the threshold dose for the management of diabetic disorders with special reference to carbohydrate metabolic and oxidative stress recovery in STZ induced diabetic rats.

Graphical representation of results

Glycogenic Biosensors:

Table 1: Ameliorative effect hydro-methanolic extract of seeds of *H. antidysenterica* on fasting blood glucose level in STZ induced diabetic male albino rat in comparison with other fraction treated group.

Groups	Fasting blood glucose level (mg/dl)						
	0 day	1 day (7^{th} day of STZ injection)	4 day	7 day	14 day	21 day	28 day
Vehicle treated control group	72.3±6.5[a]	67±3.8[a]	72±4.8[a]	68±4.0[a]	73±5.2[a]	65±4.9[a]	67±3.8[a]
Vehicle treated diabetic group	74.5±6.2[a]	328±4.2[b]	334±3.5[b]	329±3.8[b]	336±5.4[b]	339±5.6[b]	328±4.2[b]
10 mg extract treated diabetic group	72.5±6.3[a]	330.6±6.3[b]	307.3±6.4[b]	270.6±6.3[c]	255.8±6.5[c]	176.3±6.4[c]	118.2±6.4[c]
20 mg extract treated diabetic group	70.5±6.2[a]	329.4±6.5[b]	298.3±6.5[b]	261.5±6.4[c]	252.0±6.5[c]	183.7±6.3[c]	114.5±6.6[c]
40 mg extract treated diabetic group	69.1±6.3[a]	336±5.5[b]	208±2.8[d]	157±2.7[d]	126±2.7[d]	88±3.4[d]	336±5.5[b]

Data were expressed as Mean ± SEM (n = 6). Values with different superscripts (a, b, c, d) in each vertical column differ from each other significantly, $p<0.05$.

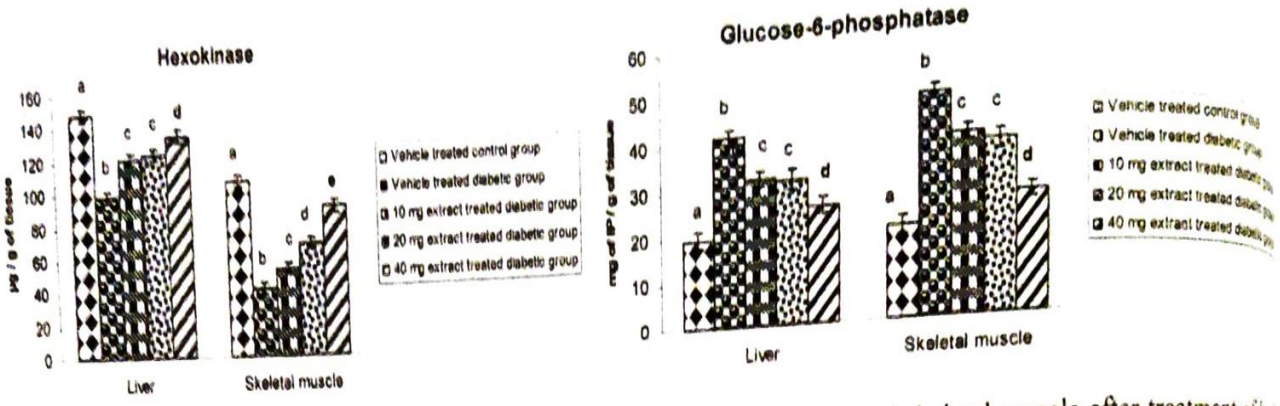


Fig 1: Resettlement in the activities of hexokinase and glucose-6-phosphatase in liver and skeletal muscle after treatment of hydro-methanol extract of seed of *H. antidysenterica* at different doses in vehicle treated diabetic rat. Values were expressed as Mean ± SEM, n=6. Bars with different superscripts (a, b, c, d, e) differ from each other significantly, $p < 0.05$

Antioxidant enzymes:

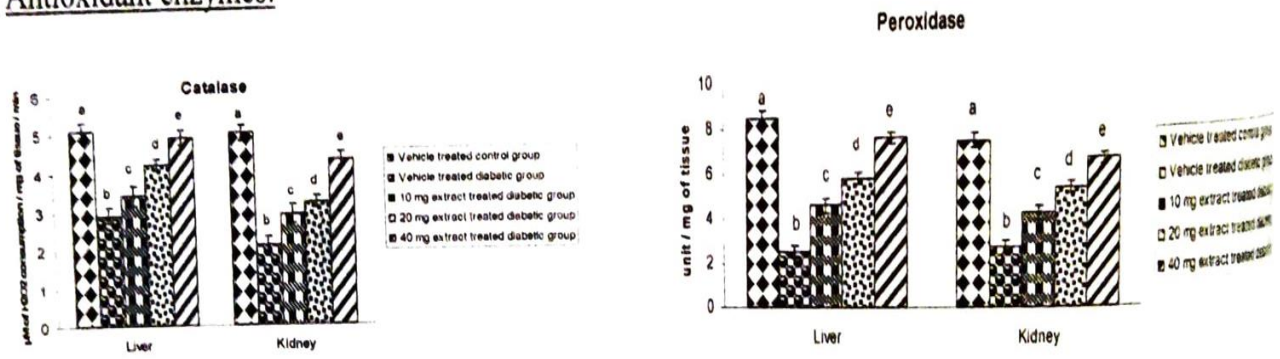


Fig 2: Amelioration in the activities of catalase and peroxidase in hepatic and renal tissue after treatment with hydro-methanol extract of seed of *H. antidysenterica* at different doses in STZ induced diabetic rat. Values were expressed as Mean ± SEM, n=6. Bars with different superscripts (a, b, c, d, e) differ from each other significantly, $p < 0.05$.

Oxidative stress end-product:

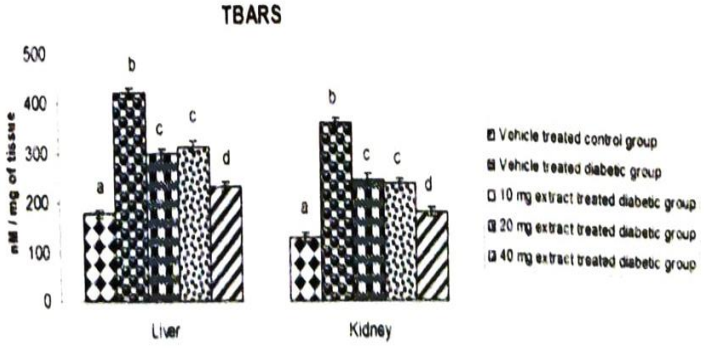


Fig 3: Remedial effect of hydro-methanol extract of seed of *H. antidysenterica* at different doses on TBARS levels in hepatic and renal tissues in comparison to vehicle treated diabetic rat. Values were represented Mean ± SEM (n=6). Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

Table 2: Corrective effect of hydro-methanol extract of seed of *H. antidysenterica* on the levels of serum uric acid, creatinine and urea in STZ induced diabetic rat.

Group	Serum uric acid (mg/dl)	Serum creatinine (mg/dl)	Serum urea (mg/dl)
Vehicle treated control group	18.08 ± 0.22^{a}	0.08 ± 0.003^{a}	35.2 ± 1.67^{a}
Vehicle treated diabetic group	28.82 ± 0.26^{b}	1.30 ± 0.012^{b}	88.1 ± 2.71^{b}
10 mg extract treated diabetic group	26.31 ± 0.25^{b}	1.19 ± 0.21^{b}	72.62 ± 2.16^{b}
20 mg extract treated diabetic group	24.51 ± 0.23^{b}	0.89 ± 0.034^{b}	61.56 ± 2.01^{b}
40 mg extract treated diabetic group	20.98 ± 0.51^{c}	0.24 ± 0.005^{c}	43.95 ± 1.51^{c}

Values express as Mean ± SEM (n = 6). 'ANOVA followed by multiple comparison Student's two tail t test'. Values with different superscripts (a, b, c) in each vertical column differ from each other significantly at $p<0.05$.

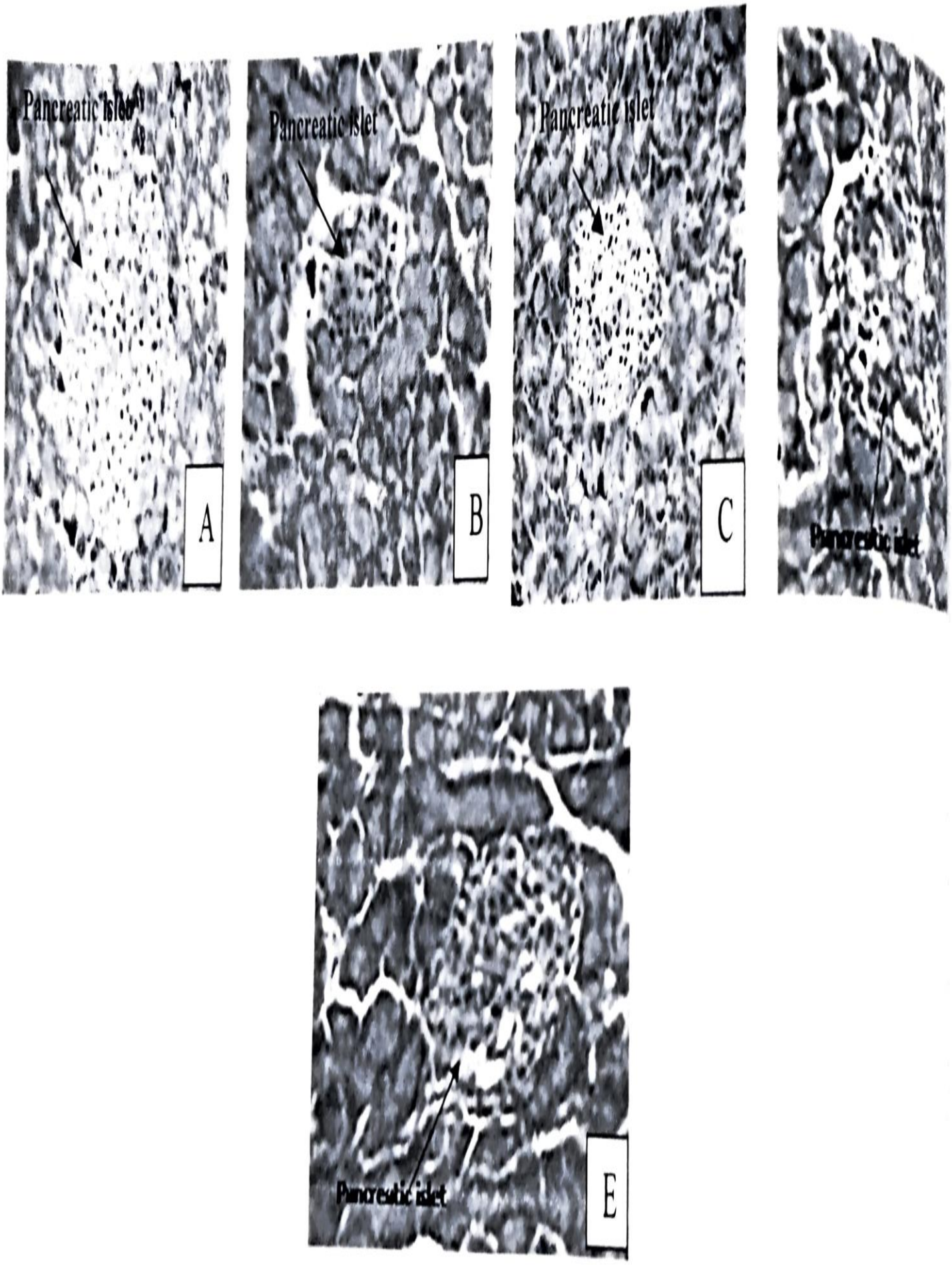

Pancreatic islet
A
Pancreatic islet
B
Pancreatic islet
C
Pancreatic islet
Pancreatic islet
E

Fig 4: Histological study of pancreatic tissue, 400 X (Haematoxylin-Eosin stain).

A: Representative sample of pancreatic tissue of vehicle treated control rat focusing on cell density in islets of Langerhans and size islet: a qualitative analysis.

B: Diminution in the cell density in representative pancreatic islet tissue sample in vehicle treated diabetic rat along with diminution in islets size by qualitative analysis.

C: Representative pancreatic tissue sample showing recovery in cell density of islet's along with elevation in the size of islet of Langerhans by qualitative analysis after administration of hydro-methanol extract of seed of *H. antidysenterica* at 10 mg dose to diabetic rat.

D: Representative sample of pancreatic tissue showing recuperation in cell density of islet's along with elevation in the size of islet of Langerhans by qualitative analysis after administration of hydro-methanol extract of seed of *H. antidysenterica* at 20 mg dose to diabetic rat.

E: Representative pancreatic tissue sample showing remarkable recovery in cell density of islet's along with elevation in the size of islet of Langerhans by qualitative analysis after administration of hydro-methanol extract of seed of *H. antidysenterica* at 40 mg dose to diabetic rat.

Group III (n-hexane fraction treated diabetic group) From the 7^{th} day of STZ injection, the diabetic rats of this group were forcefully feed by gavage with n-hexane fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 20 mg/ 0.5 ml of 3% tween 80/ 100 g body weight/ day and the drug treatment was continued for 28 days at fasting state.

EXPERIMENT NO. – 02

Antidiabetic effects of different solvent fractions of hydro-methanol :: 40:60 extract of seed of *Holarrhena antidysenterica* in stereptozotocin-induced diabetic rat: A comparative study

Animals and their maintenance

As mentioned before.

Plant materials

As mentioned before.

Induction of diabetes mellitus in rats

As mentioned before

Animal treatment

Six equal groups were made by using thirty six rats. Hydro-methanol :: 40:60 extract treatment of seeds of *H. antidysenterica* was started from the 7th day of post injection period of STZ and was considered as 1st day of treatment. The duration of treatment was 28 days.

Group I (Vehicle treated control group) Normoglycemic animals of this group was treated with citrate buffer (0.1 ml/100 g body weight/ rat) by a single intramuscular injection.

Group II (Vehicle treated diabetic group) Animals were made diabetic by a single intramuscular injection of STZ at a dose of 4 mg/ 0.1 ml citrate buffer/ 100 g body weight/ rat having high level of fasting blood sugar.

Group IV (chloroform fraction treated diabetic group) Animals belongs to this group were orally administered chloroform fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 20 mg/ 0.5 ml of 3% tween 80/ 100 g body weight/ day at fasting state for 28 days.

Group V (ethyl-acetate fraction treated diabetic group) STZ induced diabetic animals of this group were treated with oral administration of the ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 20 mg/ 0.5 ml of 3% tween 80/ 100 g body weight/ day and the drug treatment was continued for 28 days at fasting state.

Group VI (n-butanol fraction treated diabetic group) Experimental animals of this group were orally treated with n-butanol fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 20 mg/ 0.5 ml of 3% tween 80/ 100 g body weight/ day for 28 days at fasting condition.

The duration of the entire experiment was 28 days. Different fraction of above mentioned extract was given orally at 11:00 A.M. to animals (at overnight fasting state).

Fraction treatment were started from the 7^{th} day of STZ injection to the diabetic rats. Level of FBG was measured by using single touch glucometer in all the groups on every 7^{th} day (Chatterjee et al., 2009). After completion of 28 days of drug treatment, the animals were maintained in fasting condition for overnight and finally taking the body weight on 29^{th} day of post drug treatment period, then all the animals were sacrificed by decapitation using ether anaesthesia. From the dorsal aorta, blood was collected by a syringe and the serum was separated by centrifugation at 3000g for 5 minutes for the estimation of different parameters. Liver, kidnies, pancreas and skeletal muscular tissues were dissected

out from each animal, washed in normal saline, soaked in blotting paper and stored in deep freezer (-20ºC) for the measurement of different relevant biomarkers and genomic sensors in this concern.

Measurement of fasting blood glucose (FBG) level

As mentioned before.

Estimation of glycated haemoglobin (HbA1c)

Specific kits were used (Coral Clinical Systems. Verna, Goa- 403722, India) to determine the HbA1c level. Glycated haemoglobin level was expressed as GHb% **(Chandalia et al., 1980).**

Biochemical assay of serum insulin

Serum insulin level was measured using solid phase-conjugated sandwich ELISA kit (EXRMI-13K, Millipore, USA) for rat. The level of insulin was expressed in terms of ng/ml **(Pitchai et al., 2009)**.

Biochemical assay of hexokinase activity in hepatic tissue and skeletal muscle

As mentioned before.

Biochemical assay of glucose-6-phosphatase activity in hepatic tissue and skeletal muscle

As mentioned before.

Biochemical assay of superoxide dismutase (SOD) activities of liver and kidney tissue

Superoxide dismutase activities was estimated in hepatic and renal tissues by assessing the percentage of inhibition in auto-oxidation of pyrogallol by SOD according to the standard protocol. The buffered preparation was performed by 50 mM TRIS (pH 8.2). In a cuvette 2.04 ml of TRIS buffer, 20 ml of sample and 20 ml of pyrogallol were taken and mixed properly. The OD was taken at 420 nm for 3 minutes period. Each unit of SOD was defined as the enzyme efficacy that inhibits the auto-oxidation of pyrogallol by 50% **(Marklund and Marklund 1974)**.

Assessment of activity of glutathione-s-transferase (GST) in liver and renal tissue

Activity of GST was assessed in liver and renal tissues spectrophotometrically using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The assay mixture containing 0.1 ml of 1 mM CDNB in ethanol, 0.1 mM of 1 ml GSH, 2.7 ml of 100 mM potassium phosphate buffer (pH 6.5), and 0.1 ml of supernatant of the tissue homogenate. The formation of the product of CDNB-S-2, 4-dinitrophenyl glutathione was monitored by measuring the net increase in absorbance at 340 nm against the blank. The activity of the enzyme was calculated using the extinction coefficient, 6.9 M/cm and expressed in unit/mg of tissue **(Hobig et al., 1974)**.

Quantification of malondialdehyde (MDA) in hepatic and renal tissue

According to the standard method, MDA of the liver and renal tissues were quantified (Okhawa et al., 1979) in spectrophotometer cuvette and reading was taken at 535 nm.

Biochemical assay of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) activity

Activities of GOT and GPT were assessed in serum by specific kits, supplied by Span Diagnostic Ltd. Surat; India. The activities of these enzymes were expressed as IU/dl of serum **(Henry et al., 1960)**.

Western blot analysis of hepatic Bax and Bcl-2 protein

Proteomics analysis of Bax and Bcl-2 protein was carried out as per standard technique **(Maheshwari et al., 2009)**. In brief, by using 3 ml of ice-cold RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 1% SDS in PBS with protease inhibitor) per gram of tissue, frozen hepatic tissue were thawed, and the tissue was homogenized by using a homogenizer followed by incubation of ice for 30 minutes. Lysates were centrifuged at 4ºC for 10 minutes at 10000 g. By Bardford assay the protein concentration was determined **(Barford, 1976)**. For western blot study, 30-50 mg protein was resolved on a 12.5% SDS

polyacrylamide gel atb 100V in a mini-protean cell (Bio-Red). Proteins were transferred to nitrocellulose membrane by using transfer buffer (25 mM Tris base, 190 mM glycine, 20% methanol) at 100V for 1 hour in the ice-cold chamber. This membrane was blocked using PBS containing 0.05% Tween-20 (PBST) and 5% non-fat milk and then incubated in primary antibody (1:500 Bax, 1:400 Bcl-2) for overnight at 4ºC. After that phosphate buffered saline was used for washing with Tween-20 (PBST), membrane was again incubated with horseradish peroxidase-conjugated goat anti-rabbit (Santa Cruz Biotechnology) secondary antibody at 1:2000 dilutions. β-actin was used as an internal control to ensure equal protein loading. Immunodetection were visualised by using tetramethyl benzidine/ hydrogen peroxide (TMB/ H_2O_2) as substrate, and the resulting immunospecific bands were quantified by densitometric analysis.

Histological study

As mentioned before

Statistical analysis

As mentioned before.

Results

Assessment of toxicity study

No toxic reaction was found at any of the fraction treatment of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica*. During the observation period all the animals were alive, vigorous and energetic. From the toxicity analysis, it was revealed that the several fractions prepared from hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* up to the dose of 20 mg/ 100 g body weight is non-toxic. So, it may be declared that the LD50 value was above 20 mg/ 100 g body weight. As a result, it possibly confirmed that the above dose is harmless.

Body weight

A considerable diminution ($p<0.05$) in the body weight of the diabetic animals were noted in respect to the vehicle treated control animals. After the administration of different fraction produced from hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 20 mg/ 100 g body weight for 28 days to the diabetic rats, a significant elevation ($p<0.05$) in body weight was noted in respect to the diabetic animals. Treatment of ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 20 mg/ 100 g body weight/ day for 28 days to the diabetic rats resulted a significant recovery ($p<0.05$) in body weight in respect to n-hexane, chloroform, n-butanol fraction treated diabetic groups and the levels were nearly resettled to the control level. So, it may possibly stated that ethyl acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* is the potent fraction among those several fractions (Table 1).

Fasting blood glucose (FBG) level

FBG level was significantly elevated ($p<0.05$) in STZ-induced vehicle treated diabetic rats in respect to the control group. After the treatment of different fraction prepared from hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 20 mg/ 100 g body weight/ day for 28 days to the diabetic animals resulted a significant diminution ($p<0.05$) in fasting blood glucose level when comparison was made with vehicle treated diabetic group. But when comparison was made among different fractions treated to the diabetic animals, there was a significant variation was noted. In this concern ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* is the most potent fraction (Table 2).

Serum insulin

Significant ($p<0.05$) diminution of serum insulin level was observed in STZ-induced diabetic group in compare to the vehicle treated control group. After the treatment of n-hexane, chloroform, ethyl-

acetate, n-butanol prepared from hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* to diabetic animals at the dose of 20 mg/ 100 g of body weight/ day for 28 days resulted a significant elevation ($p<0.05$) in serum insulin level when compare with the vehicle treated diabetic group. Among all the fractions, ethyl-acetate fraction showed most effective outcome in this concern (Fig. 1).

Glycated haemoglobin (HbA1c)

There was a significant elevation in the level of the glycated haemoglobin (HbA1c) in the vehicle treated diabetic group in contrast with the vehicle treated control group. After treatment with the plant fractions, the levels of glycated haemoglobin were resettled towards the vehicle treated control group. All the fractions showed protective effect but ethyl-acetate fraction showed most effective result among those (Fig. 2).

Assessment of hexokinase activity

Activities of hexokinase in liver and skeletal muscle tissues were diminished significantly ($p<0.05$) in STZ induced diabetic group in compare with the vehicle treated control group. After fraction administration of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 20 mg/ 100 g body weight for 28 days to the STZ-induced diabetic animals resulted a significant elevation ($p<0.05$) in the activities of the enzymes in respect to the diabetic group. Results focused that ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at specific dose is significantly effective ($p<0.05$) in compare n-hexane, chloroform, n-butanol fraction though the levels of the said parameter was not resettled to the control level (Fig. 3).

Assessment of glucose-6-phosphatase activity

Activities of glucose-6-phosphatase in hepatic and skeletal muscle were increased significantly ($p<0.05$) in the vehicle treated diabetic animals in compared with the vehicle treated control animals. Diabetic animals treated with n-hexane, chloroform, ethyl-acetate, n-butanol fraction of hydro-

methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 20 mg/ 100g body weight/ day resulted a significant diminution ($p<0.05$) in the activities of glucose-6-phosphatase in respect to the vehicle treated diabetic group. Comparative analysis of the data in the activities of the enzyme indicated that ethyl-acetate fraction treated group showed significant effective result ($p<0.05$) in compare with another treated group (Fig. 3).

Activity of superoxide dismutase

Activities of hepatic and renal superoxide dismutase were significantly ($p<0.05$) decreased in vehicle treated diabetic group in respect to the vehicle treated control group. After the administration of n-hexane, chloroform, ethyl-acetate, n-butanol fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 20 mg/ 100g body weight/ day to diabetic group for 28 days, a significant ($p<0.05$) elevation in the activity of superoxide dismutase was noted in comparison with the vehicle treated diabetic group (Fig. 4).

Activity assessment of glutathione-s-transferase

Activities of anti-oxidant enzyme that is glutathione-s-transferase in liver and kidney tissues were decreased significantly ($p<0.05$) in vehicle treated diabetic group in respect to the vehicle treated control. Treatment with ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 20 mg/ 100g body weight/ day for 28 days to the diabetic rats showed most significant ($p<0.05$) outcome in comparison with n-hexane, chloroform, n-butanol fraction treated group (Fig. 4).

Concentration of MDA

Level of hepatic and renal MDA were initially elevated significantly ($p<0.05$) in STZ induced diabetic rats compare to the vehicle treated control rats. Fraction treated group at a particular dose showed a significant ($p<0.05$) recovery in the production of oxidative stress induced end product in both these

tissues towards diabetic rats. From investigational result, it was shown that the ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* showed the most significant ($p<0.05$) recovery in contrast with n-hexane, chloroform, n-butanol fraction treated group (Fig. 5).

Biochemical assay of glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT) activity

Activities of GOT and GPT in hepatic and renal tissue were increased significantly ($p<0.05$) in the diabetic animals in compare with the control animals. Diabetic animals treated n-hexane, chloroform, ethyl-acetate, n-butanol fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at 20 mg dose/ 100 g body weight/ day for 28 days to the diabetic rats resulted a significant diminution ($p<0.05$) in the activities of both enzymes in respect to the diabetic group. Comparative analysis of the data in the activities of the enzyme indicated that the ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* showed significantly effective result ($p<0.05$) in compare with n-hexane, chloroform, n-butanol fraction treated group (Fig. 6).

Expression of Bax, and Bcl-2 protein in hepatic tissue

Pro-apoptotic protein that is Bax (Fig. 7) expression was elevated significantly ($p<0.05$) while the expression of anti-apoptotic protein i.e. Bcl-2 (Fig. 8) was decreased significantly ($p<0.05$) in vehicle treated diabetic group in respect to the vehicle treated control group. Treatment with ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* to diabetic animal showed a significant recuperation ($p<0.05$) in the expression pattern of these parameters towards the control.

Histo-architectural assessment of pancreas

Pancreatic histological analysis showed that the diameter and the cell population density of pancreatic islet of Langerhans were significantly diminished in the diabetic group in compare with the control group. The size and cell population density of pancreatic islets were resettled significantly after the

treatment with ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* to the diabetic animals at the doses of 20 mg/ 100 b body weight. From the comparison with other fraction treated group, it was assessed that highest recovery was observed in ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* which enlighten that this fraction is the potent fraction (Fig. 9).

Discussion

The current study was conducted to delineate the potent fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* for the management of diabetes induced carbohydrate metabolic disordersin STZ induced diabetic rats which were preferred as an investigational model because it is one of the superior model to study the effect of the anti-diabetogenic agent **(Veeramani et al., 2008)**.

In the present study, ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* administered to the STZ induced diabetic rats showed effective diminution of fasting blood glucose level in respect of STZ induced diabetic rats. So, it may be said that the extract stimulates the β-cells of pancreas for the secretion of insulin or it has the capacity of restoration of pancreatic β-cells which has been projected by other, using plant products **(Chauhan et al., 2008)**. Serum insulin level of fraction treated group showed effective elevation in respect of STZ induced diabetic rats. This is possibly due to revitalization of muscle wastage through muscular protein synthesis by serum insulin which is consistent with other report **(Sellamuthu et al., 2009)**. Level of glycated haemoglobin which was significantly increased in diabetic rats was decreased by the treatment of different solvent fraction of seed of *H. antidysenterica* to diabetic rats indicated the efficacy of the extracts for glycaemic control. This may be due to the excess glucose present in blood reacts non enzymatically with haemoglobin which is an oxidative reaction and form glycated haemoglobin **(Klein, 1995; De et al., 2010)**. Here, the activity of carbohydrate metabolic enzyme and antioxidant enzyme was also studied by monitoring the appropriate biomarkers. This result was further supported from the

maximum recovery of serum insulin level at 20 mg dose of ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* which may be due to the biologically active phytomolecules present in this fraction that may stimulates the pancratic β-cell maximally in ethyl-acetate fraction or regenerate the β-cell after the treatment of this specific fraction to diabetic animals.

An important enzyme in glucose homeostasis is glucose-6-phosphatase. The enzyme is regulated by insulin in negative way. In STZ induced diabetic animals, there was an elevation in the enzyme activity in liver and skeletal muscle **(Gupta et al., 1999)**. After the application of potent fraction, a significant recovery was observed in this biosensors and this is possibly due to insulin recovery. For maintaining the blood glucose level, the role of hexokinase is crucial. The enzymatic activity of hexokinase is also insulin mediated **(Ugochukwu and Babady 2003)**. Maximum corrective efficacy exhibited after administration of ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at 20 mg dose/ 100 g body weight. That is possibly due to normal insulin secretion or by modified activity of the enzyme in positive way which was also noted in our earlier publication **(Mandal et al., 2008)**. Here, histometric observation of pancreas support the prediction about the β-cell reformative activity of ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* where haematoxylin-eosin staining of pancreas reflected the resettlement in pancreatic islet's size by the herbal extract administered in respect to the control group. Anti-diabetic efficacy of ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 20 mg/ 100 g body weight has been paying attention here from the recovery in the actions of above stated biosensors which may be due to insulinotrophic effect of the fraction.

Diabetes also develops a large amount of reactive oxygen species (ROS) generation, which may be due to the development of oxidative stress in different organs with special relevance to metabolic organs that was established in our earlier works (**Mallick et al., 2007; Ali et al., 2011)**. Superoxide dismutase and glutathione-s-transferase activity in hepatic and renal tissues were diminished and the level of

MDA was elevated in said tissues in diabetic condition that may be due hyperglycaemia influential oxidative stress in STZ induced diabetic animals which are related with the findings of our publication **(Ali et al., 2009)** in this line. After administration of the said fraction at the dose of 20 mg/ 100 g body weight, the antioxidant enzyme activities in hepatic and renal tissues were resettled that may possible due to antioxidant activity of the phytomolecule(s) present in the said fraction. These antioxidative nature have been moreover verified by quantification of lipid peroxidation by product i.e. MDA in the said tissues as there is an opposite connection between the antioxidant enzyme activities and the lipid peroxidation by product **(Pari and Saravanan, 2002; Ali et al., 2009)**.

This fraction has no adverse effect on health which has been indicated here from the enhanced body weight as well as rectification in GOT and GPT activities in hepatic and renal tissue seems to be its capability to improves glucose utilization and diminish hepato-renal dysfunction as these are the indicators of common and metabolic toxicity **(Chaterjee et al., 2009)**.

The efficacy of the fraction was further strengthened here from the densitometric analysis of protein expression of Bax and Bcl-2 in hepatic tissue through western blot, which was corrected in fraction treated diabetic animals towards control in comparison with untreated diabetic animals. Maximum efficacy was noted in ethyl-acetate fraction treated diabetic group.

Regarding the revival effect of ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* seeds, it may be stated that the fraction showed direct antioxidative activity and therefore the oxidative stress induced carbohydrate metabolic disorders is minimized. This antioxidative efficacy of the fraction probably due to the occurrence of antioxidative phytomolecule(s) in the potent fraction of *H. antidysenterica* that reduced the oxidative damage by controlling the hyperglycaemia in diabetic rats.

Conclusion

From the outcome of this experiment, it may be concluded ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* is the potent fraction for the management of diabetic disorders with special reference to genomic as well as proteomic approach and also carbohydrate metabolic enzymatic rectification in addition to oxidative stress recovery in STZ induced diabetic rats.

Graphical representation of results

Table 1: Protective efficacy of ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* on body weight in STZ induced diabetic male albino rat in comparison with other fraction treated group.

Treatment group	Body weight	
	Initial	Final
	(g)	
Vehicle treated control group	153.3±4.7[a]	165.2±3.1[a]
Vehicle treated diabetic group	156.2±5.2[a]	139.5±2.3[b]
n-hexane fraction treated diabetic group	154.3±5.1[a]	147.3±2.1[c]
Chloroform fraction treated diabetic group	153.2±4.7[a]	148.5±2.3[c]
Ethyl-acetate fraction treated diabetic group	154.7±5.4[a]	158.1±3.7[d]
n-butanol fraction treated diabetic group	155.2±5.0[a]	147.1±2.4[c]

Data were expressed as Mean ± SEM (n = 6). Values with different superscripts (a, b, c, d) in each vertical column differ from each other significantly, $p<0.05$.

Glycemic sensors:

Table 2: Ameliorative effect of ethyl-acetate fraction of hydro-methanol extract of seeds of *H. antidysenterica* on fasting blood glucose level in STZ induced diabetic male albino rat in comparison with other fraction treated group.

Groups	Fasting blood glucose level (mg/dl)						
	0 day	1 day (7^{th} day of STZ injection)	4 day	7 day	14 day	21 day	28 day
Vehicle treated control group	72.1±6.6[a]	70.3±602[a]	69.1±6.4[a]	70.6±6.5[a]	71.6±6.1[a]	69.3±6.1[a]	70.2±6.2[a]
Vehicle treated diabetic group	74.0±6.2[a]	333.4±6.4[a]	315.6±6.3[b]	310.2±6.2[b]	320.6±6.4[b]	327.8±6.2[b]	322.3±6.3[b]
n-hexane fraction treated diabetic group	72.3±6.2[a]	330.6±6.3[b]	307.3±6.4[b]	270.6±6.3[c]	255.8±6.5[c]	176.3±6.4[c]	118.2±6.4[c]
Chloroform fraction treated diabetic group	70.1±6.1[a]	329.4±6.5[b]	298.3±6.5[b]	261.5±6.4[c]	252.0±6.5[c]	183.7±6.3[c]	114.5±6.6[c]
Ethyl-acetate fraction treated diabetic group	69.0±6.4[a]	315.7±6.4[b]	269.6±6.3[c]	222.4±6.5[d]	213.4±6.5[d]	140.6±6.4[d]	88.6±6.2[d]
n-butanol fraction treated diabetic group	72.2±6.3[a]	338.2±6.2[b]	292.5±6.1[b]	250.3±6.5[c]	242.4±6.4[c]	170.2±6.6[c]	107.1±6.5[c]

Data were expressed as Mean ± SEM (n = 6). Values with different superscripts (a, b, c, d) in each vertical column differ from each other significantly, $p<0.05$.

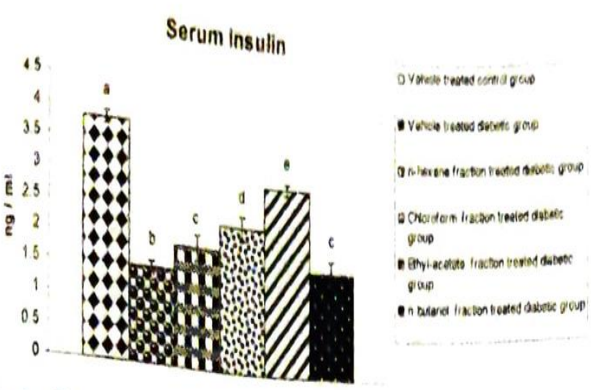


Fig 1: Changes in serum insulin level after treatment of different fraction of hydro-methanol extract of seed of *H. antidysenterica* at 20 mg / 100 g body weight /day dose in STZ-induced diabetic rat: Bars denote Mean ± SEM (n=6). ANOVA followed by 'Multiple comparisons two tail-t-test'. Bars with different superscripts (a, b, c, d, e) differ from each other significantly at $p<0.05$.

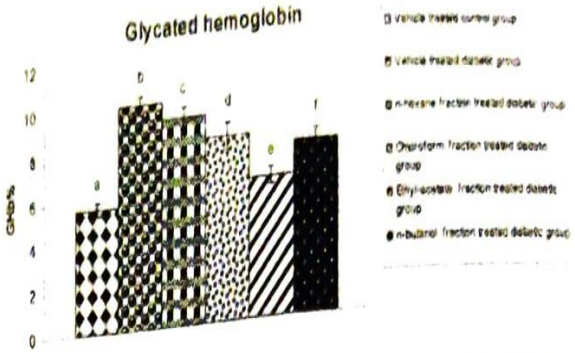
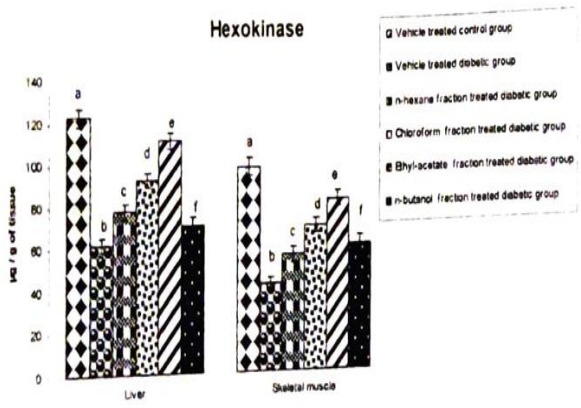


Fig 2: Glycated hemoglobin level was resetelled after treatment of different fraction of hydro-methanol extract of seed of *H. antidysenterica* at 20 mg dose / 100 g body weight /day in diabetic rat: Bars denote Mean ± SEM (n=6). ANOVA followed by 'Multiple comparisons two tail-t-test'. Bars with different superscripts (a, b, c, d, e, f) differ from each other significantly at $p<0.05$.



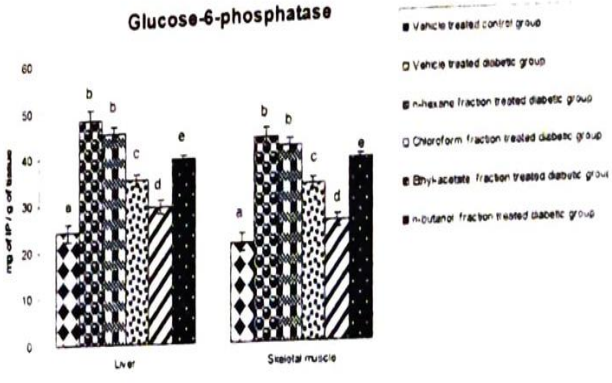


Fig 3: Resettlement in the activities of hexokinase and glucose-6-phosphatase in liver and skeletal muscle after treatment of different fraction of hydro-methanol extract of seed of *H. antidysenterica* at 20 mg / 100 g body weight /day dose in vehicle treated diabetic rat. Values were expressed as Mean ± SEM, n=6. Bars with different superscripts (a, b, c, d, e, f) differ from each other significantly, $p < 0.05$.

Antioxidant enzymes:

Fig 4: Amelioration in the activities of catalase and peroxidase in hepatic and renal tissue after treatment with hydro-methanol extract of seed of *H. antidysenterica* at different doses in STZ induced diabetic rat. Values were expressed as Mean ± SEM, n=6. Bars with different superscripts (a, b, c, d, e) differ from each other significantly, $p < 0.05$.

Oxidative stress end-product:

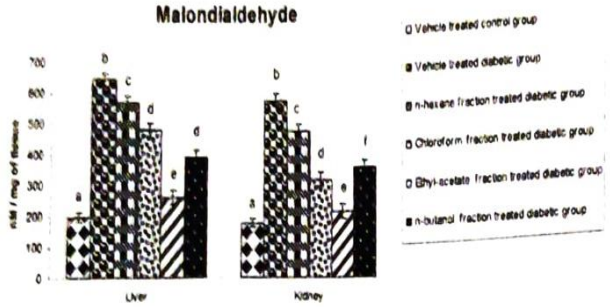


Fig 5: Corrective effect of hydro-methanol extract of seed of *H. antidysenterica* at different doses on TBARS levels in hepatic and renal tissues in comparison to vehicle treated diabetic rat. Values were represented Mean ± SEM (n=6). Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

Toxicity marker:

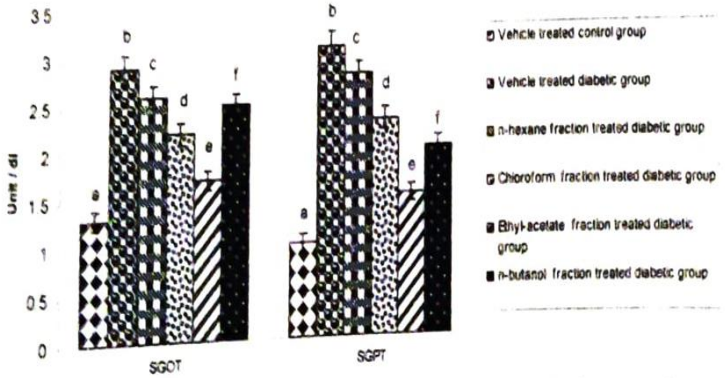


Fig 6: Assessment of serum GOT and GPT activities after hydro-methanol extract of seed of *H. antidysenterica* at different doses treatment to experimental diabetic rat. Values were expressed as Mean ± SEM, n=6. Bars with different superscripts (a, b, c, d, e differ from each other significantly, $p < 0.05$.

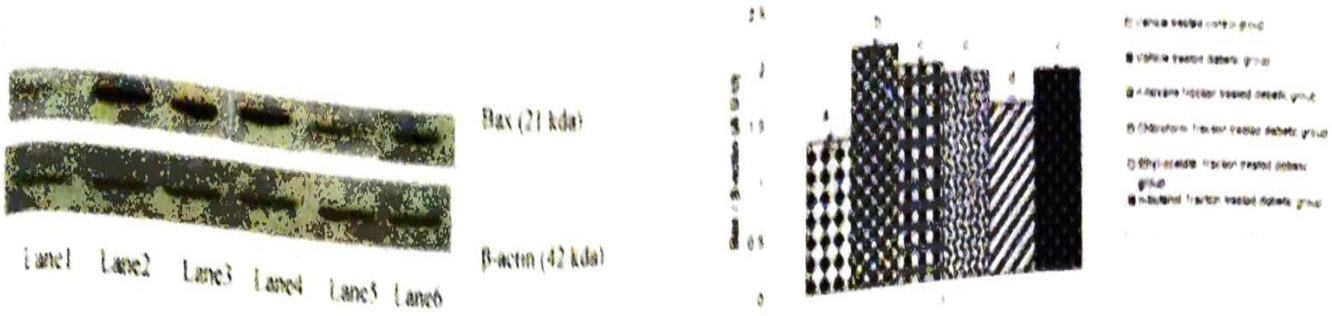


Figure 7: Representative semiquantitative result of Bax protein expression of the hepatic tissue obtained by densitometric analysis of western blots in different groups. Lane 1: Vehicle treated control rat. Lane 2: Vehicle treated diabetic rat. Lane 3: n-hexane fraction treated diabetic group. Lane 4: Chloroform fraction treated diabetic group. Lane 5: Ethyl-acetate fraction treated diabetic group. Lane 6: n-butanol fraction treated diabetic group. Analysis of variance followed by "Multiple Comparisons two tail 't' test". Bars represent mean ± SEM. (n=6). Values with significant different superscripts (a,b,c,d) in each column differ from each other significantly, $p<0.05$. A.D.U. = arbitrary densitometric units.

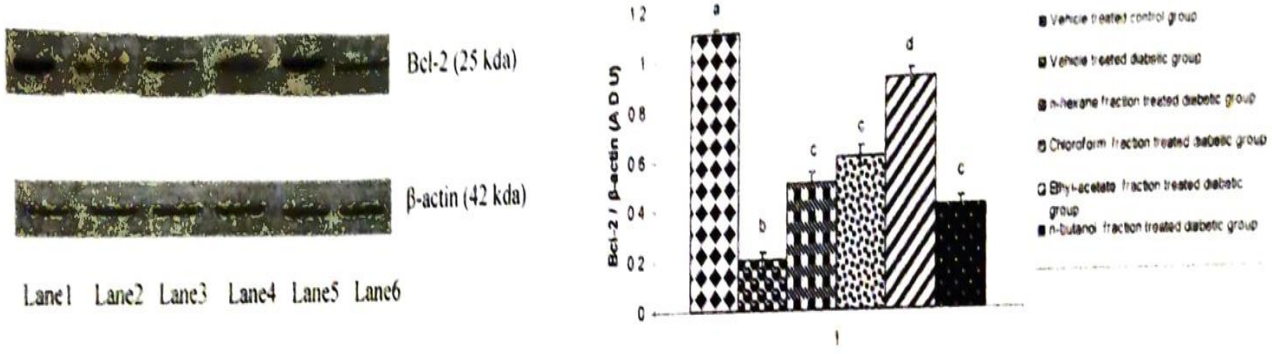


Figure 7: Representative semiquantitative result of B-cell lymphoma (Bcl-2) protein expression activity of the hepatic tissue obtained by densitometric analysis of western blots in different groups. Lane 1: Vehicle treated control rat. Lane 2: Vehicle treated diabetic rat. Lane 3: n-hexane fraction treated diabetic group. Lane 4: Chloroform fraction treated diabetic group. Lane 5: Ethyl-acetate fraction treated diabetic group. Lane 6: n-butanol fraction treated diabetic group. Analysis of variance followed by "Multiple Comparisons two tail 't' test". Bars represent mean ± SEM. (n=6). Values with significant different superscripts (a,b,c, d) in each column differ from each other significantly, $p<0.05$. A.D.U. = arbitrary densitometric units.

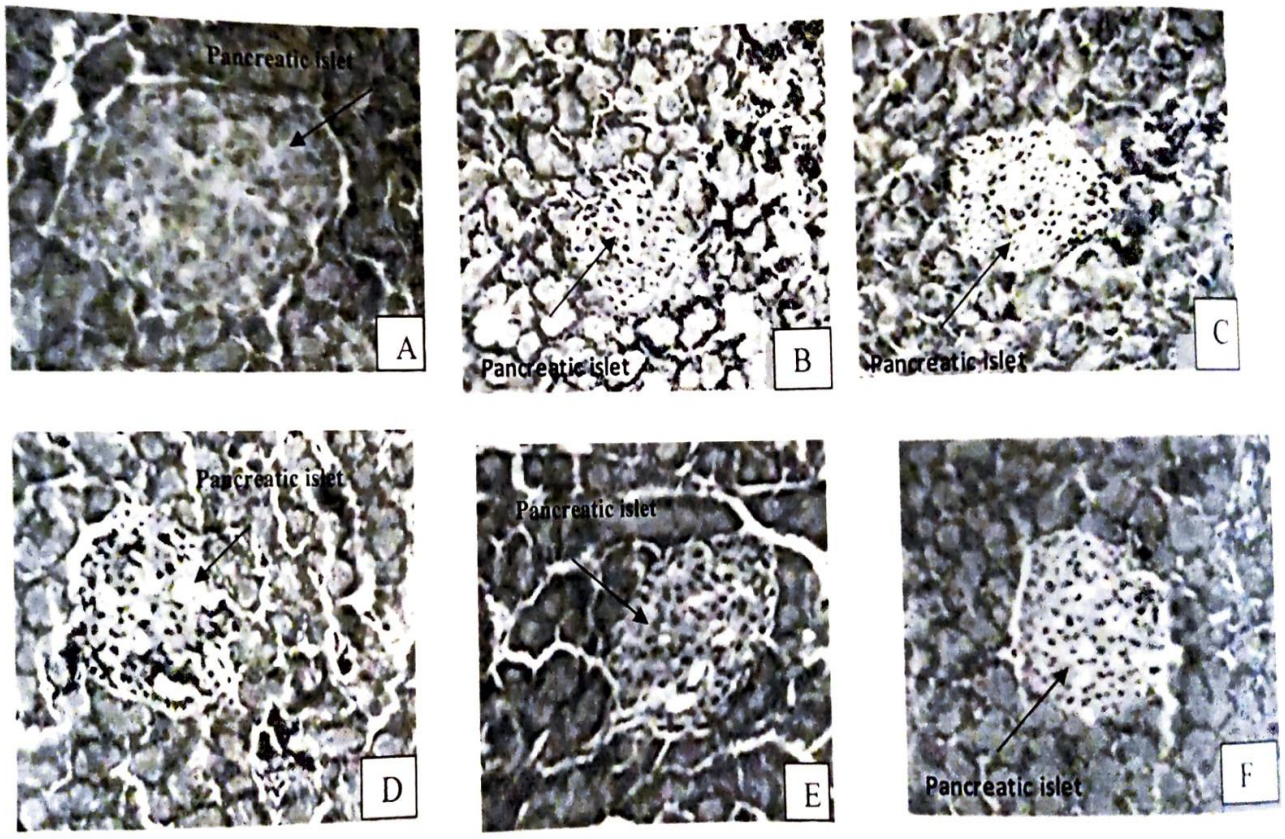


Fig 9: Histological study of pancreatic tissue, 400 X (Haematoxylin-Eosin stain).

A: Representative sample of pancreatic tissue of vehicle treated control rat focusing on cell density in islets of Langerhans and size islet: a qualitative analysis.

B: Diminution in the cell density in representative pancreatic islet tissue sample in vehicle treated diabetic rat along with diminution in islets size by qualitative analysis.

C: Representative pancreatic tissue sample showing recovery in cell density of islet's along with elevation in the size of islet of Langerhans by qualitative analysis after administration of n-hexane fraction of hydro-methanol extract of seed of *H. antidysenterica* at 20 mg dose to diabetic rat.

D: Representative sample of pancreatic tissue showing recuperation in cell density of islet's along with elevation in the size of islet of Langerhans by qualitative analysis after administration of chloroform fraction of hydro-methanol extract of seed of *H. antidysenterica* at 20 mg dose to diabetic rat.

E: Representative pancreatic tissue sample showing remarkable recovery in cell density of islet's along with elevation in the size of islet of Langerhans by qualitative analysis after administration of ethyl-acetate fraction of hydro-methanol extract of seed of *H. antidysenterica* at 20 mg dose to diabetic rat.

F: Representative pancreatic tissue sample showing remarkable recovery in cell density of islet's along with elevation in the size of islet of Langerhans by qualitative analysis after administration of n-butanol fraction of hydro-methanol extract of seed of *H. antidysenterica* at 20 mg dose to diabetic rat.

EXPERIMENT NO. – 03

Protective effect of ethyl acetate fraction of hydro-methanol :: 40:60 extracts of seeds of *Holarrhena antidysenterica* in streptozotocin-induced diabetic rat: A dose dependent analysis

Animals and their maintenance

As mentioned before.

Plant materials

As mentioned before.

Induction of diabetes mellitus in rats

As mentioned before.

Animal treatment

Six equal groups were made by using thirty six rats. Hydro-methanol :: 40:60 extract treatment of seeds of *H. antidysenterica* was started from the 7^{th} day of post injection period of STZ and was considered as 1^{st} day of treatment. The duration of treatment was 28 days.

Group I (Vehicle treated control group) Normoglycemic animals of this group was treated with citrate buffer (0.1 ml/ 100 g body weight/ rat) by a single intramuscular injection.

Group II (Vehicle treated diabetic group) Animals were made diabetic by a single intramuscular injection of STZ at a dose of 4 mg/ 0.1 ml citrate buffer/ 100 g body weight/ rat having high level of fasting blood sugar.

Group III (Ethyl acetate fraction 10 mg) From the 7th day of STZ injection, the diabetic rats of this group were forcefully feed by gavage with hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 10 mg/ 0.5 ml of 3% tween 80/ 100 g body weight/ day and the drug treatment was continued for 28 days at fasting state.

Group IV (Ethyl acetate fraction 20 mg) Animals belongs to this group were orally administered with hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 20 mg/ 0.5 ml of 3% tween 80/ 100 g body weight/ day at fasting state for 28 days.

Group V (Ethyl acetate fraction 30 mg) STZ induced diabetic animals of this group were treated with oral administration of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 30 mg/ 0.5 ml of 3% tween 80/ 100 g body weight/ day for 28 days at fasting state.

The duration of entire experiment was 28 days. Different fraction of above mentioned extract was given orally at 11:00 A.M. to animals (overnight fasting state).

Treatment was started from the 7th day of STZ injection to the diabetic rats. Level of FBG was measured by using single touch glucometer in all the groups on every 7th day **(Chatterjee et al., 2009)**. After completion of 28 days of drug treatment, the animals were maintained in fasting condition for overnight and finally taking the body weight on 29th day of post drug treatment period, then all the animals were sacrificed by decapitation using ether anaesthesia. From the dorsal aorta, blood was collected by a syringe and the serum was separated by centrifugation at 3000g for 5 minutes for the estimation of different parameters. Liver, kidnies, pancreas and skeletal muscular tissues were dissected

out from each animal, washed in normal saline, soaked in blotting paper and stored in deep freezer (-20ºC) for the measurement of different relevant biomarkers and genomic sensors in this concern.

Measurement of fasting blood glucose (FBG) level

As mentioned before.

Biochemical assay of hexokinase activity in hepatic tissue and skeletal muscle

As mentioned before.

Biochemical assay of glucose-6-phosphatase activity in hepatic tissue and skeletal muscle

As mentioned before.

Biochemical assay of catalase (CAT) activities of hepatic and renal tissues.

As mentioned before.

Biochemical assay of peroxidase activities of hepatic and renal tissues.

As mentioned before.

Estimation of lipid peroxidation from the concentration of thiobarbituric acid reactive substances

As mentioned before.

Biochemical assay of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) activity

As mentioned before.

Histological study

As mentioned before.

Statistical analysis

As mentioned before.

Results

Feeding habits

Quantity of food intake and volume of water intake were increased in STZ-induced diabetic rats in respect to vehicle treated control group. After the treatment of ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 10 mg for 28 days to diabetic rats, a significant decrease ($p<0.05$) in food and water intake was noted in respect to vehicle treated diabetic group. On the other hand, administration of ethyl-acetate fraction at the dose of 20 mg or 30 mg/ 100 g of body weight for 28 days to the diabetic rats resulted a significant recovery ($p<0.05$) in the level of the above said parameters to the control level. There was no significant variation in the levels of these parameters among 20 mg and 30 mg/ 100 g body weight of fraction treated diabetic rats (Table. 1).

Body weight

A significant diminution ($p<0.05$) in the body weight in STZ-induced diabetic rats was noted in respect to vehicle treated control rats. Administration of ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 10 mg/ 100 g body weight for 28 days to diabetic rats, a significant elevation ($p<0.05$) in body weight was observed in respect to vehicle treated diabetic control. Treatment of ethyl acetate fraction at the dose of 20 mg or 30 mg/ 100 g of body weight/ day for 28 days to the diabetic rats resulted a significant recovery ($p<0.05$) in body weight in respect to 10 mg fraction treated diabetic groups and the levels were resettled to the control level. No significant difference was found in the value of said parameter when the results in the body weights were compared among the 20 mg and 30 mg of ethyl-acetate fraction treated diabetic groups (Table. 2).

Fasting blood glucose (FBG) level

Fasting blood glucose level was significantly elevated ($p<0.05$) in STZ-induced diabetic rats in respect to vehicle treated control group. Treatment of ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seed of *H. antidysenterica* at different dose of 10 mg or 20 mg or 30 mg/ 100 g of body weight for 28 days to the diabetic animals resulted a significant diminution ($p<0.05$) in fasting blood glucose level when comparison was made with vehicle treated diabetic group. From the comparative analysis of the data it was observed that 20 mg or 30 mg fraction treated group resulted a significant diminution ($p<0.05$) of fasting blood glucose level in respect to 5 mg and 10 mg fraction treated diabetic animals though no significant variation was noted in the level of the said parameter among 20 mg, 40 mg fraction treated diabetic groups indicated that 20 mg dose is the threshold dose in this concern (Table. 3).

Activities of hexokinase in liver and skeletal muscle.

Activities of hexokinase in liver and skeletal muscle tissues were diminished significantly ($p<0.05$) in STZ induced diabetic group in compare with the vehicle treated control group. After the treatment of ethyl-acetate fraction of seed of *H. antidysenterica* at the dose of 10 mg or 20 mg or 30 mg/ 100 g of body weight for 28 days to the STZ-induced diabetic animals resulted a significant elevation ($p<0.05$) in the activities of these enzymes in respect to vehicle treated diabetic group. Results focused that 20 mg 30 mg dose significantly effective ($p<0.05$) in compare with 10 mg doses of ethyl-acetate fraction though the levels of the said sensors were not resettled to the control level. There was no significant variation in the levels of these parameters among 20 mg, 30 mg/ 100 g of body weight fraction treated diabetic groups (Fig. 1).

Assessment of glucose-6-phosphatase activity

Activities of glucose-6-phosphatase in hepatic and skeletal muscular tissues were increased significantly ($p<0.05$) in the diabetic animals in compared with the vehicle treated control animals. Vehicle treated diabetic animals treated with different dose of ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* resulted a significant diminution ($p<0.05$) in the activities of glucose-6-phosphatase in respect to the vehicle treated diabetic group. Comparative analysis of the data in the activities of the enzyme indicated that 20 mg dose treated group showed significant effective result ($p<0.05$) in compare with another treated group. There was no significant variation in the levels of these parameters among 20 mg, 30 mg/ 100 g of body weight fraction treated diabetic groups (Fig. 1).

Catalase and peroxidase activities in liver and kidney tissues

Activities of catalase and peroxidase in liver and kidney tissues were significantly diminished ($p<0.05$) in STZ treated diabetic group in compare with the vehicle treated control group. Administration of ethyl-acetate fraction of seed of *H. antidysenterica* at the dose of 10 mg/ 100 g of body weight for 28 days to the diabetic rats resulted a significant elevation ($p<0.05$) in the activities of catalase and peroxidase in liver and kidney tissues in compare with vehicle treated diabetic group. In contrast, after 28 days treatment of said fraction at the dose of 20 mg or 30 mg/ 100 g body weight to diabetic rats, a significant recovery ($p<0.05$) was noted in the activities of the concerned parameters in respect to other doses of ethyl-acetate fraction. The levels of these parameters were insignificantly differed when the results were compared among 20 mg and 30 mg/ 100 g of body weight fraction treated groups though the levels were not resettled to the control level. (Fig. 2).

Quantification of Thiobarbituric acid reactive substances (TBARS) in liver and kidney tissues

Quantity of TBARS was significantly increased ($p<0.05$) in liver and kidney tissues of STZ induced diabetic group in respect to vehicle treated control group. Treatment of ethyl-acetate fraction of seed of

H. antidysenterica at the dose of 10 mg or 20 mg or 30 mg/ 100 g body weight/ day for 28 days to the diabetic rats resulted a significant diminution ($p<0.05$) in the levels of this parameters in respect to the vehicle treated diabetic group. A significant recovery ($p<0.05$) was observed in 20 mg or 30 mg fraction treated group in compare with 10 mg fraction treated group though the levels were significantly less ($p<0.05$) than the control level. There was no significant difference among 20 mg and 30 mg/ 100 g of body weight fraction administered groups in the levels of TBARS in liver and kidney tissues (Fig. 3).

Assessment of the activities of glutamate oxaloacetate transaminase (GOT) and serum glutamate pyruvate transaminase (GPT) activity

Activities of GOT and GPT in hepatic and renal tissue were increased significantly ($p<0.05$) in the diabetic animals in compare with the control animals. Diabetic animals treated with ethyl-acetate fraction of seed of *H. antidysenterica* at the dose of 10 mg or 20 mg or 30 mg/ 100 g body weight/ day for 28 days to the diabetic rats resulted a significant diminution ($p<0.05$) in the activities of both enzymes in respect to the diabetic group. Comparative analysis of the data in the activities of the enzyme indicated that 20 mg or 30 mg fraction treated group in compare with 10 mg fraction treated group of ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* showed significantly effective result ($p<0.05$) in compare with 10 mg fraction treated group. There was no significant difference among 20 mg and 30 mg/ 100 g of body weight fraction administered groups (Fig. 4).

Histology of pancreas

Histological analysis of pancreas showed that diameter of pancreatic islets was significantly decreased in vehicle treated diabetic group in compare with vehicle treated control group. The size of pancreatic islets was recovered significantly after the treatment of ethyl acetate fraction of hydro-methanol ::

40:60 extract of seeds of *H. antidysenterica* to the diabetic animals at the doses of 10 mg or 20 mg or 30 mg/ 100 g of body weight. From the comparison, it was mentioned that maximum recovery was observed at the dose of 20 mg/ 100 g of body weight which enlighten that this dose is the threshold dose. Beyond this dose no further recovery was observed in this concern (Fig. 5).

Discussion

The present dose dependent study has been performed to find out the threshold dose of ethyl-acetate fraction of seed of *H. antidysenterica* for the management of diabetic disorders in STZ induced diabetic rats. Three different doses i.e. 10 mg, 20 mg, 30 mg were adopted in the present study.

Results focused that the significant recovery of body weight was observed in fraction treated diabetic animals at different doses in compare with vehicle treated diabetic group which may be due to recovery in glycogen content and reconstruction of muscle as supported by our previous publication **(Ali et al., 2009; De et al., 2010)**. From the comparative analysis of the data, it was observed that 20 mg dose resulted most promising results in this concern. In the present study, 20 mg dose of ethyl acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* administered to the STZ-induced diabetic rats showed effective diminution in fasting blood glucose level in respect of STZ-induced diabetic rats. So, it may be said that the extract stimulates the β-cells of pancreas for the secretion of insulin or it has the capacity of restoration of pancreatic β-cells which has been projected by other, using plant products **(Chauhan et al., 2008)**. An important enzyme in glucose homeostasis is glucose-6-phosphatase **(Berg et al., 2001)**. The enzyme is regulated by insulin in negative way **(Das 2002)**. In STZ induced diabetic animals, there was an elevation in the enzyme activity in liver and skeletal muscle **(Gupta et al., 1999)**. After the application of 20 mg dose of ethyl-acetate fraction, a significant recovery was observed in this bio-sensors and this is possibly due to insulin recovery. For maintaining the blood glucose level, the role of hexokinase is crucial **(Mayes 2000)**. The enzymatic activity of hexokinase is also insulin mediated **(Ugochukwu and Babady 2003)**. Maximum corrective

efficacy exhibited after administration of 20 mg dose/ 100 g body weight ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica*. That is possibly due to normal insulin secretion or by modified activity of the enzyme in positive way. Oxidative stress imposition is one of the pathology of diabetes **(Ghosh et al., 2009, Ali et al., 2009)**. In the present study diminution in the activities of important antioxidant enzyme i.e. catalase and peroxidase along with elevation of TBARS levels in liver and kidney tissues were observed which may be due to the hyperglycaemia induced oxidative stress in STZ induced diabetic rats **(Ali et al., 2009)**. This fraction has no adverse effect on health which has been indicated here form the enhanced body weight as well as rectification in GOT and GPT activities in hepatic and renal tissue seems to be its capability to improve glucose utilization and diminish hepato-renal dysfunction as these are the indicators of common and metabolic toxicity (**Chatterjee et al., 2009)**. Here, histometric observation of pancreas support the prediction about the β-cell reformative activity of ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* where haematoxylin-eosin staining of pancreas reflected the resettlement in pancreatic islet's size by the herbal extract administered in respect to the control group. Anti-diabetic efficacy of ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* at the dose of 20 mg/ 100 g body weight has been paying attention here from the recovery in the actions of above stated biosensors which may be due to insulinotrophic effect of the fraction. Ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* treatment to diabetic animal resulted a significant recovery after the treatment of 10 mg or 20 mg or 30 mg/ 100 g of body weight fraction treated diabetic groups and 20 mg dose resulted most promising results in compare with other doses. Higher dose above 20 mg dose did not show further rectification indicated 20 mg dose is the threshold dose in this concern. This antioxidative efficacy of the fraction may be due to the presence of antioxidative phytomolecules in ethyl-acetate fraction of hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* or fraction minimize the oxidative injury by controlling the hyperglycaemia in diabetic rats. The more higher dose of the said fraction is not more effective in this concern than the threshold

dose may be due to the saturation of the receptors of the concerned phytomolecules at the dose of 20 mg/ 100 g body weight and therefore the remedial effects are not improved by higher doses. Similarly at the sub-threshold dose, the receptors of phytomolecules are not saturated totally and therefore activities of the enzymes are not attained to the recovery level.

Conclusion

From the results of this experiment it may be concluded that 20 mg dose of ethyl-acetate fraction prepared from hydro-methanol :: 40:60 extract of seeds of *H. antidysenterica* is the threshold dose for the management of diabetic disorders with special reference to carbohydrate metabolic and oxidative stress recovery in STZ-induced diabetic rats.

Graphical representation of results

Table 1: Effect of ethyl-acetate fraction of seeds of *H. antidysenterica* on food and water intake capacity in STZ induced diabetic rats: A dose dependent response.

Groups	Food intake (g/ rat/ day)	Water intake (ml/ rat/ day)
Vehicle treated control	23.1 ± 1.41^{a}	32.4 ± 2.2^{a}
Vehicle treated diabetic	42.5 ± 1.32^{b}	56.5 ± 2.7^{b}
Ethyl acetate fraction 10 mg	30.5 ± 1.7^{c}	43.1 ± 2.1^{c}
Ethyl acetate fraction 20 mg	22.8 ± 1.12^{a}	34.2 ± 2.1^{a}
Ethyl acetate fraction 30 mg	21.7 ± 1.4^{a}	32.7 ± 2.2^{a}

Each value represents as Mean ± SEM (n = 6). "ANOVA followed by multiple comparison students two tail-t test". Values with different superscripts (a, b, c) in each vertical column differ from each other significantly, $p<0.05$.

Table 2: Recovery in the levels of body weight after the treatment of ethyl-acetate fraction of seeds of *H. antidysenterica* in STZ induced diabetic male albino rat.

Groups	Initial body weight (g)	Final body weight (g)
Vehicle treated control	148.6±3.78[a]	174.4±4.72[a]
Vehicle treated diabetic	151.4±4.02[a]	138.4±4.53[b]
Ethyl acetate fraction 10 mg	148.7±3.86[a]	161.3±3.43[c]
Ethyl acetate fraction 20 mg	152.4±4.24[a]	169.4±5.47[a]
Ethyl acetate fraction 30 mg	149.7±4.36[a]	172.7±4.58[a]

Each value represents as Mean ± SEM (n = 6). "ANOVA followed by multiple comparison students two tail-t test". Values with different superscripts (a, b, c) in each vertical column differ from each other significantly, $p<0.05$.

Glycogenic biosensors:

Table 3: Dose dependent effect of ethyl-acetate fraction of seed of *H. antidysenterica* on fasting blood glucose level in STZ-induced diabetic male albino rat.

Groups	**Fasting blood glucose level (mg/dl)**				
	1 day (7^{th} day of STZ injection)	**8^{th} day**	**15^{th} day**	**22^{nd} day**	**29^{th} day**
Vehicle treated control	70±2.8[a]	71±3.1[a]	69±3.3[a]	72±3.1[a]	71±2.6[a]
Vehicle treated	329±4.6[b]	334±5.1[b]	337±5.6[b]	337±4.8[b]	339±4.2[b]

diabetic					
Ethyl acetate fraction 10 mg	333±4.6[b]	337±5.1[b]	332±3.8[b]	329±3.5[b]	336±4.4[b]
Ethyl acetate fraction 20 mg	337±4.3[b]	236±4.2[d]	176±3.4[d]	136±3.8[d]	114±2.1[d]
Ethyl acetate fraction 30 mg	334±4.2[b]	252±4.4[c]	218±4.6[c]	192±4.5[c]	146±3.6[c]

Value represents Mean ± SEM (n = 6). "ANOVA followed by multiple comparison students two tail-t test". Values with different superscripts (a, b, c, d) in each vertical column differ from each other significantly at $p<0.05$.

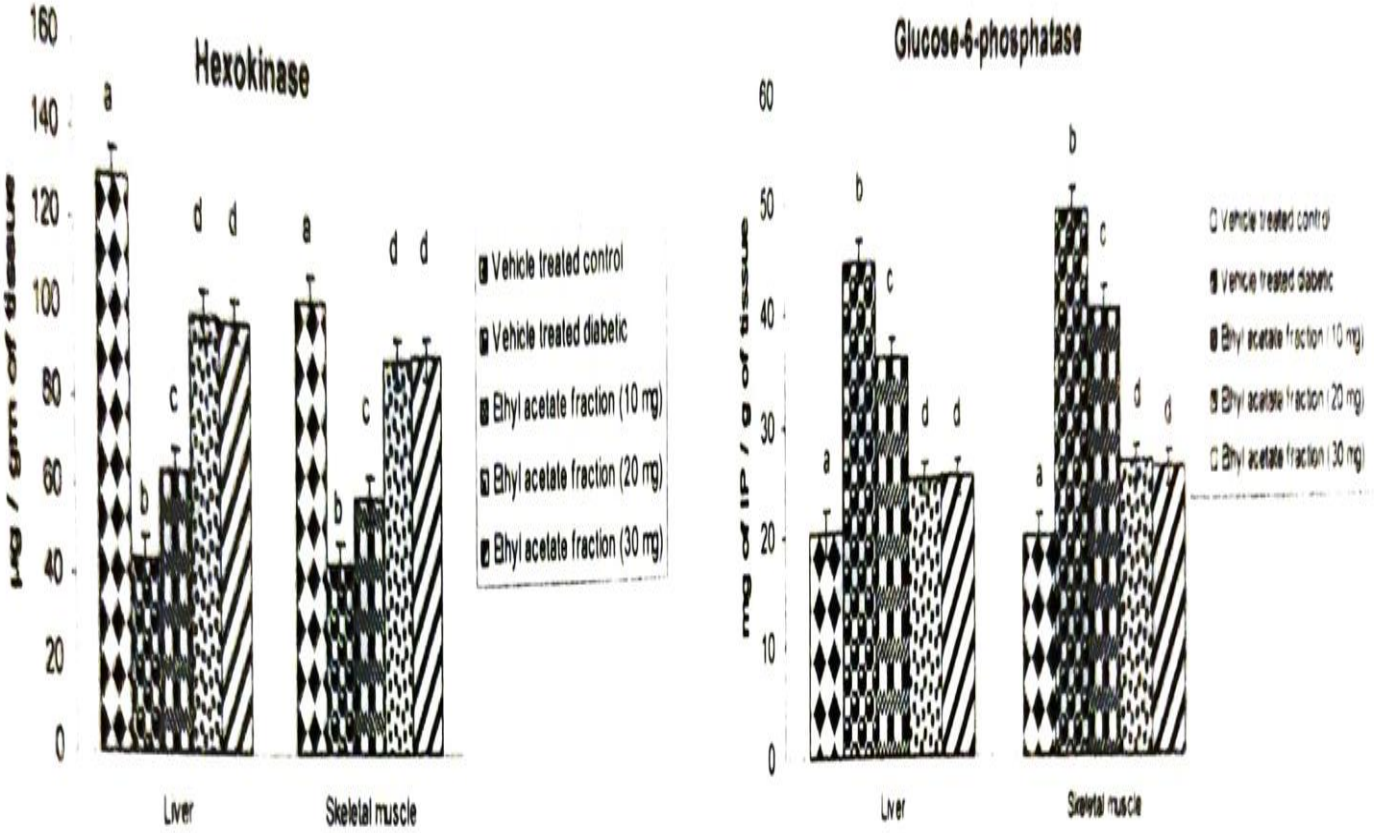


Fig 1: Resettlement in the activities of hexokinase and glucose-6-phosphatase in liver and skeletal muscle after treatment of ethyl-acetate fraction of hydro-methanol extract of seed of *H. antidysenterica* at different doses in vehicle treated diabetic rat. Values were expressed as Mean ± SEM, n=6. Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

Antioxidant enzymes:

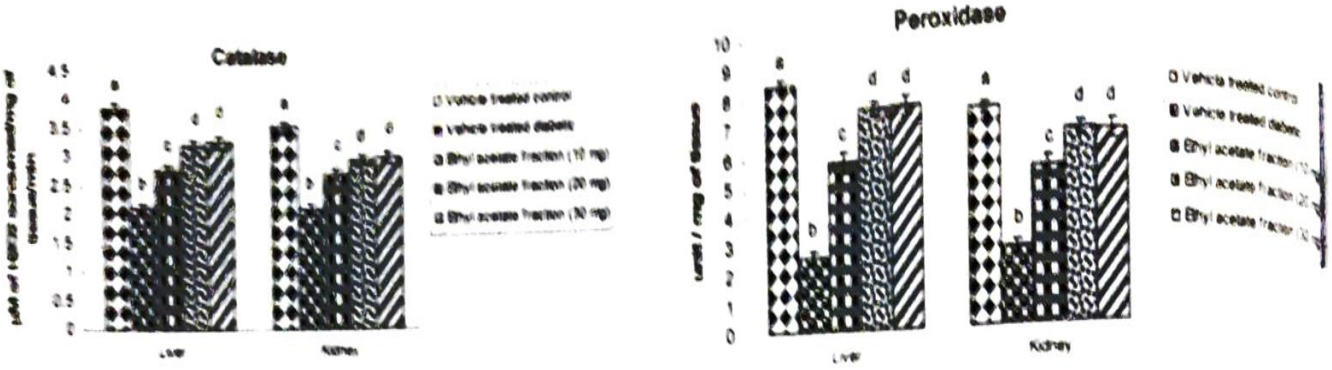


Fig 2: Amelioration in the activities of catalase and peroxidase in hepatic and renal tissue after treatment with ethyl-acetate fraction of hydro-methanol extract of seed of *H. antidysenterica* at different doses in STZ induced diabetic rat. Values were expressed as Mean ± SEM, n=6. Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

Oxidative stress end-product:

Toxicity study:

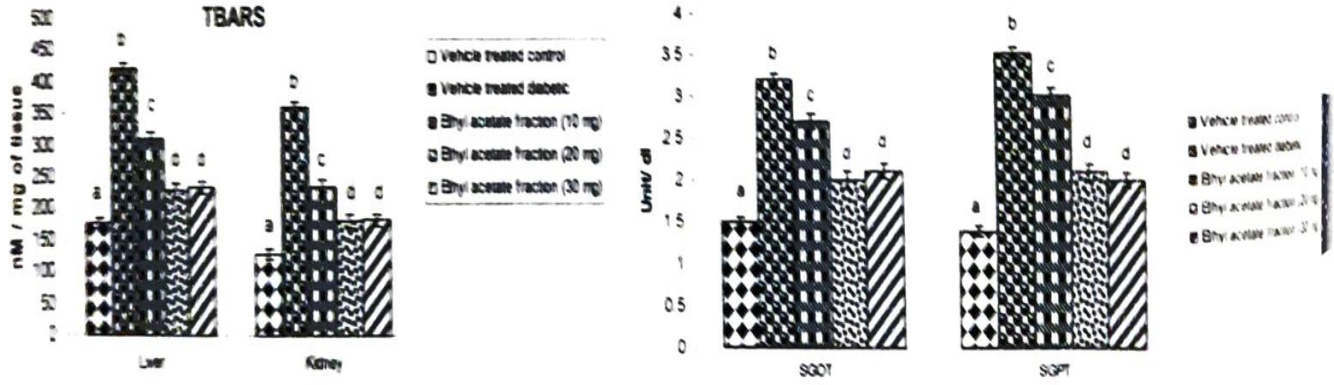
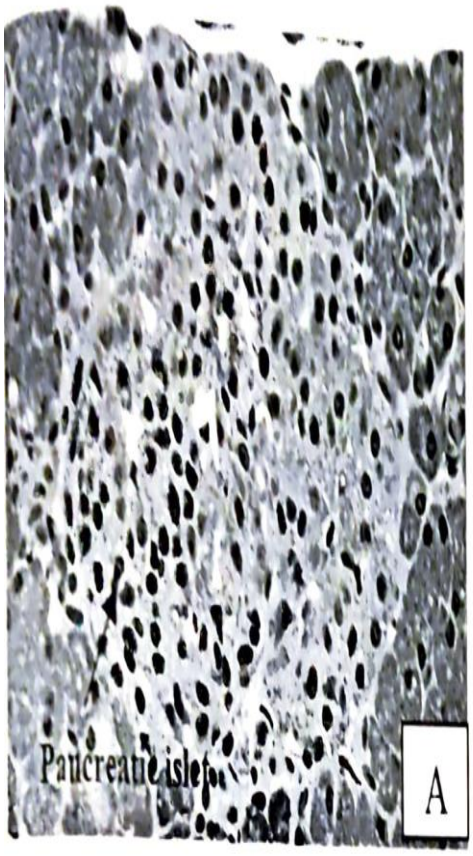


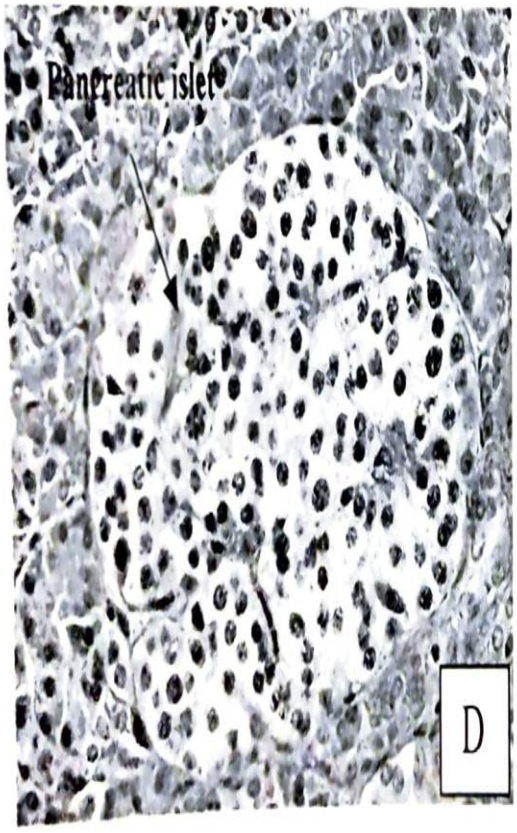
Fig 3: Remedial effect of ethyl-acetate fraction of hydro-methanol extract of seed of *H. antidysenterica* at different doses on TBARS levels in hepatic and renal tissues in comparison to vehicle treated diabetic rat. Values were represented Mean ± SEM (n=6). Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

Fig 4: Assessment of serum GOT and GPT activities after ethyl-acetate fraction of hydro-methanol extract of seed of *H. antidysenterica* at different doses treatment to experimental diabetic rat. Values were expressed as Mean ± SEM, n=6. Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.


Pancreatic islet
A


Pancreatic islet
B


Pancreatic islet
C


Pancreatic islet
D

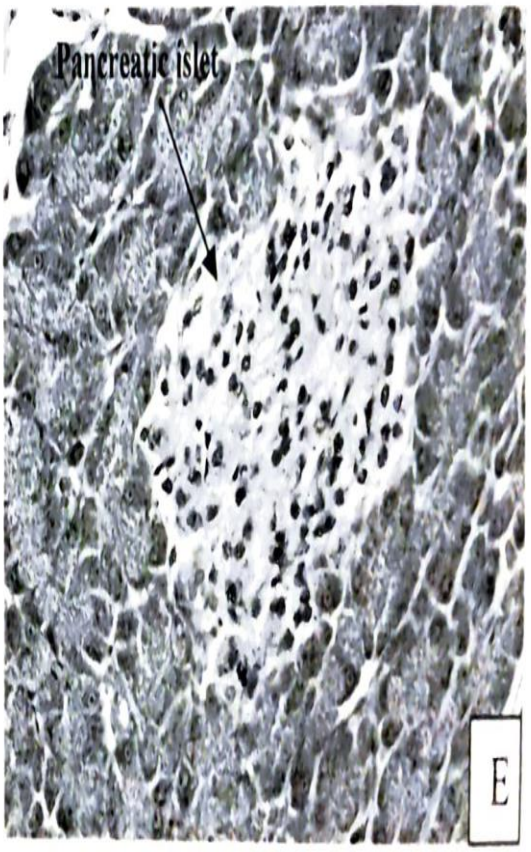

Pancreatic islet
E

Fig 5: Histological study of pancreatic tissue, 400 X (Haematoxylin-Eosin stain).

A: Representative sample of pancreatic tissue of vehicle treated control rat focusing on cell density in islets of Langerhans and size islet: a qualitative analysis.

B: Diminution in the cell density in representative pancreatic islet tissue sample in vehicle treated diabetic rat along with diminution in islets size by qualitative analysis.

C: Representative pancreatic tissue sample showing minor recovery in cell population density of islet's along with elevation in the size of islet of Langerhans by qualitative analysis after administration of 10 mg dose of ethyl-acetate fraction of hydro-methanol extract of seed of *H. antidysenterica* to diabetic rat.

D: Micro photographical representation of pancreatic tissue showing recuperation in cell density of islet's along with elevation in the size of islet of Langerhans by qualitative analysis after administration of ethyl-acetate fraction of hydro-methanol extract of seed of *H. antidysenterica* at 20 mg dose to diabetic rat.

E: Representative pancreatic tissue sample showing recovery in cell density of islet's along with elevation in the size of islet of Langerhans by qualitative analysis after administration of ethyl-acetate fraction of hydro-methanol extract of seed of *H. antidysenterica* at 20 mg dose to diabetic rat.

Experiment No 04

Revival efficacy of ethyl acetate fraction of hydro-methanol :: 40:60 extract of seed of *Holarrhena antidysenterica* (L.) in streptozotocin-induced diabetic rat: A duration dependent study

Animal Maintenance

Healthy, Normoglycemic Wistar strain adult male albino rats having 3 months of age and weighing 150±10 g were selected for the entire experimental schedules in this project work. Animals were acclimatized under standard laboratory condition i.e. 12 hrs light: 12 hrs dark and 25±2°C temperature with free access of food and water *ad libitum*. During pre-experimental period, all the animals were handled in regular basis to habituate them with the human contact and the environment of the room to minimise the physical stress imposition on them which may occur during experimentation. Prior approval was taken from the Institutional Ethic Committee to conduct the experiment as per CPCSEA guideline.

Plant Material

Holarrhena antidysenterica seeds were collected from local market of Midnapore, Paschim Medinipur, West Bengal, India. The seeds were identified by the taxonomists of the Botany and Forestry Department, Vidyasagar University, Paschim Medinipur where voucher specimen was preserved having the No Bio-Med/ V.U/ H.A/ 16/ 08.

The hydro-methanol::40:60 extract of seed of *H. antidysenterica* has been prepared as per our standard method (**Maity et al., 2004).** Fresh seeds were crushed and grinded by using electric grinder and then allowed for pulverization. About 50g powder was suspended in 250 ml of hydro-methanol:: 40:60 and kept it in room temperature, the mixture was then filtered through Whatman grade no. 3 filter paper.

Induction of Diabetes mellitus

The standard dose of streptozotocin was selected as per our previous publication of our laboratory **(Mallick et al., 2006)**. Rats fasting for 24 hours were subjected to a single intramuscular injection of Streptozotocin at the dose of 4 mg/0.1 ml of citrate buffer /100 gm body weight that produce diabetes (having fasting blood glucose level more than 300 mg/dl but less than 400 mg/dl) after 24h of STZ injection. This level of fasting blood glucose has been selected here as it represents the moderate diabetic state **(Grover et al., 2000)**. Subsequently, six days were allowed for the stability of diabetes and after that the rats were selected for the experiment those were fulfils the above criteria.

Aim of the study

This study has been performed to find out the duration dependent effect of ethyl acetate fraction prepared from aqueous-methanol (40:60) extract of seed of *H. antidysenterica* in streptozotocin induced diabetic disorders in Wistar rat and to search out the minimal but effective duration of treatment or threshold duration of treatment in this concern.

Animal Treatment

Six groups have been categorized having six animals in each group. The caging the grouping of the animals re mentioned below:

A. 14 days experimental schedule

Vehicle treated control for 14 days (Group I):

Normoglycemic animals were subjected to oral administration of 3% tween 80 forcefully by gavage method at the volume of 0.5ml /100gm of body weight / day for 14 days through intra gastric route.

Vehicle treated diabetic for 14 days (Group II):

Diabetic animals of this group were subjected to forceful feeding of 3% tween 80 for 14 days at the volume of 0.5ml /100gm of body weight / day through intra gastric route.

Ethyl acetate fraction treated diabetic group for 14 days (Group III):

Diabetic animals were treated with ethyl acetate fraction of seed of *H. antidysenterica* through intra gastric route at the dose of 20mg/0.5ml of 3% tween 80/100gm body weight / day for 14 days.

B. 21 days experimental schedule

Vehicle treated control for 21 days (Group IV):

Normoglycemic rats were subjected to oral delivery of 0.5ml of 3% tween 80/ 100gm body weight / day for 21 days through intra gastric route.

Vehicle treated diabetic for 21 days (Group V):

Diabetic rats were considered in this group and they were providing oral intubations of 3% tween 80 at the volume of 0.5 ml / 100gm body weight / day for 21 days.

Ethyl acetate fraction treated diabetic group for 21 days (Group VI):

Diabetic animals were forcefully fed by oral intubations of ethyl acetate fraction of seed of *H. antidysenterica* at the dose of 20mg / 0.5ml 3% tween 80 / 100g body weight /day for 21 days.

C. 28 days experimental schedule

Vehicle treated control for 28 days (Group VII):

Normoglycemic six rats were subjected to oral delivery of 0.5 ml of 3% tween 80/100 g of body

weight /rat/day for 28 days through intra gastric route.

Vehicle treated diabetic for 28 days (Group VIII):
Diabetic animals of this group were treated with 3% tween 80 at the volume of 0.5 ml/100 g body weight /rat/day for 28days.

Ethyl acetate fraction treated diabetic group for 28 days (Group IX):
Diabetic rats were forcefully fed by oral intubations of ethyl acetate fraction of seed of *H. antidysenterica* at the dose of 20 mg/0.5 ml of 3% tween 80/ 100gm body weight/rat/day for 28 days.

D. 35 days experimental schedule

Vehicle treated control for 35 days (Group X):

Animals of normoglycemic in nature were subjected to oral ingestion of 3% tween 80 forcefully by gavage method at the volume of 0.5ml/100 gm body weight/day for 35 days through intra gastric route.

Vehicle treated diabetic for 35 days (Group XI):

Diabetic animals of this group were subjected to forceful intake of 0.5ml of 3% tween 80/ 100g body weight for 35 days through intra gastric route.

Ethyl acetate fraction treated diabetic group for 35 days (Group XII):

Diabetic animals were treated with ethyl acetate fraction of seed of *H. antidysenterica* at the dose of 20mg/ 0.5 ml of 3% tween 80/ 100gm body weight / day for 35 days.
Animals of vehicle control group (Group I, IV, VII, X) and diabetic control group (Group II, V, VIII, XI) were subjected to gavage of 0.5 ml 3% tween 80 for 14, 21, 28 and 35 days respectively at the time of fraction treatment to the diabetic animals (Group III, VI, IX and XII), to keep all the animals under the same experimental condition and stress imposition if any due to administration of fractions and animal handling. Starting from first day of fraction treatment to diabetic rats, fasting blood glucose levels in all the groups were measured on every 7 days interval. After completion of specific experimental schedule, all the animals of respective schedule were sacrificed at fasting state by light ether anesthesia followed by decapitation after recording the final body weight. Blood was collected from dorsal aorta by a syringe and the serum was separated by centrifugation at 3000g for 5 min for the estimation of serum levels of insulin and lipid profile. The liver and skeletal muscle tissues were dissected out and stored at -20^{o}C for the assessment in the activities of carbohydrate metabolic enzymes and glycogen content. Antioxidant enzyme activities and the levels of free radical byproducts were measured in hepatic tissue.

Parameters and methods

Serum insulin level was measured using solid phase-conjugated sandwitch ELISA kit (EZRMI-13K, Millipore, USA) for rat. The level of insulin was expressed in terms of ng/ml **(Pitchai et al., 2009)**.

Activities of hexokinase **(Chou and Wilson, 1975)**, glucose-6-phosphate dehydrogenase **(Langdon, 1966)** in hepatic and skeletal muscular tissue were measured following the standard methods. Levels of glycogen in liver and skeletal muscle tissues were estimated according to standard protocol **(Sadasivam and Manickam, 2008)**. Activities of antioxidant enzymes i.e. catalase **(Beers and Sizer, 1952)** and peroxidase **(Sadasivam and Manickam, 2008)** in hepatic tissue were measured following the standard methods. The lipid peroxidation status in the said tissue was evaluated from the concentration of CD **(Slater, 1984)** and TBARS **(Okhawa et al., 1979)** following standard methods. Serum levels of triglycerides (TG) **(Desai et al., 2002)**, total cholesterol (TC) **(Allain et al., 1974)**, low density lipoprotein cholesterol (LDLc) **(Friedewald et al., 1972)**, very low density lipoprotein cholesterol (VLDLc) **(Friedewald et al., 1972)** and high density lipoprotein cholesterol (HDLc) **(Waenic and Albers, 1978)** were estimated as per standard methods using specific kits.

Results

Body weight:

A significant reduction ($p<0.05$) in the body weight was noted in vehicle treated diabetic animals of all four duration dependent groups i.e. 14, 21, 28 or 35 days experimental schedule in respect to corresponding vehicle treated control groups. The level of this parameter was significantly recovered ($p<0.05$) after the treatment of ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* at 20 mg dose for the duration of 14 days or 21 days or 28 days or 35 days in respect to vehicle treated diabetic group of respective experimental schedule. Treatment of ethyl acetate fraction of seed of *H. antidysenterica* at the dose of 20mg / 100g body weight for 28 or 35 days to the diabetic animals resulted a more significant recovery ($p<0.05$) in compare with 14 days or 21 days fraction treated groups though 35 days treatment did not resulted a significant recovery in the level of this parameter in respect to 28 days of treatment **(Table 4.1)**.

Fasting blood glucose level:

Fasting blood glucose level was significantly elevated ($p<0.05$) in STZ treated diabetic groups in different duration of experimental schedule in compare with the corresponding vehicle treated control group. Administration for 14 days or 21days of ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* to the diabetic animals resulted a significant diminution ($p<0.05$) in fasting blood glucose level in respect to the corresponding vehicle treated diabetic group. When the results of 28 days or 35 days fraction treated groups compared with 14 days or 21 days fraction treated groups, it was observed that 28 days or 35 days duration significantly effective ($p<0.05$) in this concern in compare with other duration of treatment. No significant difference in fasting blood glucose level was noted between 28 days and 35 days of fraction treatment to diabetic animals indicated that 28 days is the threshold duration as beyond this duration of treatment there was no further recovery **(Table 4.2)**.

Activities of hexokinase, glucose-6-phosphate dehydrogenase in hepatic tissue:

Activities of hepatic hexokinase and glucose-6-phosphate dehydrogenase were decreased ($p<0.05$) was increased significantly ($p<0.05$) in vehicle treated diabetic group of animals in 14 days, 21 days, 28

days and 35 days experimental schedule in compare with the vehicle treated control animals of corresponding experimental schedule. Diabetic animals treated with ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* resulted a significant elevation ($p<0.05$) in the activities of hexokinase, glucose-6-phosphate dehydrogenase in hepatic tissue in all the duration of the experiments in respect to the corresponding vehicle treated diabetic group. Comparative analysis of the data in the activities of these enzymes indicated that 28 days or 35 days fraction treated group showed significant effective result ($p<0.05$) in compare with 14 days or 21 days of duration treated group. Moreover, after administration of ethyl acetate fraction of seed of *H. antidysenterica* at the dose of 20mg / 100gm body weight for 28 and 35 days to the diabetic animals, the levels in the activities of these enzymes in above tissues were insignificantly differ from one another **(Fig. 4.1, 4.2)**.

Serum insulin:

Serum insulin level was significantly decreased ($p<0.05$) in vehicle treated diabetic group in respect to the corresponding vehicle treated control in the entire duration dependent experimental schedule. Administration of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* for 14 days or 21 days or 28 days or 35 days to the diabetic animals resulted a significant elevation ($p<0.05$) in serum insulin level in respect to the STZ-treated diabetic group of corresponding experimental schedule. It was also observed that 28 days or 35 days fraction treated groups showed promising effect significantly ($p<0.05$) than 14 days as well as 21 days fraction treated diabetic group. No significant variation was observed in serum insulin level between 28 days and 35 days fraction treated diabetic groups indicated that 28 days is the threshold duration of treatment in this aspect **(Fig. 4.3)**.

Levels of total cholesterol (TC) and triglyceride (TG) in serum:

In all the duration dependent diabetic groups, serum TC and TG levels were significantly elevated ($p<0.05$) in compare with the vehicle treated control group of respective experimental schedule. After the treatment of ethyl acetate fraction of seed of *H. antidysenterica* at the dose of 20mg/100 gm body weight to the diabetic animals for 14 or 21 or 28 days or 35 days resulted a significant diminution ($p<0.05$) in the levels of the aforesaid parameters in serum when comparison was made with the STZ-induced diabetic animals of the respective duration of treatment schedule. A significant diminution ($p<0.05$) in the levels of these parameters was noted in 28 days and 35 days of ethyl acetate fraction of seed of *H. antidysenterica* treated diabetic animals in respect to the other duration of treatment. When comparison was made in the levels of these parameters between 28 days and 35 days fraction treated groups, no significant variation was observed **(Fig. 4.4 and 4.5)**.

Serum levels of LDLc, HDLc and VLDLc:

In all the duration dependent vehicle treated diabetic groups, serum levels of VLDLc and LDLc were elevated significantly ($p<0.05$) and HDLc level was decreased significantly ($p<0.05$) in respect to the vehicle treated control group of corresponding experimental schedule. After the treatment of ethyl acetate fraction of seed of *H. antidysenterica* for 14 days or 21 days or 28 days or 35 days to the diabetic

rats, a significant diminution ($p<0.05$) in serum VLDLc and LDLc levels and a significant elevation ($p<0.05$) in HDLc level were noted in compare with the vehicle treated diabetic animals of respective duration of the experiment. Comparative study of the data in these parameters showed that 28 days or 35 days fraction treatment significantly effective ($p<0.05$) than other duration of the said fraction treatment. There was no significant variation in the levels of these parameters when comparison was made between 28 days and 35 days fraction treated groups **(Fig. 4.6, 4.7 and 4.8)**.

Activities of catalase and peroxidase in hepatic tissue:

Hepatic catalase and peroxidase activities were significantly decreased ($p<0.05$) in vehicle treated diabetic rats in different duration dependent experimental schedule in compare with vehicle treated control rats of respective duration of experiment. Diabetic animals treated with ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* at the dose of 20mg/100gm of body weight for 14 days or 21 days or 28 days or 35 days resulted a significant recovery ($p<0.05$) in the activities of these enzymes in said tissues in respect to vehicle treated diabetic group of corresponding experimental schedule. When the values were compared with each other among the different groups, it was noted that 28 days and 35 days fraction treated groups showed promising efficacy in this concern in respect to the 14 days or 21 days fraction treated group. No significant variation was noted in the levels of these parameters between 28 days and 35 days fraction treated groups which indicate that 28 days duration is the threshold duration of treatment for this purpose **(Fig. 4.9 and 4.10)**.

Quantification of CD and TBARS levels in hepatic tissue:

A significant elevation ($p<0.05$) was observed in the levels of CD and TBARS in hepatic tissue in all the duration dependent diabetic groups in respect to vehicle treated control of respective experimental schedule. After the treatment of ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* for 14 days or 21 days or 28 days or 35 days to the diabetic animals, a significant reduction ($p<0.05$) was observed in the levels of these parameters in compare with corresponding vehicle treated STZ- induced diabetic animals. Results also showed that 28 days duration or 35 days duration of treatment was significantly effective than 14 days or 21 days duration in this concern though insignificant difference was noted in the levels of these parameters when comparison was made between 28 days and 35 days of fraction treatment diabetic groups **(Fig. 4.11 and 4.12)**.

Discussion

Duration dependent study has been conducted to explore the threshold duration of treatment for the management of diabetes induced carbohydrate metabolic disorders, hyperlipidemia and oxidative stress in STZ induced diabetic animals. .Fasting blood glucose level was corrected significantly by ethyl acetate fraction of seed of *H. antidysenterica* after 14 days or 21 days or 28 days or 35 days treatment to diabetic animals indicate the antihyperglycemic efficacy of the fraction which may be due to insulin secretory activity of the fraction as the results showed that a significant recovery in serum

insulin level after the treatment of ethyl acetate fraction for the said duration. This result was consistent with the findings of our previous experiments as well as supported by our publication in this line **(Ali et al., 2009a; Ali et al., 2009b)** and by other using other plant parts **(Chauhan et al., 2008)**. The result focused that 28 days duration was the threshold duration of treatment in this concern. Activities of important carbohydrate metabolic enzyme i.e. hexokinase and glucose-6- phosphate dehydrogenase in hepatic tissue were decreased in diabetic rat at duration dependent fashion which may be due to the proportionate diminution in serum insulin level and elevated levels of oxidative stress markers as insulin is the prime regulator in both these concern **(Ali et al., 2009c; De et al., 2010)**. After treatment of ethyl acetate fraction at the dose of 20mg/100gm body weight from 14 days upto 35 days, it was observed that a gradual and significant recovery of the said biomarkers was noted towards the control upto 28 days of treatment. Therefore the threshold duration for such correction was 28 days of treatment. There was no significant variation in the levels of said biosensors between 28 days and 35 days fraction treated diabetic groups indicated that beyond 28 days treatment no further recovery was observed in the said parameters.

Oxidative stress in liver of diabetic animals was developed at different duration which has been assessed here from the diminution in the activities of catalase and peroxidase as well as significant elevation of the end products of free radical i.e. CD and TBARS in liver. After 14 days of ethyl acetate fraction treatment and continuation of treatment upto 35 days, a significant recovery was observed at different durations of treatment and the threshold duration of treatment was 28 days. This result enlighted that this recovery by this phytoingredients present in ethyl acetate fraction may be the resettlement of serum insulin as serum insulin has influence on oxidative stress induced damage repairment by enhancement of antioxidant defense system **(Jana et al., 2011)**.

Lipid profile biomarkers i.e. serum total cholesterol and triglyceride were elevated in graded manner in diabetic groups in each experimental schedule. Ethyl acetate fraction treatment can able to recover the levels of these biomarkers in all the duration of the treatment. But maximum recovery was observed in 28 days fraction treatment group as no significant recovery was observed between 28 days and 35 days fraction treated diabetic groups and hence it is established that 28 days is the threshold duration of treatment in this concern. The threshold duration of treatment focused that phytomolecules present in the said fraction of seed of *H. antidysenterica* have the potentiality to correct the diabetic disorders upto a certain promising level and if the duration of treatment is increased there is no further correction of the concerned disorders. All the concern biosensors were corrected maximally by 28 days of treatment which also focus that the phytoingredients(s) present in this fraction can able to rectify the diabetic disorders but unable to act when sensors are resettled at normal level.

Conclusion

In the light of the results of this experiment it may be concluded that 28 days duration of treatment is the threshold duration for the recovery of diabetic disorders in STZ induced diabetic Wistar rat.

Table 4.1 Protective effect of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* on body weight in streptozotocin-induced diabetic rat: A duration dependent response

Schedule of duration	Group	Initial body weight (gm)	Final body weight (gm)
14 days	Vehicle treated control	147.4±3.66[a]	162.4±4.45[a]
	Vehicle treated diabetic	149.5±4.13[a]	137.6±4.23[b]
	Diabetic + ethyl acetate fraction	150.2±4.62[a]	155.4±6.33[c]
21 days	Vehicle treated control	147.6±4.65[a]	168.5±6.12[a]
	Vehicle treated diabetic	149.7±4.88[a]	134.3±4.96[b]
	Diabetic + ethyl acetate fraction	148.6±4.66[a]	159.7±4.62[c]
28 days	Vehicle treated control	151.2±5.54[a]	171.4±4.37[a]
	Vehicle treated diabetic	149.7±5.23[a]	128.7±5.69[b]
	Diabetic + ethyl acetate fraction	148.6±4.42[a]	167.4±5.67[d]
35 days	Vehicle treated control	150.7±4.73[a]	176.4±6.64[a]
	Vehicle treated diabetic	151.6±5.35[a]	131.7±5.76[b]
	Diabetic + ethyl acetate fraction	150.6±5.31[a]	168.6±5.67[d]

Each value represent Mean ± SEM (n=6). ANOVA followed by 'Multiple Comparison Two tail-*t*-test'. Values with different superscripts (a, b, c, d) in each vertical column differ from each other significantly at $p<0.05$.

Table 4.2 Duration dependent effect of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* on FBG in streptozotocin-induced diabetic rat

Duration of schedule	Group	Fasting blood glucose level (mg/dl)					
		1st day (7th day of STZ injection)	8th day	15th day	22nd day	29th day	36th day
14 days	Vehicle treated control	78.6±5.4^{a}	76.4±6.1^{a}	79.6±5.2^{a}	-	-	-
	Vehicle treated diabetic	352.6±7.5^{b}	347.4±6.3^{b}	343.4±6.7^{b}	-	-	-
	Diabetic + ethyl acetate fraction	347.6±6.9^{b}	257.4±5.6^{c}	186.5±7.4^{d}	-	-	-
21 days	Vehicle treated control	78.1±6.1^{a}	74.7±5.7^{a}	77.9±6.1^{a}	76.5±4.9^{a}	-	-
	Vehicle treated diabetic	344.5±7.2^{b}	351.5±6.9^{b}	342.7±7.3^{b}	346.6±7.3^{b}	-	-
	Diabetic + ethyl acetate fraction	347.5±8.4^{b}	261.5±6.6^{c}	196.6±6.9^{d}	127.5±6.4^{e}	-	-
28 days	Vehicle treated control	77.6±4.7^{a}	75.7±4.9^{a}	78.6±5.5^{a}	77.4±5.4^{a}	78.6±5.2^{a}	-
	Vehicle treated diabetic	346.3±7.5^{b}	353.5±6.8^{b}	346.7±6.7^{b}	346.5±6.8^{b}	343.6±5.7^{b}	-
	Diabetic + ethyl acetate fraction	347.6±6.8^{b}	249.5±7.3^{c}	194.6±5.7^{d}	129.7±5.7^{e}	96.4±4.2^{f}	-
35 days	Vehicle treated control	77.6±5.3^{a}	69.7±6.1^{a}	77.8±5.3^{a}	76.6±5.4^{a}	79.6±4.3^{a}	78.6±5.3^{a}
	Vehicle treated diabetic	344.6±6.9^{b}	349.8±5.6^{b}	349.6±5.7^{b}	344.5±7.5^{b}	346.7±5.4^{b}	346.7±5.3^{b}
	Diabetic + ethyl acetate fraction	348.3±6.4^{b}	253.5±5.7^{c}	197.6±7.2^{d}	129.7±7.6^{e}	97.3±5.3^{f}	94.6±2.8^{f}

Each value represent Mean ± SEM (n=6). ANOVA followed by 'Multiple Comparison Two tail-t-test'. Values with different superscripts (a, b, c, d, e, f) in each vertical column differ from each other significantly at $p<0.05$.

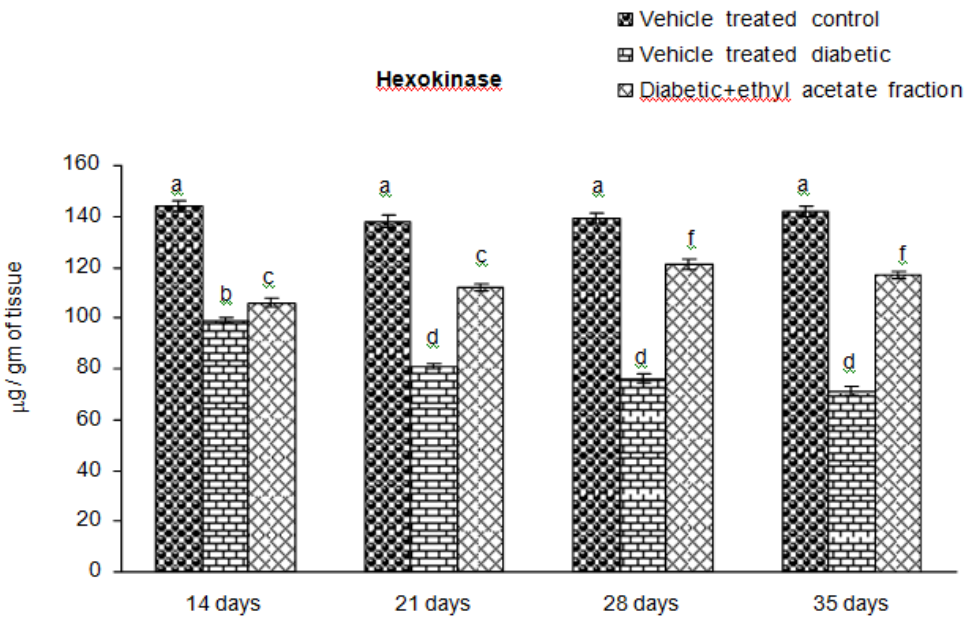


Fig 4.1 Hexokinase activity in hepatic tissue after the treatment of ethyl acetate fraction of seed of H. antidysenterica in STZ induced diabetic male albino rat at different duration. Bars indicate Mean ± SEM (n= 6). ANOVA followed by 'Multiple Comparison Two tail-t test'. Bars with different superscript (a, b, c, d, e, f) differ from each other significantly at $p < 0.05$

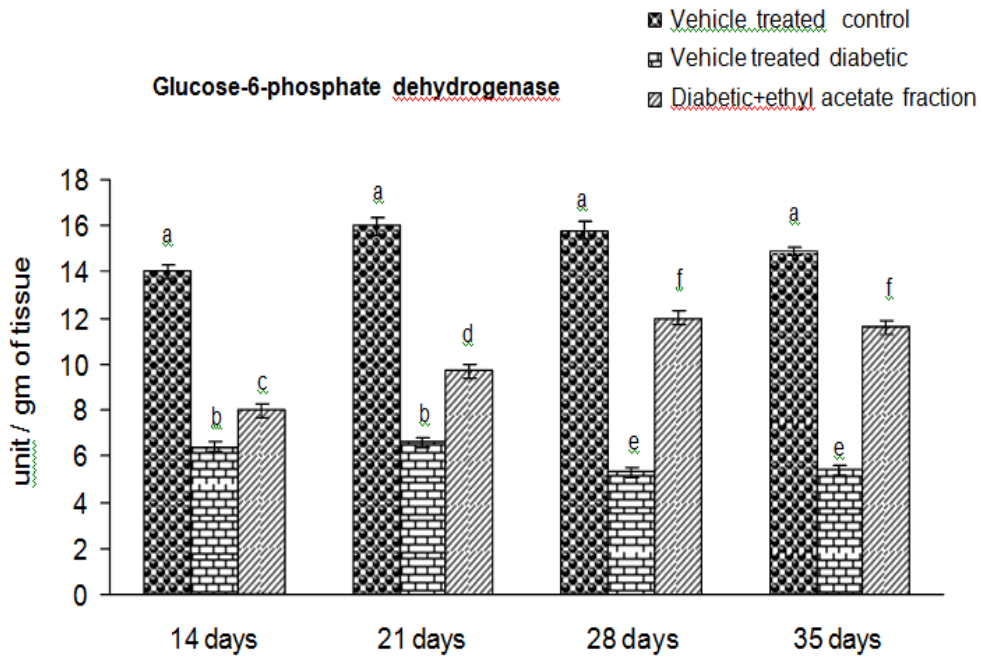


Fig 4.2 Glucose-6-phosphate dehydrogenase activity in hepatic tissue after the treatment of ethyl acetate fraction of seed of *H. antidysenterica* in STZ induced diabetic male albino rat at different duration. Bars indicate Mean ± SEM (n= 6). ANOVA followed by 'Multiple Comparison Two tail-t test'. Bars with different superscript (a, b, c, d, e, f) differ from each other significantly at $p < 0.05$

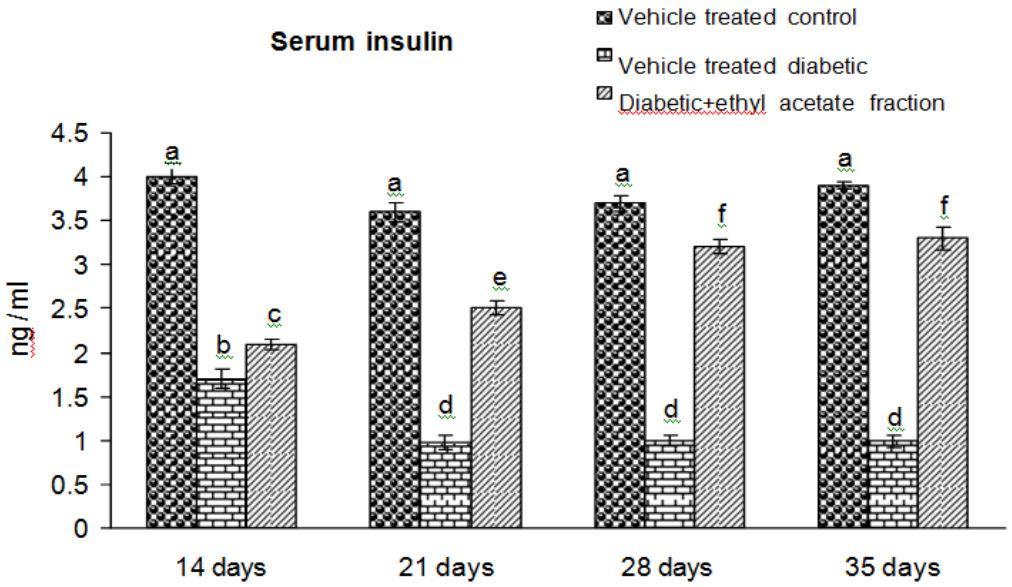


Fig 4.3 Serum insulin after the treatment of ethyl acetate fraction of seed of *H. antidysenterica* in STZ induced diabetic male albino rat at different duration. Bars indicate Mean ± SEM (n= 6). ANOVA followed by 'Multiple Comparison Two tail-t test'. Bars with different superscript (a, b, c, d, e, f) differ from each other significantly at $p < 0.05$

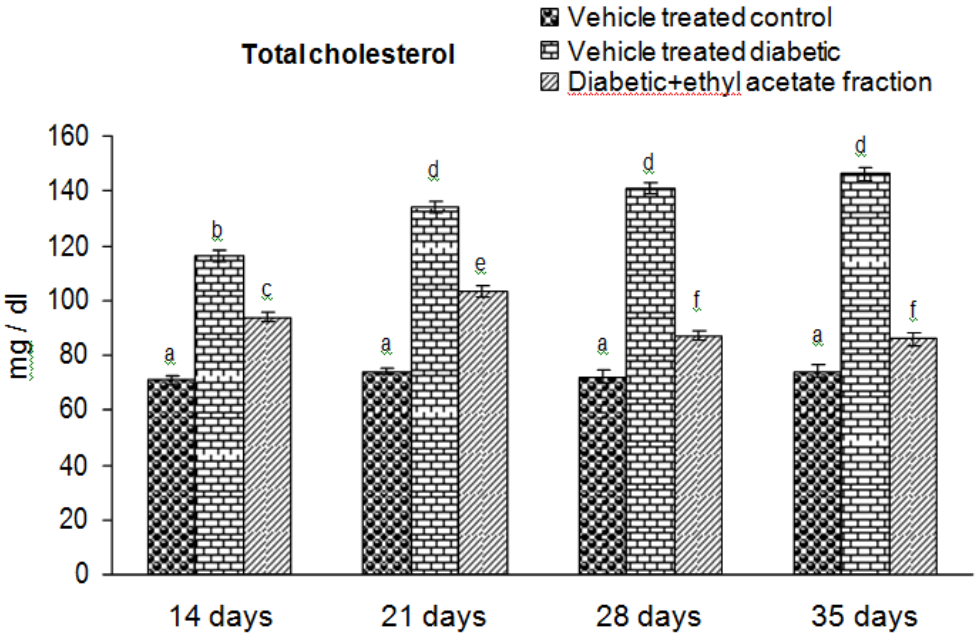


Fig 4.4 Serum total cholesterol level after the administration of ethyl acetate fraction of seed of *H. antidysenterica* in STZ induced diabetic male albino rat at different duration. Bars indicate Mean ± SEM (n= 6). ANOVA followed by 'Multiple Comparison Two tail-t test'. Bars with different superscript (a, b, c, d, e, f) differ from each other significantly at $p < 0.05$

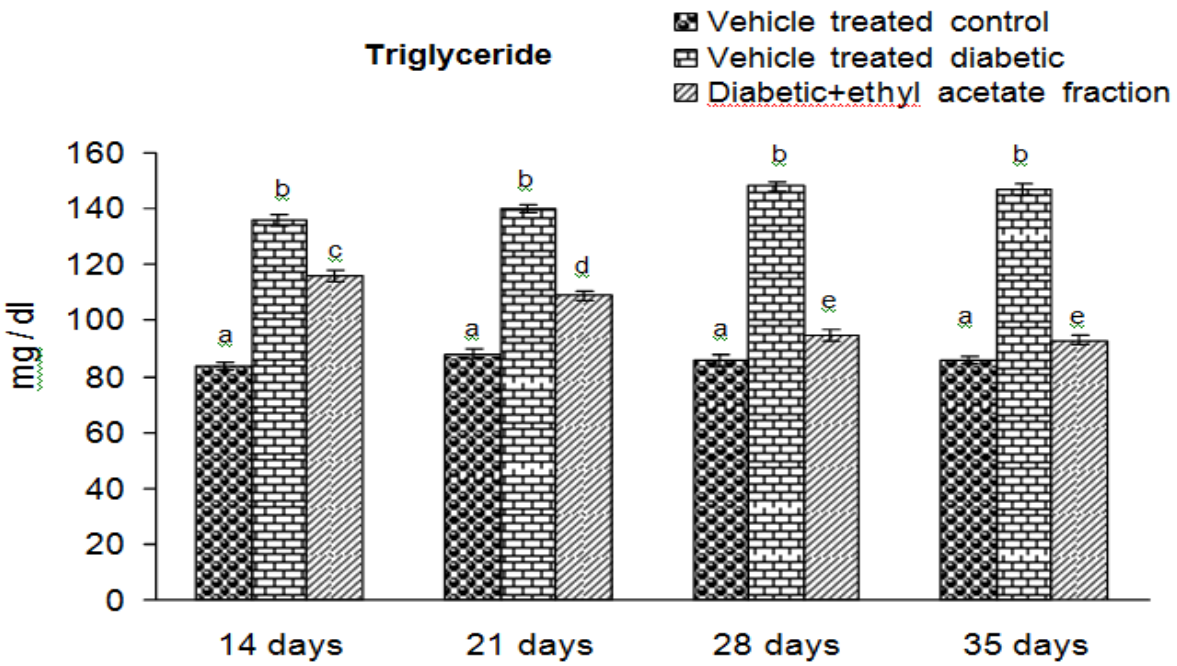


Fig 4.5 Protective effect of ethyl acetate fraction of seed of *H. antidysenterica* for different duration of treatment on serum triglyceride level in STZ-induced diabetic rat. Bars indicate Mean ± SEM (n=6). ANOVA followed by 'Multiple Comparison Two tail-t- test'. Bars with different superscripts (a, b, c, d, e) differ from each other significantly at $p<0.05$.

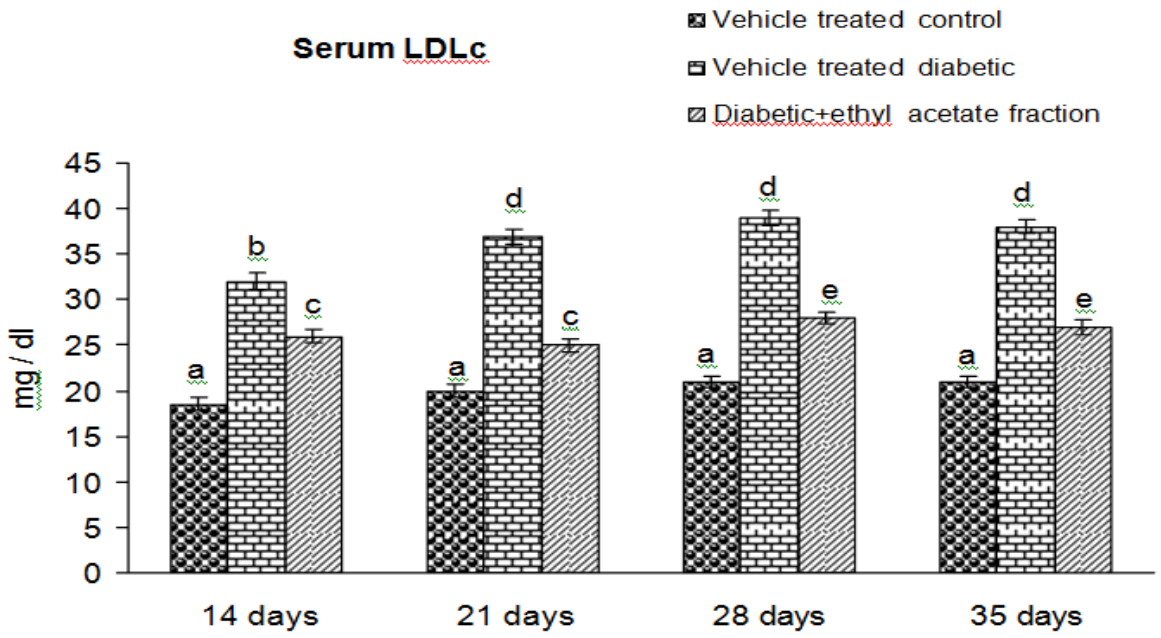


Fig 4.6 Ameliorative effect of ethyl acetate fraction of seed of *H. antidysenterica* on serum LDLc level in STZ-induced diabetic rats: Duration dependent response. Bars denote Mean ± SEM (n=6). ANOVA followed by 'Multiple Comparison Two tail-t-test'. Bar with different superscripts (a, b, c, d, e) differ from each other significantly at $p<0.05$.

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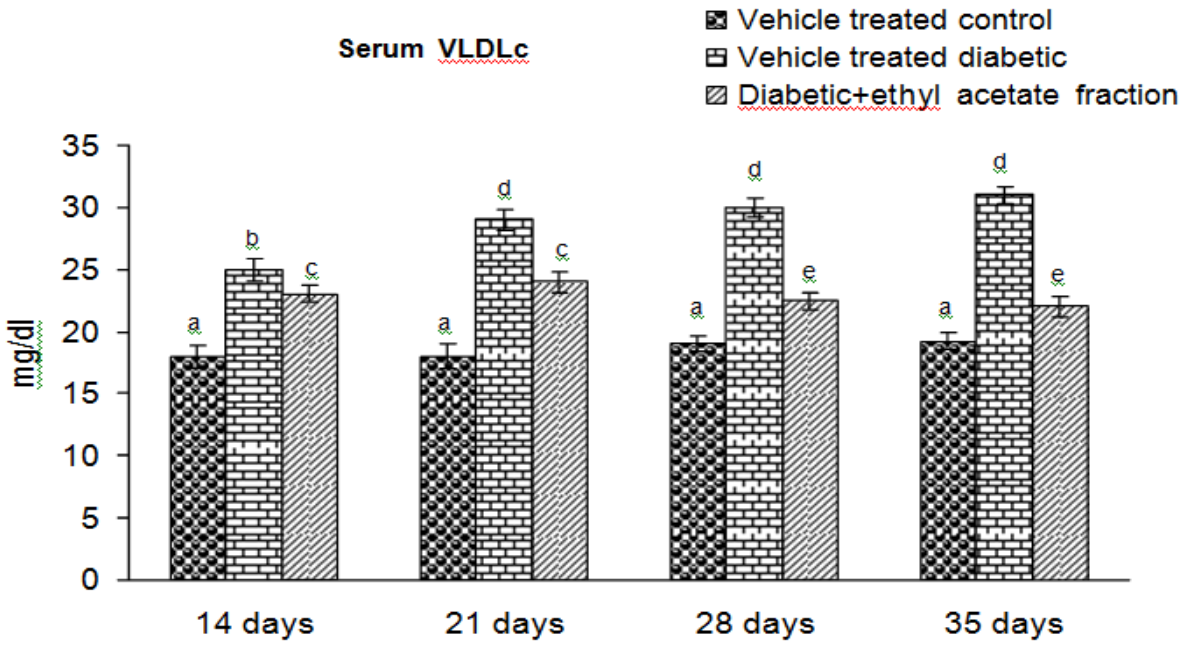

Fig 4.7 Duration dependent protective effect of ethyl acetate fraction of seed of *H. antidysenterica* on serum VLDLc level in STZ induced diabetic rat. Bars indicate Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Bar with different superscripts (a, b, c, d, e) differ from each other significantly at $p<0.05$.

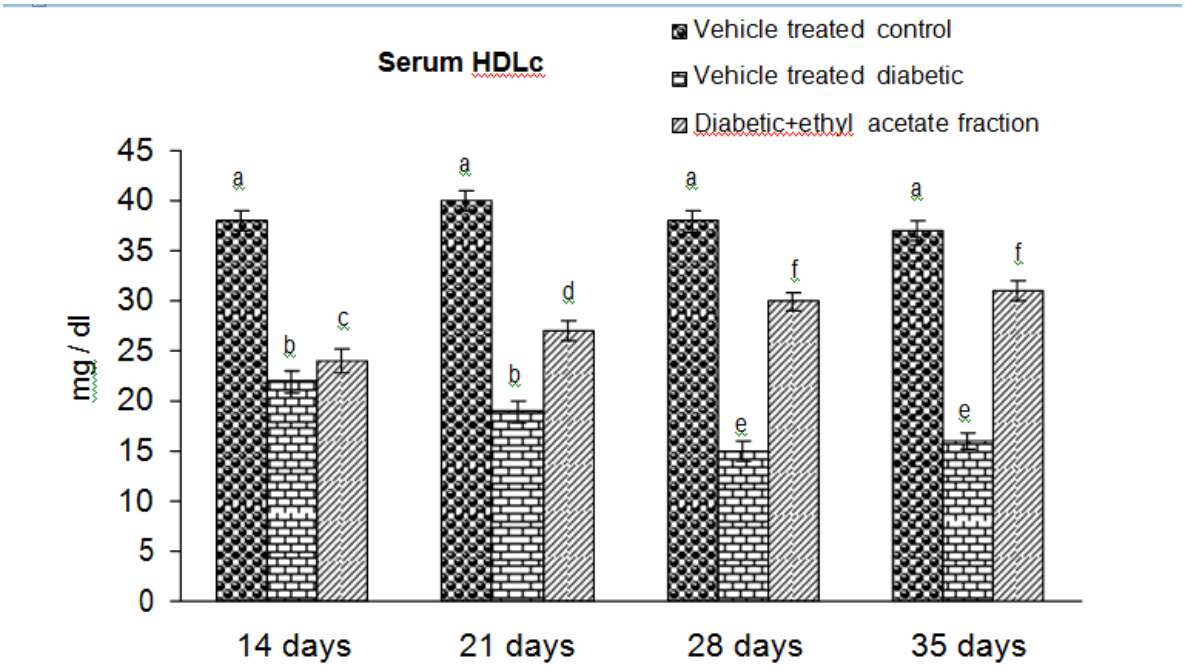

Fig 4.8 A duration dependent response in the level of serum HDLc in ethyl acetate fraction of seed of *H. antidysenterica* treated STZ-induced diabetic rat. Bars indicate Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Bars with different superscripts (a, b, c, d, e, f) differ from each other significantly at $p<0.05$.

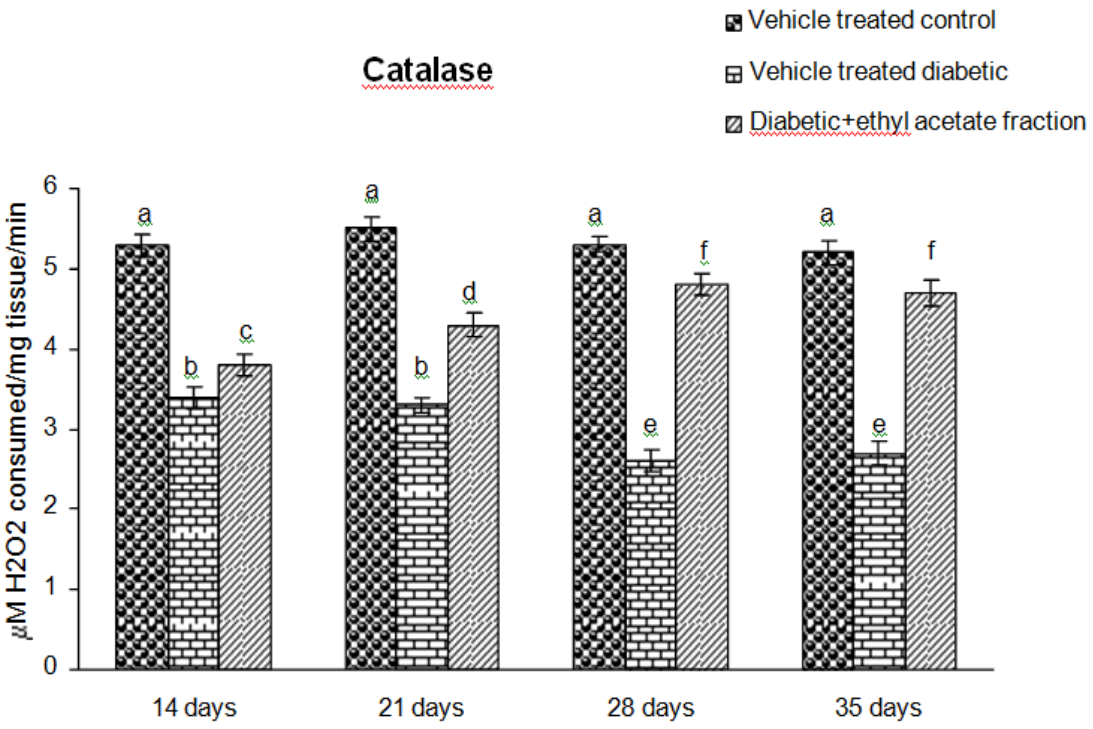

Fig 4.9 A duration dependent response in the level of serum HDLc in ethyl acetate fraction of seed of *H. antidysenterica* treated STZ-induced diabetic rat. Bars indicate Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Bars with different superscripts (a, b, c, d, e, f) differ from each other significantly at $p<0.05$.

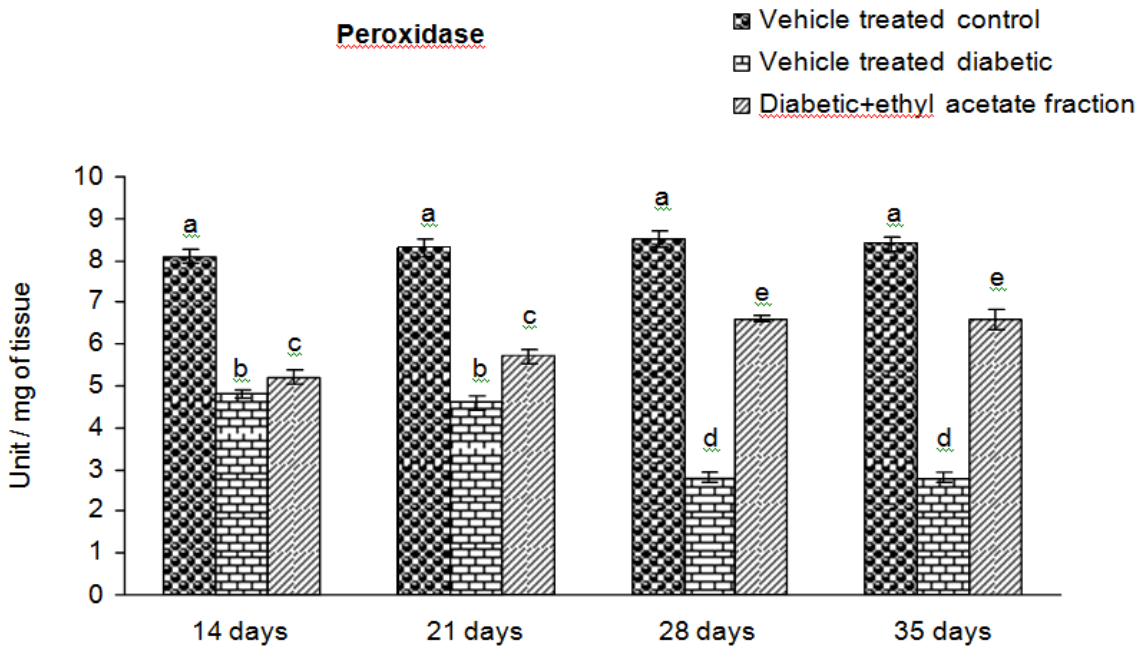

Fig 4.10 A duration dependent response in peroxidase activity in ethyl acetate fraction of seed of *H. antidysenterica* treated STZ-induced diabetic rat. Bars indicate Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Bars with different superscripts (a, b, c, d, e) differ from each other significantly at $p<0.05$.

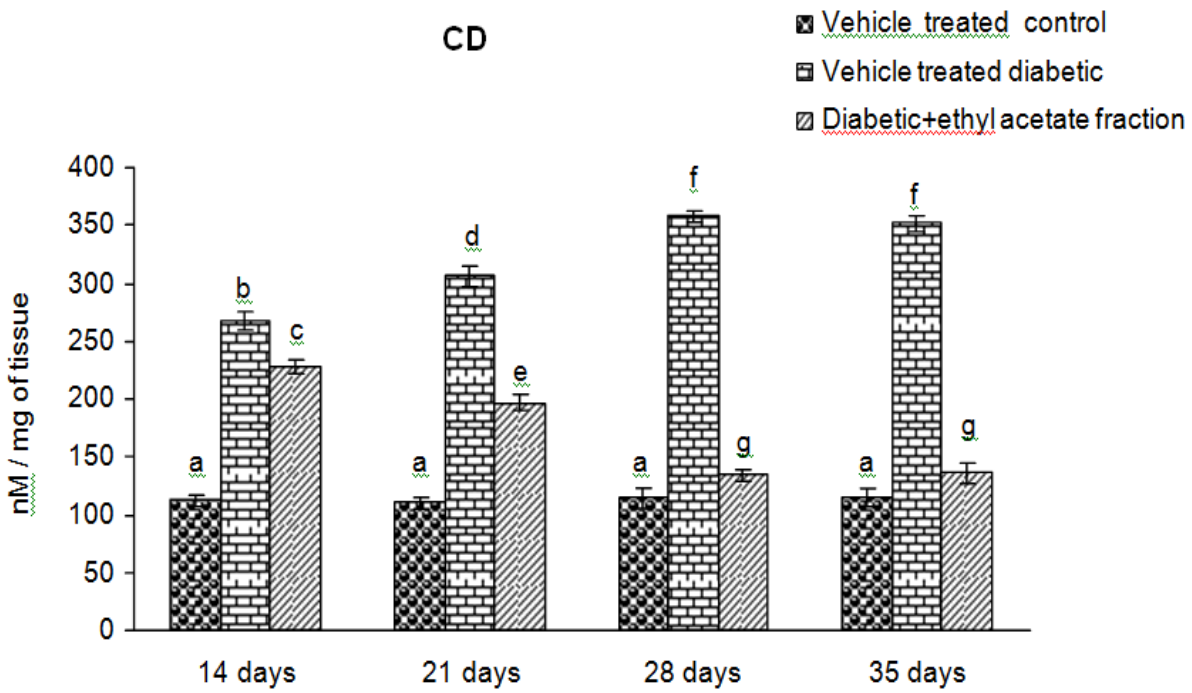

Fig 4.11 Duration dependent effect of ethyl acetate fraction of seed of *H. antidysenterica* on the levels of CD in hepatic tissue of different experimental group of animals. Bars denote Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Bar with different superscripts (a, b, c, d, e, f, g) differ from each other significantly at $p<0.05$.

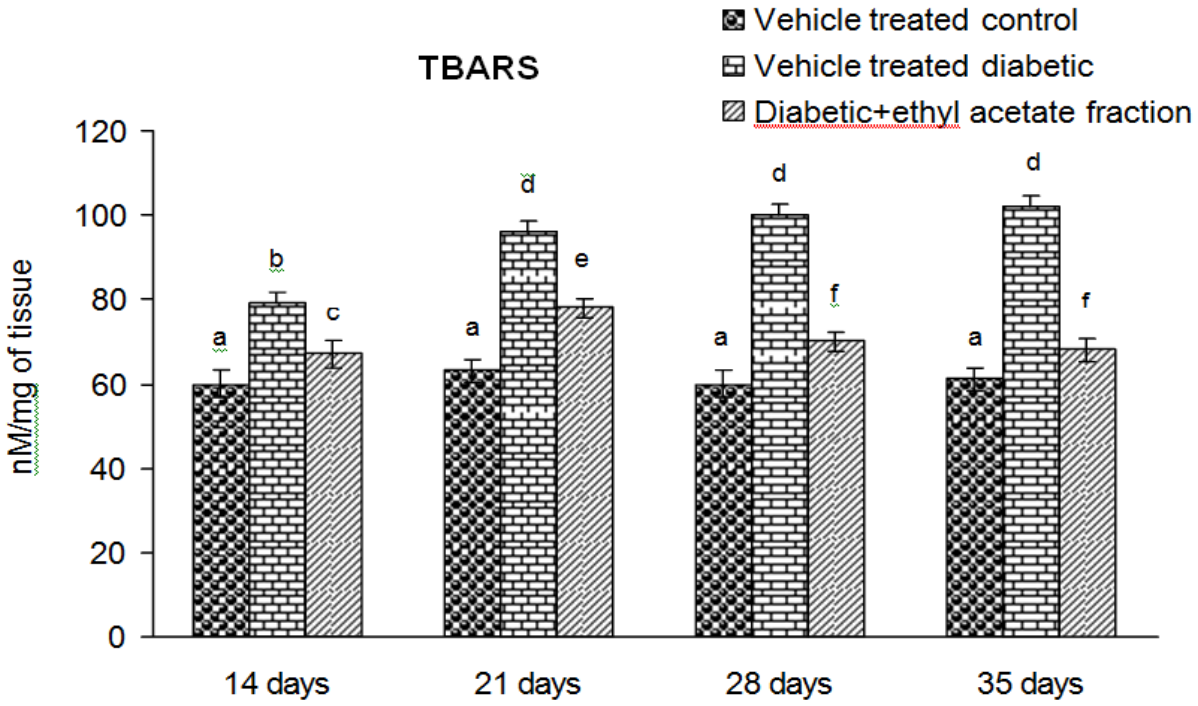

Fig 4.12 Remedial effect of ethyl acetate fraction of seed of *H. antidysenterica* on the levels of TBARS in hepatic tissue: A duration dependent response. Bars indicate Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Bar with different superscripts (a, b, c, d, e, f) differ from each other significantly at $p<0.05$.

Experiment No 05

Effect of pre-treatment followed by treatment of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *Holarrhena antidysenterica* in streptozotocin induced diabetic male albino rat

Aim of the study

This experiment has been carried out to delineate whether there is any protective effect of the pre-treatment of ethyl acetate fraction prepared from aqueous-methanol (40:60) extract of seed of *H. antidysenterica* against diabetic induction in streptozotocin treated rat as well as recovery from diabetic complication.

Experimental design

To fulfill the aim of the study, forty eight Wistar strain, healthy normoglycemic (FBG 65- 75 mg/dl) male albino rats, three months of age having body weight 150±10gm were selected. Animals were acclimated for 15 days in disease free well ventilated animal house prior to start the experiment. The animals were kept overnight fasting but allowed to free access to water. Institutional Animal Ethics Committee (IAEC) guidelines were followed throughout the experimentation. Eighteen healthy animals were made diabetic by a single intramuscular injection of STZ at the dose of 4mg / 100gm body weight as per standard method **(Mallick et al., 2007a; Ali et al., 2009a)** described in methodology section. Stable diabetic state was confirmed on 7^{th} day of STZ injection by monitoring the fasting blood glucose (FBG)level. Side by side, twelve normoglycemic rats treated with ethyl acetate fraction for previous 15 days were also induced diabetes in the same way. In case of eighteen untreated healthy animals treated with STZ, twelve animals having FBG levels 300-350mg/dl were selected for the study like previous experiments in this study. In case of fraction pre-treated normoglycemic healthy rats followed by STZ injection, rats having FBG levels 200-250 mg/dl were used in this study as the rats of this group already treated with the fraction prior STZ injection therefore the FBG levels was not developed >300mg/dl like fraction untreated rats. Out of eighteen fresh untreated rats, twelve normoglycemic healthy rats were included in vehicle treated control and fraction treated control groups.

Vehicle treated control (Group I):

Rats of this group were subjected to treatment with 0.5ml of 3% tween 80 / 100g body weight / day for 28 days at the time of fraction treatment to respective group.

Fraction treated control (Group II):

Normoglycemic healthy rats of this group were treated with ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* at the dose of 20mg/0.5 ml of 3% tween 80/ 100g body weight /day for 28 days.

Vehicle treated diabetic (Group III):

STZ induced diabetic animals of this group were treated with 0.5ml of 3% tween 80 / 100g body weight / day for 28 days.

Fraction pre-treated diabetic (Group IV):

Animals of this group were treated with ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* at the dose of 20mg / 0.5ml of 3% tween 80/ 100g body weight / day for 15 days prior to the STZ injection. Then, the animals were made diabetic by STZ injection following the standard protocol **(Mallick et al., 2007a; Ali et al., 2009a)**. The fraction pre treated diabetic animals were then subjected to treatment of vehicle i.e. 0.5ml of 3% tween 80 /100gm body weight / day for 28 days.

Fraction treated diabetic (Group V):

Diabetic animals of this group were treated with ethyl acetate fraction prepared from aqueous-methanol (40:60) extract of seed of *H. antidysenterica* at the dose of 20mg / 0.5ml of 3% tween 80 / 100gm body weight /day for 28 days.

Fraction pre-treated diabetic followed by fraction treatment (Group VI):

Fraction pre-treated diabetic animals of this group (like Gr. IV) were subjected to treatment of ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* at the dose of 20mg / 0.5 ml of 3% tween 80 / 100gm body weight /day for 28 days.

Parameters and methods

Serum insulin was measured by sandwitch ELISA kit (EZRMI-13K, Millipore, USA) for rat and the result was expressed in terms of ng/ml **(Pitchai et al., 2009)**. Activities of glucose-6-phosphatase **(Swanson, 1955)**, glucose-6-phosphate dehydrogenase **(Langdon, 1966)** and hexokinase **(Chou and Wilson, 1975)** in liver along with glycogen content in skeletal muscle were assessed **(Sadasivam and Manickam, 2008a)**. Estimation of serum levels of TC **(Allain et al., 1974)**, TG **(Desai et al., 2002)**,

LDLc **(Friedewald et al., 1972)**, VLDLc **(Friedewald et al., 1972)**, HDLc **(Waenic and Albers, 1978)**, urea **(Chaney and Marbach, 1962)** and creatinine **(Bartels et al., 1972)** were performed. Hepatic and renal tissues were used for the biochemical assay of the activities of catalase **(Beers and Sizer, 1952)** and peroxidase **(Sadasivam and Manickam, 2008b)** along with the quantification of free radical byproduct i.e. thiobarbituric acid reactive substances following the standard biochemical methods **(Okhawa et al., 1979)**.

Results

Body weight:

A significant diminution ($p<0.05$) in body weight was observed in vehicle treated diabetic (Gr. III) and fraction pre-treated diabetic (Gr. IV) groups in respect to vehicle treated control (Gr. I) but the level of diminution in body weight was significantly less ($p<0.05$) in fraction pre-treated diabetic group (Gr. IV) in compare with vehicle treated diabetic group (Gr. III). After the treatment of ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* to the diabetic animals (Gr. V) the level of this parameter was recovered significantly ($p<0.05$) to the control level. Pre-treatment of ethyl acetate fraction to diabetic animal followed by fraction treatment (Gr. VI) resulted a significant recovery ($p<0.05$) in the level of body weight in compare with vehicle treated diabetic group (Gr. III) or fraction pre-treated diabetic group (Gr. IV). No significant difference ($p<0.05$) was observed in body weight among fraction treated diabetic group (Gr. V), fraction pre-treated diabetic followed by fraction treatment group (Gr. VI), and vehicle treated control (Gr. I). Normoglycemic animal treated with ethyl acetate fraction (Gr. II) focused no significant variation in the level of this parameter in compare with vehicle treated control (Gr. I) **(Table 5.1).**

Fasting blood glucose level:

Fasting blood glucose level was increased significantly ($p<0.05$) in vehicle treated diabetic (Gr. III) and fraction pre-treated diabetic animals (Gr. IV) in respect to the vehicle treated control (Gr. I). The elevated level of fasting blood glucose was significantly higher ($p<0.05$) in vehicle treated diabetic group (Gr. III) in compare with fraction pre-treated diabetic group (Gr. IV). After treatment of ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* to the diabetic rats (Gr. V), the level of this parameter was significantly recovered ($p<0.05$) towards the control level. When rats were subjected for pre-treatment by the said fraction followed by fraction treatment after diabetes induction (Gr. VI), fasting blood glucose level was not differ significantly ($p<0.05$) with the control level (Gr. I) which indicate the protective efficacy of the fraction against STZ induced hyperglycemia. On 29^{th} day of the experiment, no significant variation in the level of the said parameter was noted between fraction pre-treated diabetic rats followed by fraction treatment after diabetes induction (Gr. VI) and vehicle treated control group (Gr. I) or fraction treated control group (Gr. III). Results of vehicle treated control rats (Gr. I) insignificantly differ when comparison was made with fraction treated control rats (Gr. II) **(Table 5.2)**.

Serum insulin level:

Serum insulin level was significantly reduced ($p<0.05$) in vehicle treated diabetic (Gr. III) and fraction pre-treated diabetic group (Gr. IV) in respect to vehicle treated control group (Gr. I). When the results of vehicle treated diabetic group (Gr. III) was compared with the fraction pre-treated diabetic group (Gr. IV), it was observed that the diminution in the serum insulin level was significantly high ($p<0.05$) in vehicle treated diabetic rats (Gr.III) in compare with fraction pre-treated diabetic rats (Gr. IV). Treatment of ethyl acetate fraction of seed of *H. antidysenterica* to diabetic animals (Gr. V) and fraction pre-treated diabetic animals (Gr. VI) resulted a significant recovery ($p<0.05$) towards control in compare with vehicle treated diabetic animals (Gr. III). When the result of fraction treated diabetic animals (Gr. V) was compared with fraction pre-treated diabetic animals followed by fraction treatment (Gr. VI), it was noted that fraction pre-treatment followed by fraction treatment after diabetes induction (Gr. VI) resulted a significant recovery ($p<0.05$) in this concern. No significant variation was observed in serum insulin level between vehicle treated control (Gr. I) and fraction pre-treated followed by fraction treatment diabetic animals (Gr. VI). There was no significant variation between the fraction treated control group (Gr. II) and vehicle treated control group (Gr. I) **(Fig. 5.1)**.

Glucose-6-phosphatase, hexokinase and glucose-6-phosphate dehydrogenase activities in liver:

There was no significant variation in the activities of these enzymes between the fraction treated control animals (Gr. II) and vehicle treated control animals (Gr. I). Hepatic glucose-6-phosphatase activity was elevated significantly ($p<0.05$) and the activities of hexokinase and glucose-6-phosphate dehydrogenase in the said tissue were decreased significantly ($p<0.05$) in vehicle treated diabetic (Gr. III) and fraction pre-treated diabetic group (Gr. IV) in compare with vehicle treated control group (Gr. I). Levels of elevation in the activity of hepatic glucose-6-phosphatase was significantly high ($p<0.05$) and activities of hexokinase and glucose-6-phosphate dehydrogenase in the said tissue were significantly reduced ($p<0.05$) in vehicle treated diabetic group (Gr. III) when comparison was made with fraction pre-treated diabetic group (Gr. IV). The activities of these enzymes in hepatic tissue were significantly recovered ($p<0.05$) towards the control level after the treatment of ethyl acetate fraction to the diabetic rats (Gr. V) as well as fraction pre-treated followed by fraction treatment to diabetic rats (Gr. VI) in respect to the vehicle treated diabetic (Gr. III) or fraction pre-treated diabetic animals (Gr. IV). From comparative analysis of the data, it was observed that the activities of the said enzymes were recovered significantly ($p<0.05$) in fraction pre-treated cum fraction treated diabetic group (Gr. VI) in compare with fraction treated diabetic group (Gr. V) **(Table 5.3)**.

Liver and skeletal muscular glycogen content:

There was no significant variation in the levels of glycogen content in liver and skeletal muscle between the fraction treated control (Gr. II) and vehicle treated control groups (Gr. I). Glycogen levels in liver and skeletal muscular tissues were significantly reduced ($p<0.05$) in vehicle treated diabetic (Gr. III) and fraction pre-treated diabetic group (Gr.III) in respect to vehicle treated control (Gr. I). Comparative analysis showed that a significant diminution ($p<0.05$) in the levels of the said biomarker

in both the tissues of vehicle treated diabetic animals (Gr. III) in respect to fraction pre-treated diabetic animals (Gr. IV). Treatment of ethyl acetate fraction prepared from aqueous-methanol (40:60) extract of seed of *H. antidysenterica* to diabetic animals (Gr. V) or fraction pre-treatment to diabetic animals followed by fraction treatment (Gr. VI) resulted a significant recovery ($p<0.05$) in the level of said biosensor when comparison was made with vehicle treated diabetic animals (Gr. III). Insignificant difference was noted in the levels of hepatic and skeletal muscular glycogen content between vehicle treated control (Gr. I) and fraction pre-treated followed by fraction treated diabetic groups (Gr. VI). No significant variation was noted in the level of this parameter between fraction treated control (Gr. II) and fraction pre-treated followed by post diabetic treated groups (Gr. VI) **(Fig. 5.2)**.

Serum lipid profile*:*

Levels of TC, TG, LDLc, VLDLc in serum were significantly elevated ($p<0.05$) and HDLc level was significantly reduced ($p<0.05$) in vehicle treated diabetic (Gr. III) and fraction pre-treated diabetic group (Gr. IV) in respect to vehicle treated control (Gr. I). The levels of serum TG, TC, LDLc and VLDLc were significantly low ($p<0.05$) and HDLc was significantly high ($p<0.05$) in fraction pre-treated diabetic group (Gr. IV) in compare with vehicle treated diabetic group (Gr. III). A significant reduction ($p<0.05$) in the levels of serum TC, TG, LDLc, VLDLc and a significant elevation ($p<0.05$) in the level of HDLc in serum were noted in ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* treated diabetic rats (Gr. V) or fraction pre-treated diabetic followed by fraction treatment diabetic groups (Group VI) in compare with vehicle treated diabetic (Gr. III) or fraction pre-treated diabetic animals (Gr. IV). When the comparison was made between fraction treated diabetic group (Gr. V) and fraction pre-treated followed by fraction treatment diabetic group (Gr. VI), it was noted that fraction pre-treated followed by fraction treated diabetic group (Gr. VI) resulted a significant recovery ($p<0.05$) than fraction treated diabetic group (Gr. V). There was insignificant difference in the levels of these parameters between fraction pre-treated followed by fraction treated diabetic group (Gr. VI) and vehicle treated control group (Gr. I). Normoglycemic animals treated with the said fraction (Gr. II) showed insignificant variation in the levels of these parameters in comparison with vehicle treated control group (Gr. I) **(Table 5.4)**.

Activities of catalase and peroxidase in liver and kidney tissues:

Activities of antioxidant enzymes i.e. catalase and peroxidase in hepatic and renal tissues were decreased significantly ($p<0.05$) in vehicle treated diabetic rats (Gr. III) and fraction pre-treated diabetic animals (Gr. IV) in compare with vehicle treated control (Gr. I). Results also focused a significant diminution ($p<0.05$) in the activities of these enzymes in vehicle treated diabetic group (Gr. III) in compare with fraction pre-treated diabetic group (Gr. IV). After the treatment of ethyl acetate fraction of the said plant part to diabetic animals (Gr. V) or fraction pre-treated followed by fraction treated diabetic animals (Gr. VI), a significant increase ($p<0.05$) in the activities of these enzymes were noted in compare with vehicle treated diabetic group (Gr. III). Fraction pre-treated followed by fraction treated diabetic (Gr. VI) showed a significant recovery ($p<0.05$) in this concern in respect to fraction treated diabetic group (Gr. V). No significant variation was noted when comparison was made between fraction

pre-treated followed by fraction treated diabetic group (Gr. VI) and vehicle treated control group (Gr. I). Comparative analysis of the data between fractions treated control (Gr. II) and vehicle treated control groups (Gr. I) showed an insignificant variation in the activities of these enzymes **(Fig. 5.3 and 5.4)**.

Levels of TBARS:

An insignificant variation was observed in the levels of TBARS in hepatic and renal tissues when the result of vehicle treated control group (Gr. I) was compared with fraction treated normoglycemic animals (Gr. II). Levels of TBARS in hepatic and renal tissues were increased significantly ($p<0.05$) in vehicle treated diabetic (Gr. III) and fraction pre-treated diabetic animals (Gr. IV) in compare with vehicle treated control (Gr. I). Levels in the elevation of TBARS both in hepatic and renal tissues was significantly high ($p<0.05$) in vehicle treated diabetic group (Gr. III) in respect to fraction pre-treated diabetic group (Gr. IV). After the treatment of ethyl acetate fraction for 28 days to STZ induced diabetic animals (Gr. V) or fraction pre-treated diabetic animals (Gr. VI), a significant diminution ($p<0.05$) in the level of this biomarker towards control was observed when comparison was made with vehicle treated diabetic group (Gr. III). Fraction pre-treated followed by fraction treated diabetic group (Gr. VI) resulted a significant recovery ($p<0.05$) in this concern in compare with fraction treated diabetic group (Gr. V). No significant difference was observed in this aspect between fraction pre- treated followed by fraction treated diabetic group (Gr. VI) and vehicle treated control (Gr. I) **(Fig. 5.5)**.

Serum urea, uric acid and creatinine:

Levels of serum urea, creatinine and uric acid were not significantly altered ($p<0.05$) in fraction treated control (Gr. II) in compare with vehicle treated control group (Gr. II). Serum urea and creatinine levels were significantly elevated ($p<0.05$) and uric acid was significantly reduced ($p<0.05$) in vehicle treated diabetic (Gr. III) and fraction pre-treated diabetic groups (Gr. IV) in respect to vehicle treated control (Gr. I). Comparative analysis focused that the urea and creatinine levels in serum were significantly low ($p<0.05$) and uric acid was significantly high ($p<0.05$) in vehicle treated diabetic animals (Gr. III) in compare with fraction pre-treated diabetic group (Gr. IV). After the treatment of ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* to diabetic animals (Gr. V) or fraction pre-treated cum fraction treated diabetic animals (Gr. VI), the levels of these parameters were significantly recovered ($p<0.05$) towards control level in respect to the vehicle treated diabetic (Gr. III) or fraction pre-treated diabetic animals (Gr. IV). Comparative analysis showed that no significant variation in the levels of these parameters between fraction pre-treated followed by fraction treated diabetic group (Gr. VI) and vehicle treated control group (Gr. I) **(Table 5.5)**.

Discussion

Present experiment was conducted to find out whether the fraction has any activity to protect the STZ-induced diabetic complications, preconditioning of the rat by this fraction treatment followed by diabetes induction by injection of STZ was considered in the present study. Therefore, the effect of ethyl acetate fraction on diabetic rats as well as fraction pre-treated diabetic rats were studied here and the results were compared between fraction treated diabetic rats and fraction pre-treated diabetic cum fraction treated diabetic rats.

Carbohydrate metabolic enzyme i.e. glucose-6-phosphatase activity in liver was increased along with diminution in the activities of hepatic glucose-6-phosphate dehydrogenase and hexokinase in STZ induced vehicle treated diabetic rat. This may be due to low plasma level of insulin as the enzymes hexokinase and glucose-6-phosphate dehydrogenases are regulated by insulin in positive way and glucose-6-phosphatase in negative way **(Mitra et al., 1996; Prince et al., 1998**). The results focused that there was a significant protection of these parameters in fraction pre-treated diabetic group in compare with vehicle treated diabetic group. As, the above said enzymes are under the positive control of insulin so it may be predicted that pre-treatment of the rat by ethyl acetate fraction followed by diabetic induction can able to resists at a significant level in variation in the activities of above said enzymes by protecting the β-cell damage partially after STZ injection and thereby recovered serum insulin level which was supported by previous findings in this line using other plant products **(Mallick et al., 2009; Sellamuthu et al., 2009; Bera et al., 2011)**.

Antidiabetic efficacy of the ethyl acetate fraction has been further supported here from the recovery of glycogen levels in liver and in skeletal muscular tissue which are under positive control of insulin **(Shih et al., 1997)**. Results of fasting blood glucose level which was significantly recovered in fraction treated diabetic animals and fraction pre- treated followed by fraction treated diabetic animals also proposed that the phytomolecule present in this fraction can able to resists the β-cell degeneration and or regenerate the β-cell. This protective nature of the phytomolecule present in the ethyl acetate fraction was supported by our previous publication **(Panda et al., 2009)**.

Diabetes also develops oxidative stress in different organs with special reference to metabolic organs that was established by our previous works **(Mallick et al., 2007b)**. Activities of catalase and peroxidase in liver and kidney tissues were decreased in diabetic condition and were increased significantly by this fraction treatment. This supports the antioxidant activities that may protects the catalytic protein in cells from diabetes induced oxidative cell injury **(Prakasam et al., 2003; El-Beshbishy et al., 2006)**. Pre-treatment of ethyl acetate fraction followed by diabetes induction can able to protect the activities of these enzymes significantly in hepatic and renal tissues in respect to vehicle treated diabetic group which further suggest that the oxidative stress protection by ethyl acetate fraction, may be due to the recovery of FBG level as hyperglycemia is one of the important factor for oxidative stress imposition in diabetes **(Kuyvenhoven and Meinders, 1999)**. This antioxidative activity has been strengthen here from the recovery of TBARS levels in above said tissues as there is an inverse relationship between the activities of antioxidant enzymes and quantity of free radical byproduct i.e. TBARS, as proposed by other **(Pari and Saravanan., 2002)** as well as by our previous work **(Mallick et al., 2007b)**. Hyperlipidemia is one of the consequences of metabolic disorders in diabetes **(Ali et al., 2009b)**. In diabetic condition the alteration in the levels of serum lipid profile biosensors like total TC, TG, HDLc, VLDLc and LDLc were noted in our previous experiments

(Experiment No. III and V) as well as our previous publication in this line **(Mallick et al., 2007b; Ali et al., 2009b; De et al., 2010)**. Pre-treated animal by ethyl acetate fraction followed by experimental diabetes induction by STZ can able to resists the alteration in the level of these parameters partially in respect to vehicle treated diabetic animals but at significant level. This result was further supported from the significant recovery of these parameters in fraction pre-treated cum fraction treated rats in compare with fraction treated rats. As lipid metabolism is also regulated by insulin **(Gupta et al., 2005; Ali et al., 2009a)** so the protection in serum lipid profile level by pre-treatment of ethyl acetate fraction may be due to the recovery of serum insulin level which is in parallel with other report **(Mandal et al., 2008)**. This nature of recovery in serum lipid profile level in diabetic condition was supported by our previous publications **(Chatterjee et al., 2009; Ali et al., 2009a)**. In diabetes serum uric acid has been decreased significantly as uric acid is also used as free radical scavenger and serum urea and creatinine were increased significantly **(Mallick et al., 2009)**. Preconditioning of animals by ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* followed by diabetes induction can able to protect these parameters at significant level in compare with non preconditioned diabetic animals which further support the protective efficacy of the phytomolecule(s) present in ethyl acetate fraction from diabetic induced protein metabolic disorders and the results were supported with published report in this line **(De et al., 2010)**. Regarding the mechanism of action of ethyl acetate fraction of seed of *H. antidysenterica* for the management of diabetes following two hypotheses may be postulated. **One hypothesis for the correction of diabetic disorders is insulinotrophic effects of this fraction by regeneration of beta-cells which has been supported here by the significant recovery of serum insulin. This is supported by other report as investigated by our previous work using other plant (Mallick et al., 2006). Another possibility is the diabetes induced oxidative stress correction by antioxidative potency of the ingredients present in the fraction like other phytochemicals investigated by us (Maiti et al., 2004) and also by others (Borchers et al., 1999; Shao et al., 2004)**. Pre- treatment of the said fraction before diabetes induction by STZ followed by treatment of the fraction to STZ-induced diabetic rat resulted maximum levels of recovery in all the sensors in respect to other groups. This may be due to protection of pancreatic beta-cells from STZ induced injury as well as stimulation of insulin synthesis from existing beta-cells by treatment of the fraction in diabetic animals. So the phytomolecules present in the fraction may have some beta-cell protective effect as well as beta-cell stimulating and regenerative effect.

Conclusion

The results of this experiment indicated that pre-treatment of ethyl acetate fraction followed by diabetes induction in animal can able to minimize the diabetic complications. So, the study highlighted that pre-treatment of ethyl acetate fraction is more effective to prevent the diabetes in compare with non pretreated diabetic animal.

Table 5.1 Effect of treatment of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* in different conditions on body weight in STZ-induced diabetic rat.

Group	Body weight (gm)	
	Initial	Final
Vehicle treated control (Group I)	124.53±3.2[a]	143.42±4.3[a]
Fraction treated control (Group II)	121.65±4.6[a]	141.87±3.6[a]
Vehicle treated diabetic (Group III)	127.62±4.9[a]	120.65±6.1[b]
Fraction pre-treated diabetic (Group IV)	126.14±3.1[a]	133.24±3.3[c]
Fraction treated diabetic (Group V)	122.27±3.4[a]	144.37±4.1[a]
Fraction pre-treated followed by fraction treated diabetic (Group VI)	122.26±2.7[a]	145.35±4.4[a]

Data indicate Mean ±SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Values with different superscripts (a, b, c) in each vertical column significantly differ from each other at $p<0.05$.

Table 5.2 Protective effect of ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* on blood glucose level in STZ-induced diabetic male albino rat: A comparative study between pre-treatment followed by fraction treatment in diabetic rat with other conditioned based fraction treated diabetic group.

Group	Fasting blood glucose (mg/dl)				
	1st day (7th day of STZ injection)	8th day	15th day	22nd day	29th day
Vehicle treated control (Group I)	74.3 ± 4.5[a]	73.7 ± 5.7[a]	76.27 ± 5.2[a]	71.31 ± 4.3[a]	74.34 ± 4.8[a]
Fraction treated control (Group II)	71.3 ± 3.6[a]	75.54 ± 4.6[a]	74.34 ± 4.3[a]	70.41 ± 3.7[a]	75.43 ± 4.2[a]
Vehicle treated diabetic (Group III)	313.5 ± 1.7[b]	292.3 ± 5.5[b]	304.1 ± 1.8[b]	309.2 ± 3.6[b]	311.1 ± 1.6[b]
Fraction pre-treated diabetic (Group IV)	221.3 ± 4.8[c]	218.2 ± 4.8[c]	209.3 ± 5.1[c]	211.5 ± 4.7[c]	214.2 ± 4.6[c]
Fraction treated diabetic (Group V)	311.8 ± 4.8[b]	261.4 ± 4.7[d]	152.2 ± 4.6[d]	123.5 ± 5.2[d]	97.44 ± 5.3[d]
Fraction pre treated followed by fraction treated diabetic (Group VI)	221.6 ± 1.5[c]	179.6 ± 5.3[e]	128.5 ± 1.6[e]	91.35 ± 5.2[e]	71.56 ± 1.7[a]

Data indicates Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Values with different superscripts (a, b, c, d, e) in each vertical column significantly differ from each other at $p<0.05$.

Table 5.3 Activities of hepatic glucose-6-phosphatase, hexokinase and glucose-6-phosphate dehydrogenase and its correction by the treatment of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* in different experimental conditions in rat

Group	Glucose-6-phosphatase (mg of IP/gm of tissue)	Hexokinase (µg/gm of tissue)	Glucose-6-phosphate dehydrogenase (unit/gm of tissue)
Vehicle treated control (Group I)	23.58 ± 1.3^{a}	142.24 ± 3.1^{a}	14.52 ± 0.5^{a}
Fraction treated control (Group II)	21.5 ± 1.2^{a}	138.87 ± 2.7^{a}	15.73 ± 0.6^{a}
Vehicle treated diabetic (Group III)	40.72 ± 1.4^{b}	76.57 ± 2.4^{b}	5.1 ± 0.3^{b}
Fraction pre-treated diabetic (Group IV)	33.41 ± 2.1^{c}	98.31 ± 3.4^{c}	8.1 ± 0.7^{c}
Fraction treated diabetic (Group V)	28.35 ± 1.3^{d}	114.56 ± 3.2^{d}	11.72 ± 0.8^{d}
Fraction pre-treated followed by fraction treated diabetic (Group VI)	21.24 ± 1.8^{a}	141.37 ± 3.7^{a}	14.23 ± 0.8^{a}

Data denote Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Values with different superscripts (a, b, c, d) in each vertical column significantly differ from each other at $p<0.05$.

Table 5.4 Different conditions based treatment for remedial effects of ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* on serum lipid profile level in STZ-induced diabetic male albino rat.

Groups	Serum lipid profile (mg/dl)				
	TC	TG	LDLc	VLDLc	HDLc
Vehicle treated control (Group I)	78.55 ± 3.2^{a}	87.53 ± 4.4^{a}	59.4 ± 1.7^{a}	18.65 ± 1.1^{a}	60.23 ± 1.6^{a}
Fraction treated control (Group II)	76.44 ± 3.7^{a}	88.21 ± 3.9^{a}	61.5 ± 1.8^{a}	19.78 ± 1.2^{a}	62.58 ± 1.2^{a}
Vehicle treated diabetic (Group III)	152.5 ± 3.7^{b}	218.61 ± 4.3^{b}	114.5 ± 2.1^{b}	63.64 ± 1.2^{b}	24.46 ± 1.1^{b}
Fraction pre-treated diabetic (Group IV)	136.6 ± 3.9^{c}	187.72 ± 3.8^{c}	97.5 ± 1.6^{c}	49.36 ± 1.2^{c}	37.56 ± 1.8^{c}
Fraction treated diabetic (Group V)	98.53 ± 3.8^{d}	105.56 ± 3.8^{d}	73.43 ± 1.4^{d}	28.13 ± 1.2^{d}	47.54 ± 1.4^{d}
Fraction pre-treated followed by fraction treated diabetic (Group VI)	80.42 ± 3.2^{a}	90.52 ± 4.8^{a}	61.7 ± 1.8^{a}	20.53 ± 1.2^{a}	59.87 ± 1.5^{a}

Data represents Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Values with different superscripts (a, b, c, d) in each vertical column significantly differ from each other at $p<0.05$.

Table 5.5 Protective effect of time dependent different treatment schedule of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* on serum urea, uric acid and creatinine levels in STZ-induced diabetic rat.

Group	Urea (mg/dl)	Creatinine (mg/dl)	Uric acid (mg/dl)
Vehicle treated control (Group I)	39.1±1.18^{a}	0.69±0.04^{a}	6.12±0.27^{a}
Fraction treated control (Group II)	41.4±1.12^{a}	0.67±0.3^{a}	5.76±0.22^{a}
Vehicle treated diabetic (Group III)	66.36±1.08^{b}	2.32±0.10^{b}	2.87±0.19^{b}
Fraction pre-treated diabetic (Group IV)	55.6±1.05^{c}	1.78±0.03^{c}	3.41±0.22^{c}
Fraction treated diabetic (Group V)	48.2±1.11^{d}	1.10±0.04^{d}	4.12±0.27^{d}
Fraction pre-treated followed by fraction treated diabetic (Group VI)	40.4±1.07^{a}	0.71±0.02^{a}	5.86±0.27^{a}

Data indicates Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Values with different superscripts (a, b, c, d) in each vertical column significantly differ from each other at $p<0.05$.

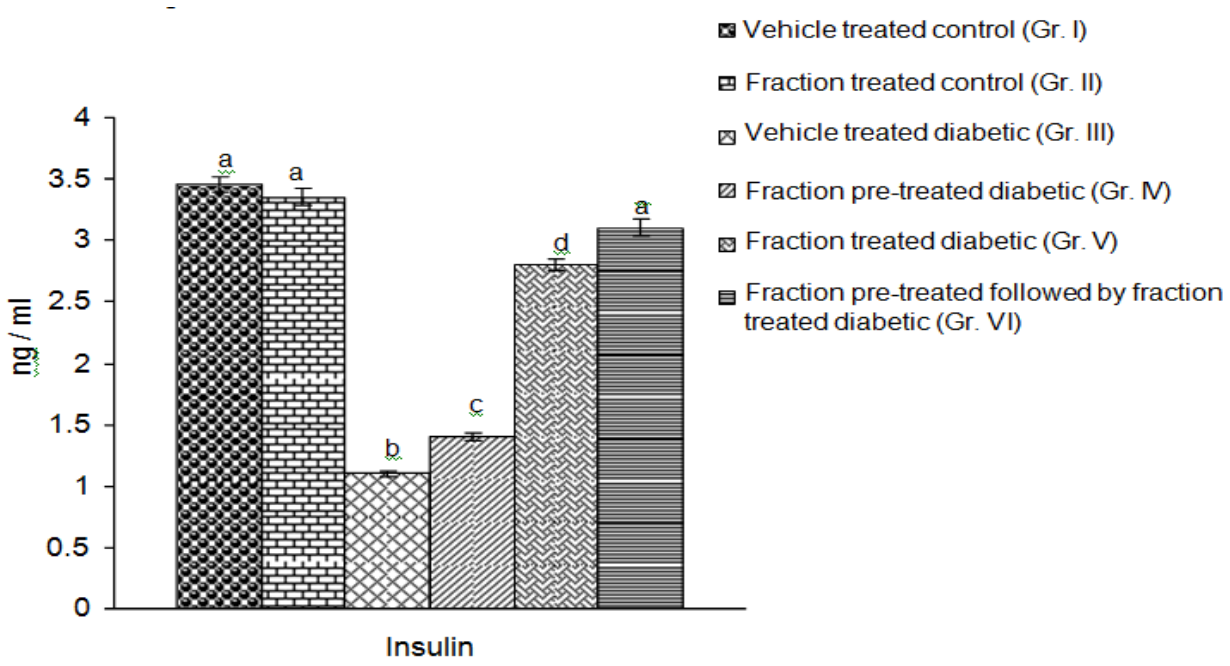

Figure 5.1 Change in serum insulin level after the treatment of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* in STZ-induced diabetic male albino rat in different experimental conditions. Bars denote Mean±SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Bars with different superscripts (a, b, c, d) significantly differ from each other at $p<0.05$.

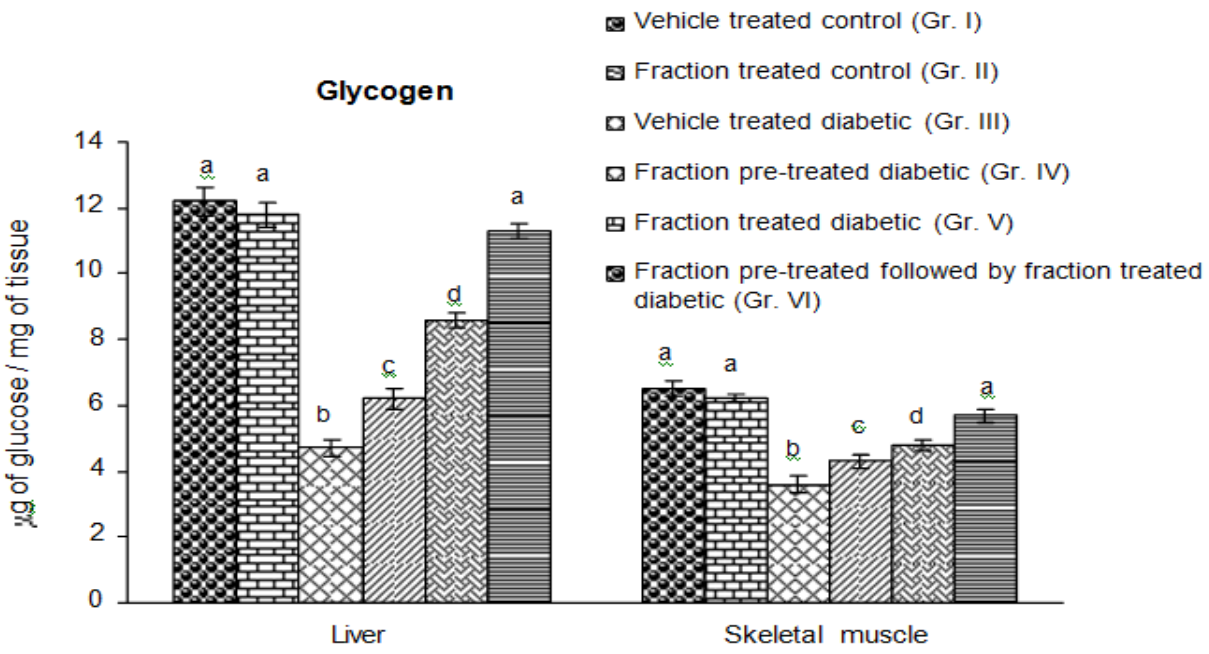

Figure 5.2 Hepatic and skeletal muscular glycogen content in ethyl acetate fraction of aqueous- methanol :: 40:60 extract of seed of *H. antidysenterica* treated diabetic and fraction pre-treated cum post diabetes fraction treated male albino rat. Bars denote Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Bars with different superscripts (a, b, c, d) significantly differ from each other at $p<0.05$.

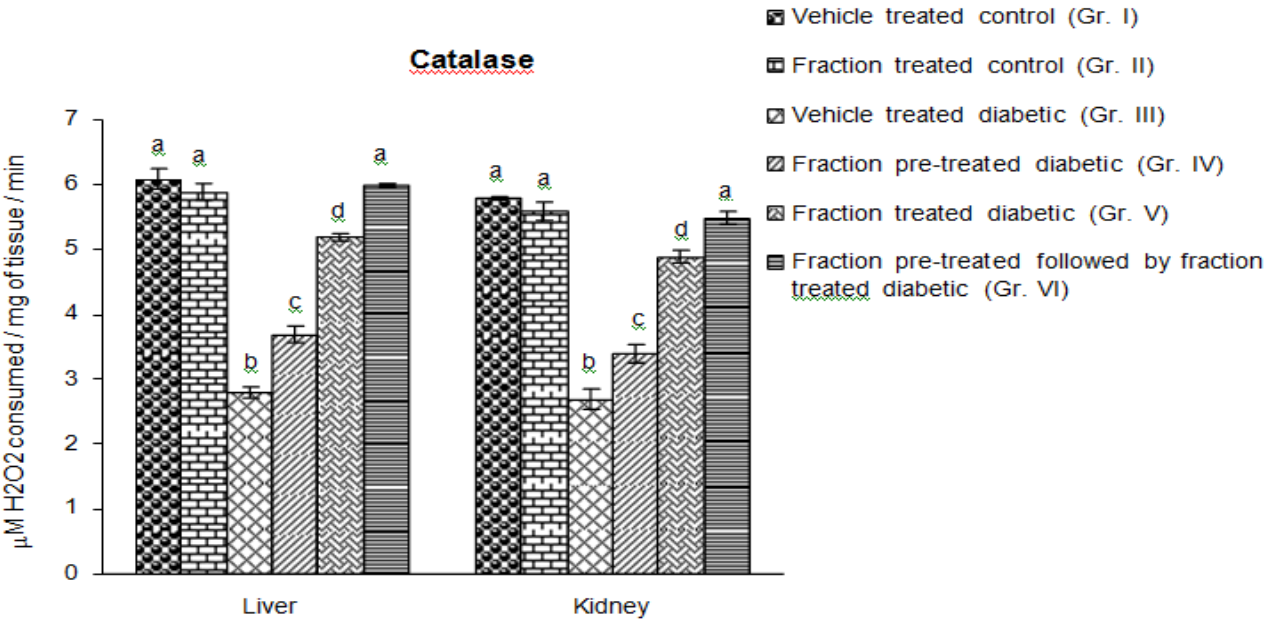

Figure 5.3 Effect of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* on the activities of catalase in hepatic and renal tissues of diabetic male albino rat in different treatment period of the fraction. Bars denote Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Bars with different superscripts (a, b, c, d) significantly differ from each other at $p<0.05$.

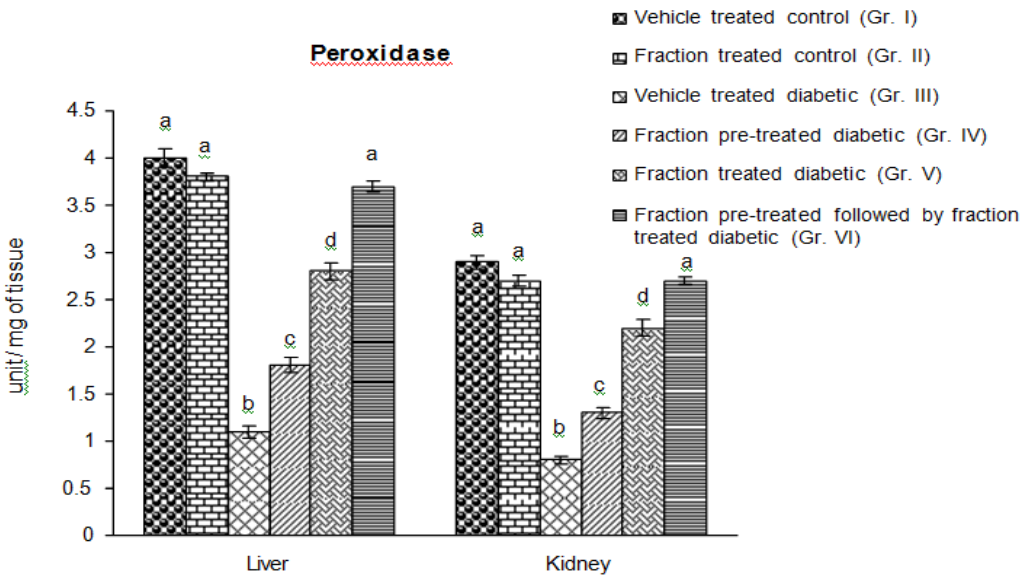

Figure 5.4 Remedial effect of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* on peroxidase activities in hepatic and renal tissues of diabetic and fraction pre-treated cum fraction treated diabetic animals. Bars indicate Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Bars with different superscripts (a, b, c, d) significantly differ from each other at $p<0.05$.

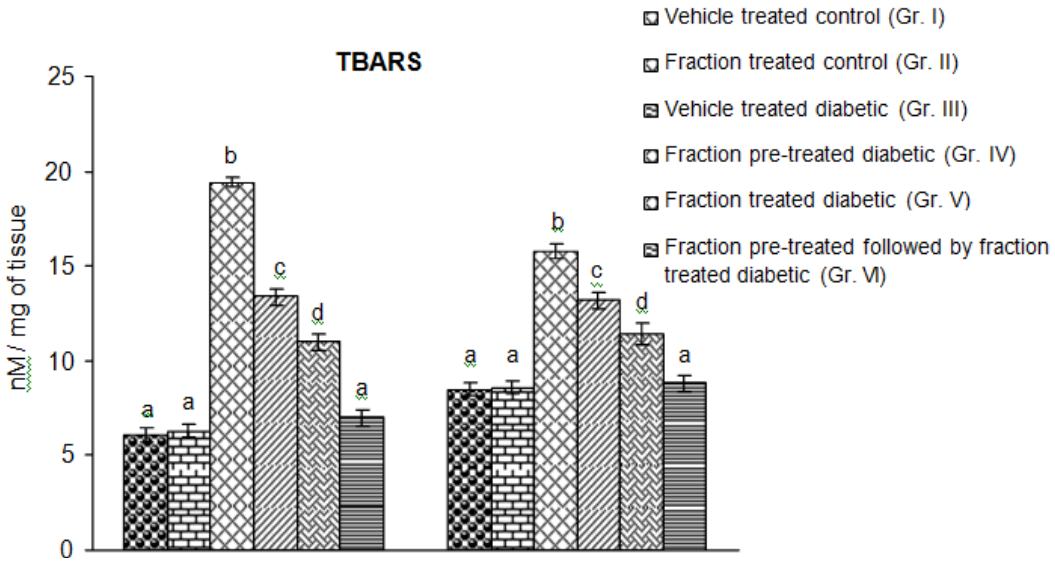

Figure 5.5 Protective effect of the treatment of ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* in different time period on TBARS levels in liver and kidney tissues of STZ-induced diabetic animals. Bars denote Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Bars with different superscripts (a, b, c, d) differ significantly from each other at $p<0.05$.

Experiment No 06

Effect of ethyl acetate fraction prepared from aqueous-methanol :: 40:60 extract of seed of *Holarrhena antidysenterica* in streptozotocin-induced diabetic male albino rat: A Duration dependent withdrawal study

Aim of the study

This study has been conducted to search out the reversible or irreversible nature of recovery from STZ induced diabetic complication in rat by the treatment of ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica*.

Experimental design

To fulfill the aim of this study, sixty six normoglycemic, healthy Wistar strain male albino rats having 3 months of age and body weight 150 ± 10 gm were selected. Six animals were housed in each clean and dry polypropylene made cage and acclimated for 15 days in our well ventilated light controlled (12h L: 12h D) animal house prior to start the experiment. Research on animal model was conducted according to the instructions of our Institutional Animal Ethics Committee (IAEC). Standard environment in the animal house was maintained with room temperature 25±2^{o}C and relative humidity 45%-60%. Animals have free access to standard feed and drinking water adequately. Forty eight overnight fasted animals were subjected to a single intramuscular injection of STZ in 0.1 M citrate buffer at the dose of 4 mg/100 gm body weight as per standard method **(Mallick et al., 2007a)** that produce diabetes having fasting blood glucose level 300-350 mg/dl after 48h of STZ injection. The animals were observed up to 7th day in post injection period to confirm the stability of diabetic state considering the blood glucose level as one of the biomarkers. Thirty six animals stable in diabetic state at our desired level were selected for this study out of forty eight STZ treated animals and rest mild diabetic rats were not considered for this experiment. Eighteen normoglycemic healthy animals having FBG level 65–75 mg / dl were used in this experiment as vehicle treated control in different duration of withdrawal schedule.

Finally the rats were divided into nine groups having six animals in each group –

A. 14 days withdrawal schedule

Vehicle treated control (Group I):

Normoglycemic animals of this group were subjected to forceful feeding of 0.5ml 3% tween

80/100gm of body weight / day for 28 days followed by withdrawal of vehicle treatment for the next 14 days.

Vehicle treated diabetic (Group II):

Diabetic animals were subjected to oral intubations of 0.5ml of 3% tween 80 / 100gm of body weight/day for 28 days. These animals kept without vehicle treatment for next 14 days like control group.

Fraction treated diabetic followed by 14 days withdrawal (Group III):

Diabetic animals of this group were subjected to treatment of ethyl acetate fraction prepared from aqueous-methanol (40:60) extract of seed of *H. antiidysenterica* at the dose of 20mg / 0.5ml 3% tween 80 / 100gm body weight / day for 28 days by gavage method followed by cessation of the said fraction treatment for next 14 days.

B. 28 days withdrawal schedule

Vehicle treated control (Group I):

Healthy normoglycemic animals were subjected to feeding of 0.5ml of 3% tween 80/ 100gm body weight / day for 28 days using feeding canula followed by withdrawal of vehicle treatment for the next 28 days.

Vehicle treated diabetic (Group II):

Streptozotocin induced diabetic animals were subjected to oral intubations of 0.5ml 3% tween 80/100gm body weight / day for 28 days followed by cessation of the treatment for next 28 days.

Fraction treated diabetic followed by 28 days withdrawal (Group III):

Six diabetic animals of this group were subjected to treatment of ethyl acetate fraction of seed of *H. antidysenterica* at the dose of 20mg / 0.5ml 3% tween 80 / 100gm body weight/day for 28 days through oral route followed by withdrawal of the said fraction for next 28 days.

C. 56 days withdrawal schedule

Vehicle treated control (Group I):

Six normoglycemic animals were subjected to administration of 0.5ml 3% tween 80 / 100gm of body weight / day for 28 days followed by withdrawal of vehicle treatment for the next 56 days.

Vehicle treated diabetic (Group II):

Diabetic animals were subjected to treatment of 0.5ml of 3% tween 80 / 100gm body weight / day for 28 days orally using feeding canula followed by withdrawal of vehicle treatment for next 56 days.

Fraction treated diabetic followed by 56 days withdrawal (Group III):

Diabetic animals of this group were subjected to treatment of ethyl acetate fraction prepared from aqueous-methanol (40:60) extract of seed of *H. antidysenterica* at the dose of 20mg / 0.5ml 3% tween 80 / 100gm body weight / day for 28 days through oral route followed by withdrawal of said fraction treatment for next 56 days.

After completion of 28 days of fraction treatment followed by 14 days or 28 days or 56 days of withdrawal of 3% tween 80 or ethyl acetate fraction prepared from aqueous- methanol (40:60) extract of seed of *H. antidysenterica* in respective group, all the animals were sacrificed at fasting state by light ether anesthesia. Blood was collected from dorsal aorta by sterilized syringe and serum was separated by centrifugation for the measurement of insulin level. Liver, kidney and skeletal muscular (Gastrocnemius) tissues were dissected out and stored at -20^{o}C for the assessment of activities of carbohydrate metabolic enzymes, antioxidant enzymes, quantification of glycogen content and free radical byproducts in respective tissue sample.

Parameters and Methods

Fasting blood glucose level was measured by single touch glucometer (Chatterjee et al., 2009). Serum insulin level was measured using sandwich ELISA kit **(Pitchai et al., 1988)**. Activities of glucose-6-phosphatase **(Swanson, 1955**), glucose-6-phosphate dehydrogenase **(Langdon, 1966)** and hexokinase **(Chou and Wilson, 1966)** in liver along with glycogen contents **(Sadasivam and Manickam, 2008a)** in liver and skeletal muscular tissue were measured biochemically. Assessment of antioxidant enzymes catalase **(Beers and Sizer, 1952)** and peroxidase **(Sadasivam and Manickam, 2008b**) activities as well as quantification of free radical byproducts from the concentration of conjugated diene **(Slater, 1984)** and thiobarbituric acid reactive substances **(Okhawa et al., 1979)** were assessed in hepatic tissue and in renal tissue.

Results

Fasting blood glucose level:

A significant ($p<0.05$) elevation in the level of fasting blood glucose was observed in the diabetic groups of different experimental schedules in compare with the respective vehicle treated control group. The levels of elevation was significantly high ($p<0.05$) in vehicle treated diabetic group of 28 days and 56 days withdrawal schedule in compare with 14 days withdrawal schedule. Treatment of ethyl acetate fraction prepared from aqueous-methanol (40:60) extract of seed of *H. antidysenterica* for 28 days followed by withdrawal of the said fraction treatment for 14 days or 28 days or 56 days resulted a significant recovery ($p<0.05$) in fasting blood glucose level towards control in respect to vehicle treated diabetic group of respective experimental schedule. There was no significant variation ($p<0.05$) in the level of fasting blood glucose level among 14 days or 28 days or 56 days withdrawal groups after the completion of ethyl acetate fraction treatment for 28 days to diabetic animals **(Table 6.1)**.

Serum insulin:

Serum insulin level was diminished significantly ($p<0.05$) in all the diabetic groups in respect to vehicle treated control of respective experimental schedule. After the treatment

of ethyl acetate fraction of seed of *H. antidysenterica* for 28 days to the diabetic animals followed by cessation of said fraction treatment to diabetic animals for 14 days or 28 days or 56 days resulted a significant recovery ($p<0.05$) in compare with vehicle treated diabetic control. The level of this parameter was not significantly differed between the 14 days withdrawal and 28 days withdrawal groups though a significant diminution ($p<0.05$) in serum insulin level was observed in 56 days withdrawal group when comparison was made with 14 days or 28 days withdrawal groups **(Fig. 6.1)**.

Hexokinase, glucose-6-phosphate dehydrogenase and glucose-6- phosphatase activity in hepatic tissue:

Activities of hepatic hexokinase and glucose-6-phosphate dehydrogenase were significantly reduced ($p<0.05$) in vehicle treated diabetic group in different duration of withdrawal schedule in respect to the corresponding control. The diminution in the activities of these enzymes was significantly high ($p<0.05$) in vehicle treated diabetic group in 56 days withdrawal schedule. Ethyl acetate fraction prepared from aqueous- methanol (40:60) extract of seed of *H. antidysenterica* treatment to diabetic animals for 28 days followed by cessation of the said fraction treatment for 14 or 28 or 56 days to the corresponding STZ-induced diabetic group resulted a significant elevation ($p<0.05$) in the activities of these enzymes. Hepatic glucose-6-phosphatase activity was significantly elevated ($p<0.05$) in vehicle treated diabetic rats in all duration dependent withdrawal schedule. A significant diminution ($p<0.05$) was noted in the activity of this enzyme after 28 days fraction treatment to diabetic animals followed by cessation of the said fraction treatment for 14 or 28 or 56 days in comparison to the vehicle treated diabetic group. Comparative analysis of the data revealed that there was no significant variation in the level of the said sensors among the groups of 14 days, 28 days and 56 days withdrawal of the fraction treatment. In all these withdrawal schedule, the levels of the enzymes were not resettled to the control or the levels did not deviate further after cessation of treatment **(Fig. 6.2, 6.3 and 6.4)**.

Liver and skeletal muscle glycogen:

Hepatic glycogen content was significantly diminished ($p<0.05$) in STZ induced diabetic group of 14 days, 28 days and 56 days withdrawal schedule in comparison with corresponding vehicle treated control group. The level of diminution was significantly high ($p<0.05$) in 56 days withdrawal group in compare with 14 days or 28 days withdrawal schedule. Administration of ethyl acetate fraction of hydro-methanolic (40:60) extract of seed of *H. antidysenterica* to the diabetic animals for 28 days followed by 14 or 28 or 56 days withdrawal of the said fraction to the respective diabetic animals resulted a significant recovery ($p<0.05$) in respect to diabetic control. Comparative analysis of the data focused that there was no significant variation in the level of this parameter after withdrawal of fraction treatment for 14 days, 28 days and 56 days diabetic groups **(Fig. 6.5)**.

Catalase and peroxidase activities in hepatic and renal tissues:

A significant diminution ($p<0.05$) in the activities of catalase, peroxidase in hepatic and renal tissues were noted in different duration dependent diabetic group in compare with corresponding vehicle treated control group but the levels of diminution was significantly high ($p<0.05$) in vehicle treated diabetic group of 56 days withdrawal schedule in compare with diabetic group under 14 days or 28 days withdrawal schedule. Treatment of ethyl acetate fraction prepared from aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* to diabetic animals followed by withdrawal of the said fraction for 14 days or 28 days or 56 days resulted a significant recovery ($p<0.05$) in the activities of these enzymes both in hepatic and renal tissues towards control level but not to the control level **(Table 6.2)**.

Levels of CD and TBARS in hepatic and renal tissues:

Levels of CD and TBARS in hepatic tissue of diabetic animals in different duration of withdrawal treatment regimen were significantly elevated ($p<0.05$) when comparison was made with the control group of respective treatment regimen. The elevation of these biomarkers was significantly high ($p<0.05$) in diabetic group of 56 days withdrawal schedule in compare with other duration of withdrawal schedule. A significant reduction ($p<0.05$) towards control in the levels of these parameters was observed after ethyl acetate fraction treatment to diabetic animals for 28 days followed by cessation of fraction treatment for 14 days or 28 days or 56 days. Insignificant difference was noted in the levels of these said biosensors among 14 days, 28 days and 56 days fraction treated withdrawal groups **(Table 6.3)**.

Serum lipid profile:

Serum TC, TG, LDLc, and VLDLc levels were increased significantly ($p<0.05$) in STZ- induced vehicle treated diabetic animals of different duration of withdrawal experimental schedule when compare with the vehicle treated control group. Elevated levels of these parameters were significantly high ($p<0.05$) in diabetic group of 56 days withdrawal schedule in compare with 14 days or 28 days withdrawal schedule. After the treatment of ethyl acetate fraction to diabetic animal of different experimental schedule, a significant recovery ($p<0.05$) was noted in respect to vehicle treated diabetic group of the respective schedule. When comparison was made among the 28 days fraction treated group followed by withdrawal of fraction treatment in different duration of experimental schedule, no significant variation was observed in the levels of these biosensors **(Table 6.4)**.

Level of HDLc was decreased significantly ($p<0.05$) in vehicle treated diabetic group of different experimental schedule in respect to vehicle treated control. Level of diminution was significantly high ($p<0.05$) in vehicle treated diabetic group in 56 days withdrawal experimental schedule in compare with others schedule. Treatment of ethyl acetate fraction to diabetic animals for 28 days followed by cessation of fraction treatment for 14 days or 28 days or 56 days resulted a significant recovery ($p<0.05$) in respect to vehicle treated diabetic group in respective experimental schedule. From the comparative analysis of the data, no significant variation was observed in the level of this parameter among the fraction treated groups in different duration of withdrawal schedule **(Table 6.4)**.

Discussion

Present experiment leads to point out the reversible or irreversible nature of recovery by ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* on diabetic disorders in model animal.

Fasting blood glucose level, activities of hexokinase, glucose-6-phosphatase and glucose- 6-phosphate dehydrogenase in hepatic tissue were significantly recovered in fraction treated diabetic group and this recovery stage was stable after 28 days of treatment followed by cessation of same fraction treatment even upto 56 days to diabetic animals. These results were further supported from the recovery of glycogen content in liver and skeletal muscular where it was found that after cessation of the fraction treatment the levels of these parameters were not recurrent to the diabetic state up to 56 days of withdrawal of fraction treatment which indicating the irreversible nature of antidiabetic efficacy of the ethyl acetate fraction of the said plant.

As from previous experiments, it has been focused that antioxidative efficacy of the said fraction is one of the way for the management of diabetes so, to find out the pattern of recovery of oxidative stress related biomarkers, antioxidant enzyme activities like catalase and peroxidase in hepatic and renal tissues as well as levels of TBARS and CD in the said tissue were measured. It was observed a significant recovery in the activities of said enzymes in fraction treated group in respect to diabetic group and it was stable even upto 56 days of withdrawal of treatment which focused the irreversible or permanent recovery in this oxidative stress domain by the phytoingredient(s) present in ethyl acetate fraction of seed of *H. antidysenterica*. To strengthen these results, levels of CD and TBARS in the said tissue were assessed as there is an inverse relationship between antioxidant enzyme activities and the levels of free radical byproducts **(Mallick et al., 2007b; Ali et al., 2009c)**. Here, fraction treated diabetic animals also resulted significant recovery in the levels of these parameters and this condition was existed after cessation of the treatment up to 56 days of the fraction treatment.

Serum lipid profile biomarkers like total cholesterol and triglyceride levels were significantly recovered in 28 days fraction treated diabetic group in respect to vehicle treated diabetic group. After the completion of the fraction treatment for 28 days followed by cessation of the fraction treatment to diabetic animals for 14 days or 28 days or 56 days resulted the existence of recovery state of these parameters. This recovery may be due to long term recovery in pancreas by the said fraction that can reset the serum insulin level as insulin is one of the important regulators for the correction of lipid profile in diabetes **(Maiti et al., 2005)**. Similarly permanent recovery of oxidative stress in this concern may be another possible way for lipid profile correction as there is a close relation between oxidative stress and hyperlipidemia **(Pari and Saravanan, 2002; Mallick et al., 2007b)**.

Conclusion

From the results of this fraction treatment followed by withdrawal schedule of experiment, it may be concluded that ethyl acetate fraction prepared from aqueous- methanol (40:60) extract of seed of *H. antidysenterica* can able to minimize the diabetic disorders in irreversible manner.

Table 6.1 Fasting blood glucose level after the duration dependent withdrawal treatment of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* in STZ induced diabetic male albino rat.

Withdrawal schedule	Group	Fasting blood glucose level (mg/dl)			
		28th day of treatment	14th day withdrawal	28th day withdrawal	56th day withdrawal
14 days	Vehicle treated control	72.5±1.1[a]	74.2±3.5[a]		
	Vehicle treated diabetic	326.7±3.8[b]	318.4±5.3[b]	-	-
	Fraction treated diabetic followed by 14 days withdrawal	91.3±5.3[c]	93.2±4.7[c]	-	-
28 days	Vehicle treated control	75.8±3.7[a]	75.7±3.9[a]	76.3±4.1[a]	
	Vehicle treated diabetic	313.8±5.2[b]	316.5±4.9[b]	334.7±5.3[d]	-
	Fraction treated diabetic followed by 28 days withdrawal	88.6±3.9[c]	90.4±4.1[c]	89.5±4.3[c]	-
56 days	Vehicle treated control	74.2±2.8[a]	76.4±3.6[a]	74.2±4.7[a]	76.7±4.4[a]
	Vehicle treated diabetic	317.6±3.5[b]	314.5±4.3[b]	337.7±5.1[d]	336.7±5.5[d]
	Fraction treated diabetic followed by 56 days withdrawal	91.5±4.1[c]	89.6±3.4[c]	90.4±5.7[c]	92.6±4.3[c]

Data represents Mean ± SEM (n=6). ANOVA followed by 'Multiple comparisons two tail-t-test'. Values with different superscripts (a, b, c) in each vertical column differ from each other significantly at $p<0.05$.

Table 6.2 Activities of antioxidant enzymes i.e. catalase and peroxidase in hepatic and renal tissues after duration dependent withdrawal treatment of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* in STZ-induced diabetic male albino rat.

Withdrawal schedule	Groups	Catalase (µM H_2O_2 consumed/mg of tissue/min)		Peroxidase (Unit/mg of tissue)	
		Liver	Kidney	Liver	Kidney
14 days	Vehicle treated control	5.98 ± 0.15[a]	4.92 ± 0.08[a]	4.72 ± 0.19[a]	3.82 ± 0.07[a]
	Vehicle treated diabetic	3.27 ± 0.11[b]	3.14 ± 0.12[b]	2.31 ± 0.11[b]	2.03 ± 0.11[b]
	14 days withdrawal	3.77 + 0.21[c]	3.41 + 0.21[c]	3.01 + 0.19[c]	2.84 + 0.06[c]
28 days	Vehicle treated control	6.10 ± 0.21[a]	4.83 ± 0.07[a]	4.77 ± 0.14[a]	3.94 ± 0.08[a]
	Vehicle treated diabetic	3.21 ± 0.13[b]	3.02 ± 0.14[b]	2.24 ± 0.12[b]	2.08 ± 0.14[b]
	28 days withdrawal	3.69 ± 0.27[c]	3.47 ± 0.18[c]	3.16 ± 0.21[c]	2.91 ± 0.09[c]
56 days	Vehicle treated control	5.94 ± 0.19[a]	4.89 ± 0.06[a]	4.82 ± 0.16[a]	4.01 ± 0.12[a]
	Vehicle treated diabetic	2.56 ± 0.15[d]	2.37 ± 0.21[d]	1.71 ± 0.15[b]	1.13 ± 0.12[d]
	56 days withdrawal	3.58 ± 0.19[c]	3.43 ± 0.18[c]	3.19 ± 0.13[c]	2.96 ± 0.08[c]

Data represents Mean ± SEM (n=6). ANOVA followed by 'Multiple comparisons two tail-t-test'. Values with different superscripts (a, b, c, d) in each vertical column differ from each other significantly at $p<0.05$.

Table 6.3 Levels of free radical byproducts i.e. CD and TBARS in hepatic and renal tissues after duration dependent withdrawal treatment of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* in STZ-induced diabetic male albino rat.

Withdrawal schedule	Groups	CD (nM / mg of tissue)		TBARS (nM / mg of tissue	
		Liver	Kidney	Liver	Kidney
14 days	Vehicle treated control	272.5 ± 4.7^{a}	304.6 ± 3.2^{a}	8.16 ± 0.57^{a}	9.67 ± 0.53^{a}
	Vehicle treated diabetic	359.4 ± 5.2^{b}	371.7 ± 4.6^{b}	13.84 ± 0.62^{b}	16.48 ± 0.74^{b}
	14 days withdrawal	308.7 ± 4.3^{c}	338.6 ± 3.7^{c}	10.43 ± 0.43^{c}	13.12 ± 0.59^{c}
28 days	Vehicle treated control	277.4 ± 5.2^{a}	309.3 ± 3.4^{a}	8.24 ± 0.68^{a}	9.46 ± 0.41^{a}
	Vehicle treated diabetic	362.5 ± 4.6^{b}	368.8 ± 4.7^{b}	14.02 ± 0.59^{b}	17.18 ± 0.62^{b}
	28 days withdrawal	311.6 ± 4.5^{c}	333.4 ± 3.8^{c}	9.94 ± 0.49^{c}	14.03 ± 0.59^{c}
56 days	Vehicle treated control	269.6 ± 4.6^{a}	313.5 ± 4.2^{a}	7.87 ± 0.57^{a}	8.92 ± 0.54^{a}
	Vehicle treated diabetic	396.7 ± 5.2^{d}	398.6 ± 5.2^{d}	18.26 ± 0.61^{d}	19.23 ± 0.64^{d}
	56 days withdrawal	304.7 ± 5.1^{c}	326.5 ± 4.2^{c}	9.64 ± 0.52^{c}	13.22 ± 0.49^{c}

Data denotes Mean ± SEM (n=6). ANOVA followed by 'Multiple comparisons two tail-t-test'. Values with different superscripts (a, b, c, d) in each vertical column differ from each other significantly at $p<0.05$.

Table 6.4 Levels of serum lipid profile (TC, TG, LDLc, VLDLc and HDLc) in duration dependent withdrawal treatment of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* in STZ induced diabetic male albino rat.

Withdrawal schedule	Groups	Serum lipid profile (mg/dl)				
		TC	TG	HDLc	LDLc	VLDLc
14 days	Vehicle treated control	64.7 ±2.8^{a}	54.6 ±2.3^{a}	35.6 ±1.2^{a}	15.5 ±1.1^{a}	10.8 ±0.76^{a}
	Vehicle treated diabetic	106.5 ±1.8^{b}	118.4 ±2.7^{b}	21.5 ±1.4^{b}	62.5 ±0.85^{b}	25.8±0.70^{b}
	14 days withdrawal	77.6 ±1.8^{c}	88.7 ± 1.8^{c}	25.3 ±1.4^{c}	25.8 ±0.83^{c}	18.4±.95^{c}
28 days	Vehicle treated control	66.8 ±3.1^{a}	57.2 ± 1.8^{a}	37.4 ±1.4^{a}	16.1 ±1.4^{a}	9.8 ±1.8^{a}
	Vehicle treated diabetic	110.5 ±2.7^{b}	123.4 ±2.6^{b}	19.7 ±1.8^{b}	64.1 ±2.2^{b}	24.3 ± 1.4^{b}
	28 days withdrawal	74.8 ±2.6^{c}	91.3 ± 2.3^{c}	26.3 ±1.7^{c}	23.6 ± 1.7^{c}	16.3 ± 1.1^{c}
56 days	Vehicle treated control	65.4 ±2.6^{a}	58.3 ± 1.4^{a}	36.4 ±2.1^{a}	14.7 ±1.7^{a}	10.7 ±1.4^{a}
	Vehicle treated diabetic	134.7 ±1.9^{d}	137.4 ±3.1^{d}	14.7 ±1.8^{d}	76.8 ±2.4^{d}	35.4 ± 1.7^{d}
	56 days withdrawal	72.5 ±2.8^{c}	84.6 ± 2.6^{c}	24.8 ±1.4^{c}	24.8 ± 1.3^{c}	17.5 ± 1.6^{c}

Data represents Mean ± SEM (n=6). ANOVA followed by 'Multiple comparisons two tail-t-test'. Values with different superscripts (a, b, c, d) in each vertical column differ from each other significantly at $p<0.05$.

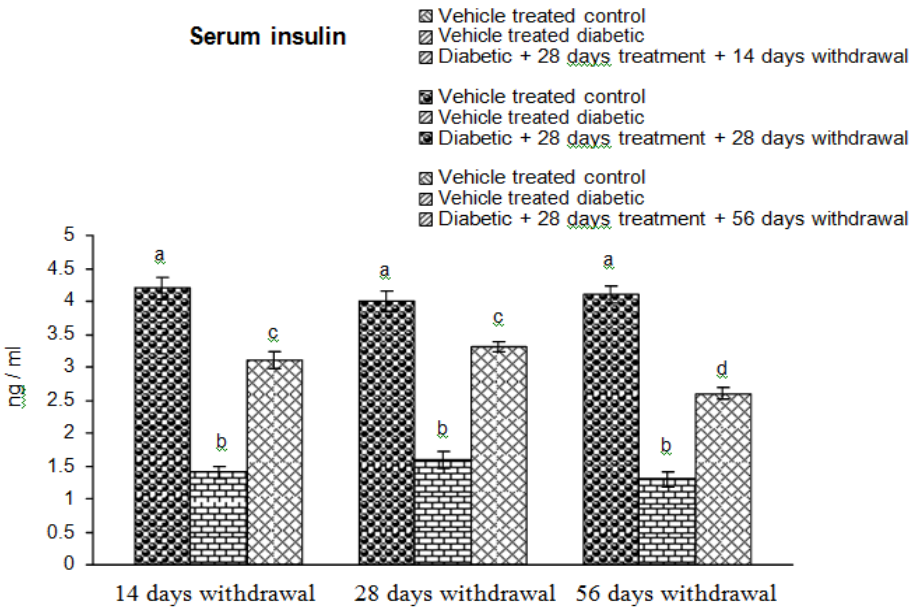

Figure 6.1 Serum insulin level in 28 days fraction treatment followed by 14 days or 28 days or 56 days withdrawal of fraction treatment in STZ induced diabetic rats. Bar represent as Mean ± SEM (n=6). ANOVA followed by 'Multiple comparisons two tail-t- test'. Bars with different superscripts (a, b, c, d) differ from each other significantly at $p<0.05$.

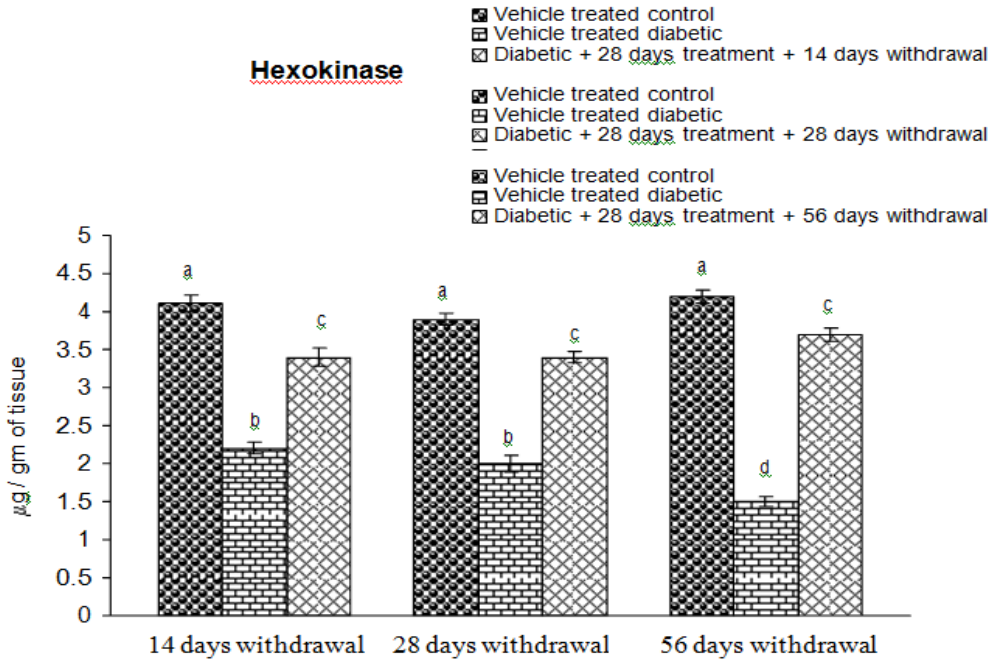

Figure 6.2 Activities of hexokinase after 28 days of fraction treatment followed by 14 days or 28 days or 56 days withdrawal of fraction treatment in STZ induced diabetic groups. Bar represent as Mean± SEM (n=6). ANOVA followed by 'Multiple comparisons two tail-t-test'. Bars with different superscripts (a, b, c, d) differ from each other significantly at $p<0.05$.

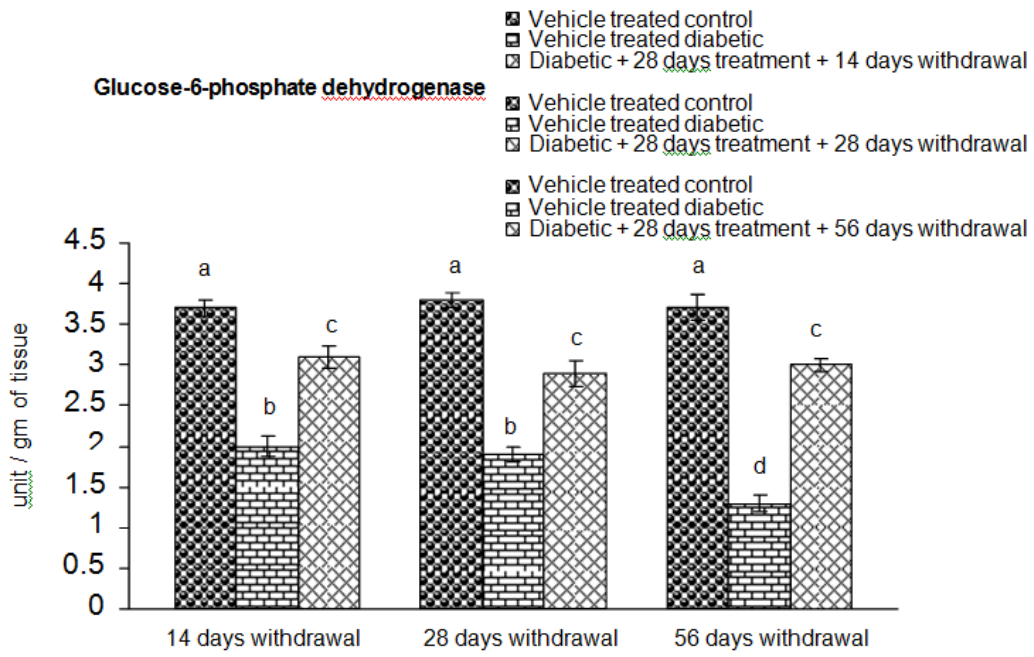

Figure 6.3 Effect of 28 days fraction treatment followed by withdrawal of fraction treatment for 14 days or 28 days or 56 days on hepatic glucose-6-phosphate dehydrogenase activity in STZ induced diabetic rat. Bars represent as Mean± SEM (n=6). ANOVA followed by 'Multiple comparisons two tail-t-test'. Bars with different superscripts (a, b, c, d) differ from each other significantly at $p<0.05$.

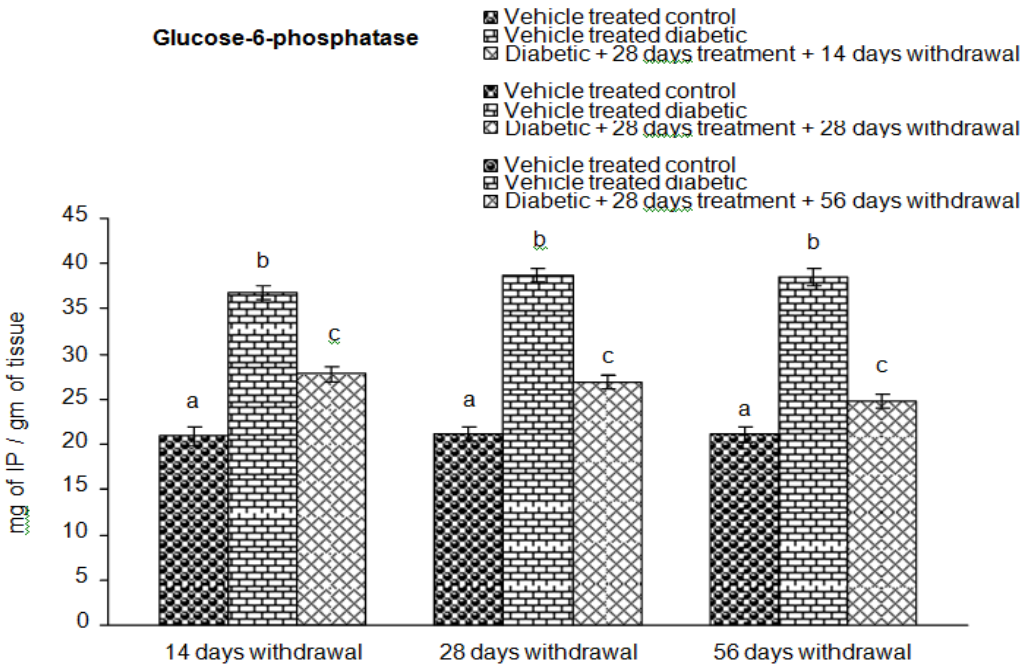

Figure 6.4 Activities of glucose-6-phosphatase in hepatic tissue after 28 days fraction treatment followed by withdrawal of fraction treatment for 14 days or 28 days or 56 days in STZ-induced diabetic group. Bars represents Mean ± SEM (n=6). ANOVA followed by 'Multiple comparisons two tail-t-test'. Bars with different superscripts (a, b, c, d) differ from each other significantly at $p<0.05$.

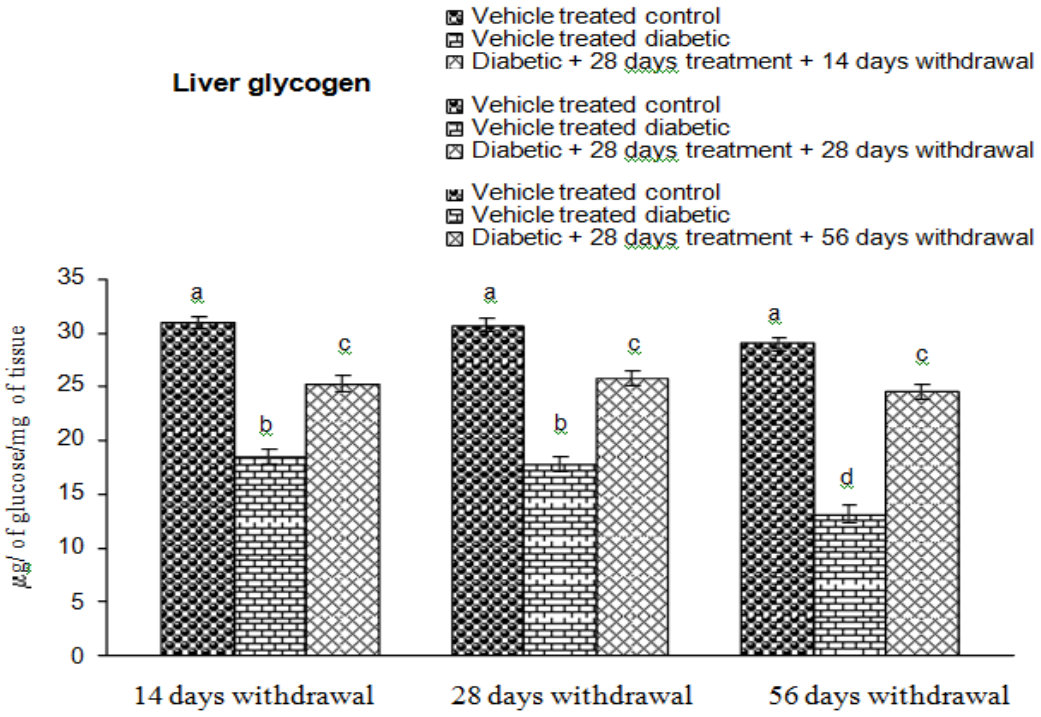

Figure 6.5 Comparative analysis of duration dependent fraction withdrawal treatment in liver glycogen content after 28 days of fraction treatment in STZ induced diabetic rat. Bar represent as Mean ± SEM (n=6). ANOVA followed by 'Multiple comparisons two tail-t- test'. Bar with different superscripts (a, b, c, d) differ from each other significantly at $p<0.05$.

Experiment No 07

Direct effect of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *Holarrhena antidysenterica* on the activities of key carbohydrate metabolic enzymes and oxidative stress markers in streptozotocin-induced diabetic male rat: An in vitro approach

Aim of the study

This study was carried out to delineate the direct effect of ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* on the activities of key carbohydrate metabolic enzymes and antioxidant enzymes in hepatic tissue as well as levels of free radical byproducts in the said tissue in STZ induced diabetic male albino rat.

Experimental design

In this study, eighteen Wistar strain matured healthy normoglycemic (65-75 mg/dl) male albino rats of 3 months of age having body weight 150 ± 10gm were used. They were kept in polypropylene made cage with six animals in each cage. Before starting the experiment all the animals were acclimated for 15 days in our well hygienic animal house having room temperature of 24±2°C, humidity 45-60% with 12 hour light: 12 hour dark cycle. The animals were supplied with standard animal feed and water *ad libitum.* After that ten rats were made diabetic by single intramuscular injection of streptozotocin at the dose of 4mg / 0.1ml citrate buffer / 100gm body weight as per standard method **(Mallick et al., 2007a; Ali et al., 2009a)**. Six animals were subjected to control group and they were injected with citrate buffer at the dose of 0.1ml / 100gm body weight/rat. Out of ten STZ treated animals, six animals having FBG level 300-350mg/dl were selected for this study. To test the stability of diabetes, FBG level was further measured on 7th day. Six animals having FBG at that desirable range were considered here and rest four animals were not included in this experiment. After that these **animals were kept in diabetic condition for next 28 days without any fraction treatment.** All the control and diabetic animals were sacrificed on 29th day. Liver tissue was dissected out from each animal of control group and cut into small pieces with 2-2.5 mm thickness. **Two liver slices were used from each animal and these two slices were placed in two separate test tubes of 10 ml Kreb's Ringer Bicarbonate (KRB) Buffer solution.** One test tube for vehicle treated control and another one for fraction treated control.

In the same way liver tissue of each diabetic animal was dissected out and cut into small pieces, and these pieces were subjected to in vitro treatment as per the design framed for the control group. **Each group composed of 6 test tubes and therefore total 24 test tubes containing liver slices were used for this in-vitro study** and schedule of grouping were as follows -

Vehicle treated control tissue (Group I):

In each test tube, 0.2 ml of 3% tween 80 was mixed with 10 ml of KRB solution. One slice of liver tissue of each control animal was placed in each test tube. Six tubes were taken in this group for six control animals.

Ethyl acetate fraction treated control tissue (Group II):

Here, in each test tube, 2 mg of ethyl acetate fraction in 0.2 ml of 3% tween 80 was mixed with 10 ml of KRB solution. One slice of liver of each control animal was placed in each tube. Six test tubes were included in this group for six animals.

Vehicle treated diabetic tissue (Group III):

In this group, one slice of liver tissue from each diabetic animal was placed in each test tube containing 0.2 ml of 3% tween 80% with 10 ml of KRB solution. Six tubes were taken for liver tissues of six diabetic animals.

Ethyl acetate fraction treated diabetic tissue (Group IV):

Ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* at 2mg / 0.2ml 3% tween 80 was added with 10ml of KRB solution in each test tube. One piece of liver slice of each diabetic animal placed in each tube. Six test tubes were used for six diabetic animals in this group.

All these test tubes were incubated at 37°C for 2 hours. Incubation of tissue slices was performed allowing a gas mixture of 95% O_2 and 5% CO_2 in the form of bubbles **(Brady, 1951)**. After the completion of incubation period, liver slices were transferred separately to the tubes containing 1 ml of sodium phosphate buffer solution (pH 7.4) for washing.

Parameters and methods

In-vitro study was performed in glass test tubes using Kreb's Ringer Bicarbonate (KRB) Buffer solution having pH – 7.4 (NaCl-8.0 gm, $CaCl_2$ – 0.20 gm, $MgCl_2, 6H_2O$ – 0.10 gm, Na_2HCO_3 – 1.0 gm, NaH_2PO_4-0.05 gm, Glucose -1.00 g in 1000 ml deionized water) **(Brady, 1951)**. After 2 hours of incubation followed by washing in sodium phosphate buffer solution, tissue slices were used for the measurement of glucose-6-phosphatase **(Swanson, 1955)** and hexokinase **(Chou and Wilson, 1975)** activities biochemically. Assessment in the activities of catalase **(Beers and Sizer, 1952)** and peroxidase **(Sadasivam and Manickam, 2008b)** as well as levels of CD **(Slater, 1984)** and TBARS **(Okhawa et al., 1979)** in the tissue samples were also performed.

Results

Activities of hexokinase and glucose-6-phosphatase:

Hepatic hexokinase activity was decreased significantly ($p<0.05$) and the hepatic glucose-6-phosphatase activity was elevated significantly ($p<0.05$) in vehicle treated diabetic tissue in compare with vehicle treated control tissue. Exposure of ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterca* for two hours on liver tissue of diabetic

animals resulted no significant effect in the activities of these enzymes in compare with vehicle treated diabetic tissue. Comparative analysis of the data indicated that there was an insignificant variation in the levels of this parameter between vehicle treated control tissue and fraction treated control tissue **(Fig. 10.1 and 10.2)**.

Antioxidant enzyme catalase and peroxidase activities:

Activities of catalase and peroxidase were decreased significantly ($p<0.05$) in vehicle treated diabetic tissue in respect to vehicle treated control tissue. Addition of ethyl acetate fraction to the tissue of diabetic animals followed by incubation for 2 hours not showed any significant recovery ($p<0.05$) in the activities of these enzymes when comparison was made with vehicle treated diabetic tissue. No significant variation was observed in the activities of these said enzymes between vehicle treated control tissue and fraction treated control tissue **(Fig. 10.3 and 10.4)**.

Levels of CD and TBARS:

A significant elevation ($p<0.05$) was noted in the levels of CD and TBARS in vehicle treated diabetic hepatic tissue in compare with vehicle treated control tissue. There was no significant alteration ($p<0.05$) in the levels of these parameters when diabetic tissues were exposed in presence of ethyl acetate fraction in compare with vehicle treated diabetic tissue. Levels of CD and TBARS in liver tissue did not differ significantly in ethyl acetate fraction treated control tissue when comparison was made with vehicle treated control tissue **(Fig. 10.5 and 10.6)**.

Discussion

Results of the experiments suggested that the phytomolecule(s) present in ethyl acetate fraction may stimulate the β-cell regeneration or β-cell excitation which is consistent with the results of other workers using other natural products **(Prince et al., 1998; Shao et al., 2004)**. To explore whether there is any direct effect or nongenomic effect of the phytomolecule(s) present in this fraction on the activities of hepatic hexokinase and glucose-6-phosphatase as well as antioxidant enzymes like catalase and peroxidase in liver, this in-vitro model of experiment was conducted where the fraction was directly exposed on the tissue.

Results of this experiment revealed that the activities of carbohydrate metabolic enzymes were not affected by ethyl acetate fraction directly in control and diabetic tissues. Similarly, the activities of catalase and peroxidase in liver were not affected significantly by the phytoingredient(s) present in ethyl acetate fraction both in control and diabetic tissues which focused the inability of the phytoingredient(s) present in this fraction for the modulation of antioxidant enzyme activities directly. This result was supported by the insignificant alteration in the levels of TBARS and CD both in control and diabetic tissues with or without exposure of the said fraction as activities of antioxidant enzymes control the levels of these free radical byproduct in inverse manner **(Pari and Saravanan, 2002)**.

Conclusion

Results of this experiment may lead to conclude us that the ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* has no direct moduulatory role for the recovery of diabetes induced altered carbohydrate metabolic enzyme activities as well as oxidative stress markers in STZ model diabetic male rat.

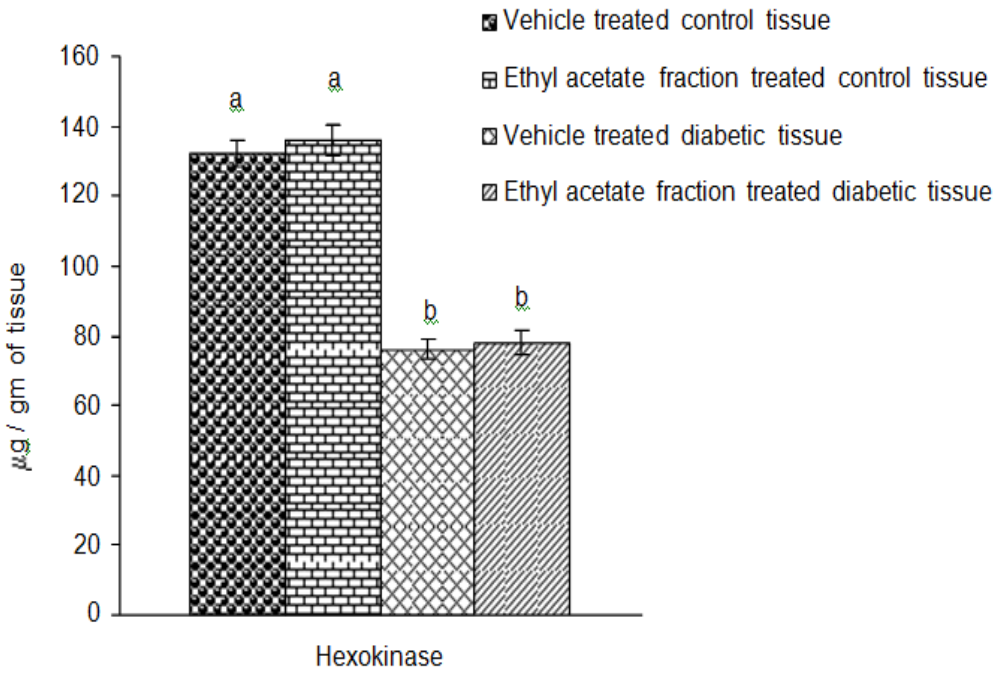

Fig 7.1 Direct effect of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* on hexokinase activity in liver tissue of control and diabetic animal. Bars indicate Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Bars with different superscripts (a, b) differ from each other significantly at $p<0.05$.

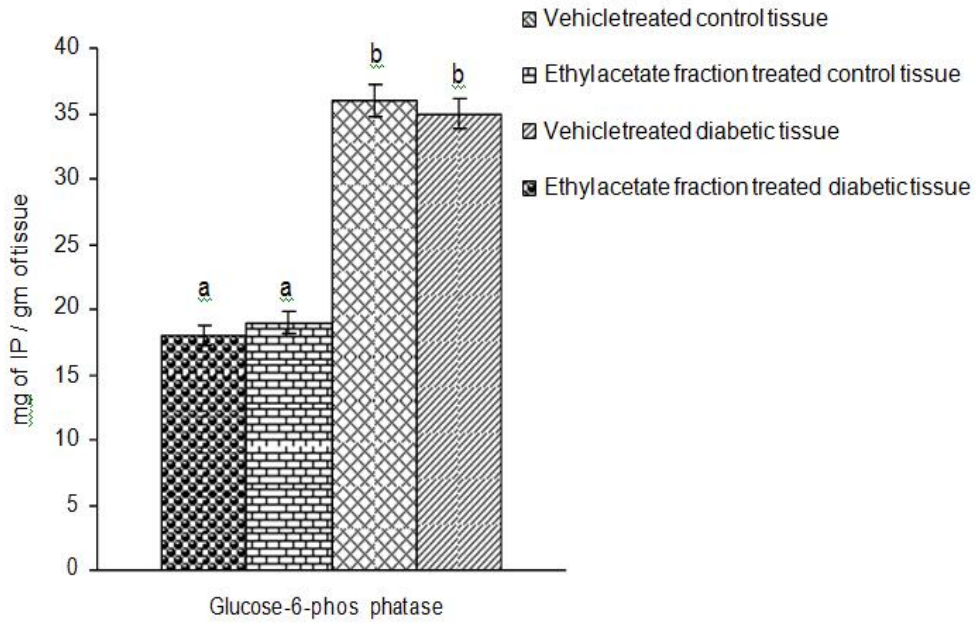

Fig 7.2 Glucose-6-phosphatase activity in ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* exposed control and diabetic liver tissue. Bars denote Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t- test'. Bars with different superscripts (a, b) differ from each other significantly at $p<0.05$.

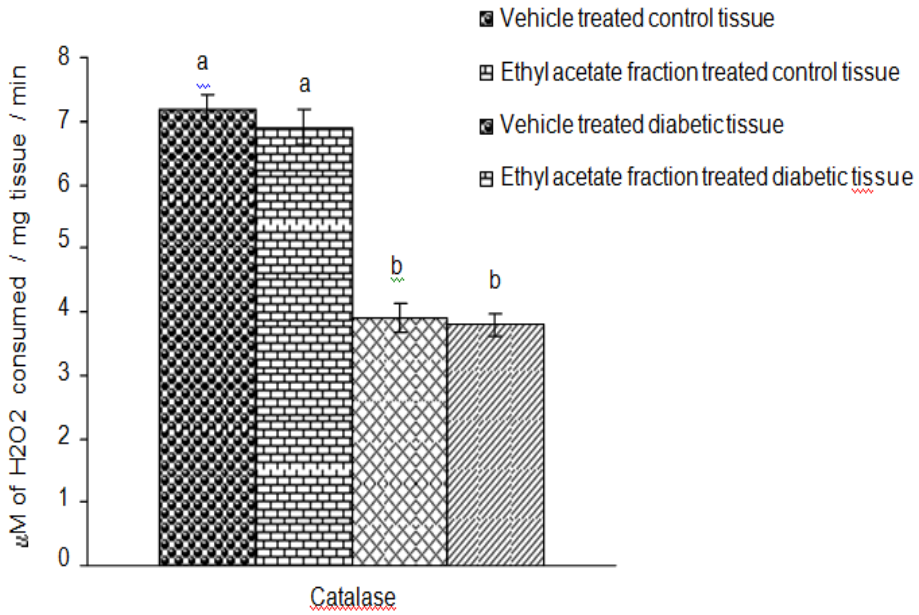

Fig 7.3 In-vitro effect of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* on catalase activity in liver tissue of control and diabetic animal. Bars indicate Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Bars with different superscripts (a, b) differ from each other significantly at $p<0.05$.

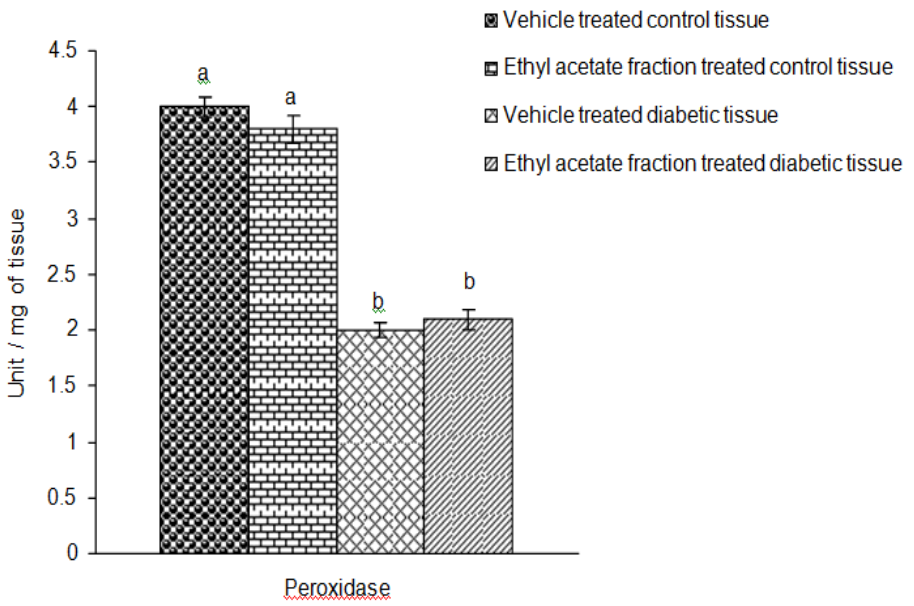

Fig 7.4 Direct exposure of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* on hepatic peroxidase activity. Bars indicate Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Bars with different superscripts (a, b) differ from each other significantly at $p<0.05$.

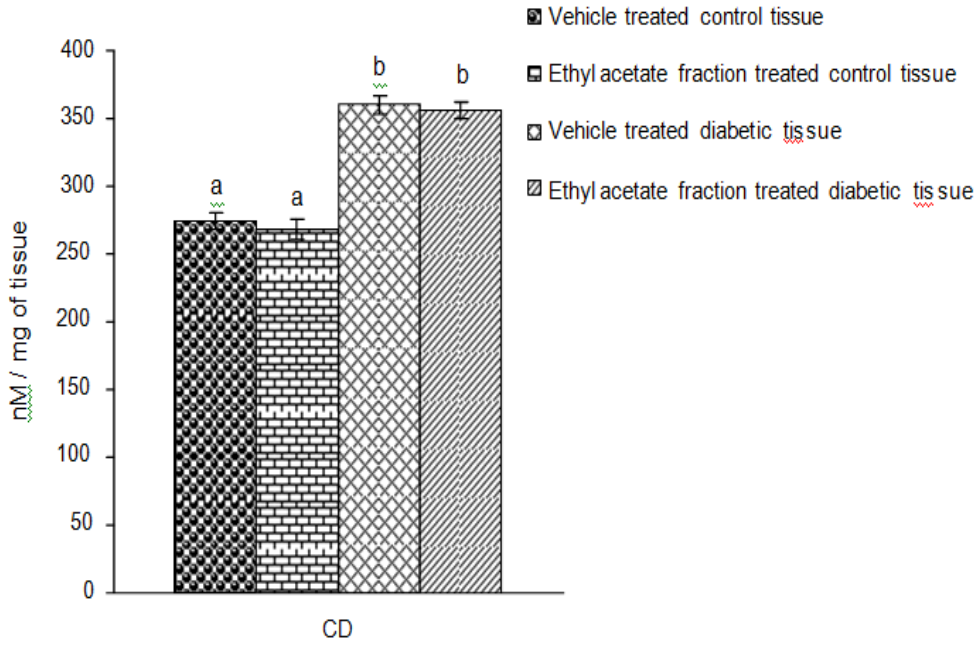

Fig 7.5 In-vitro effect of ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* on the level of CD in liver tissue of control and diabetic animal. Bars indicate Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Bars with different superscripts (a, b) differ from each other significantly at $p<0.05$.

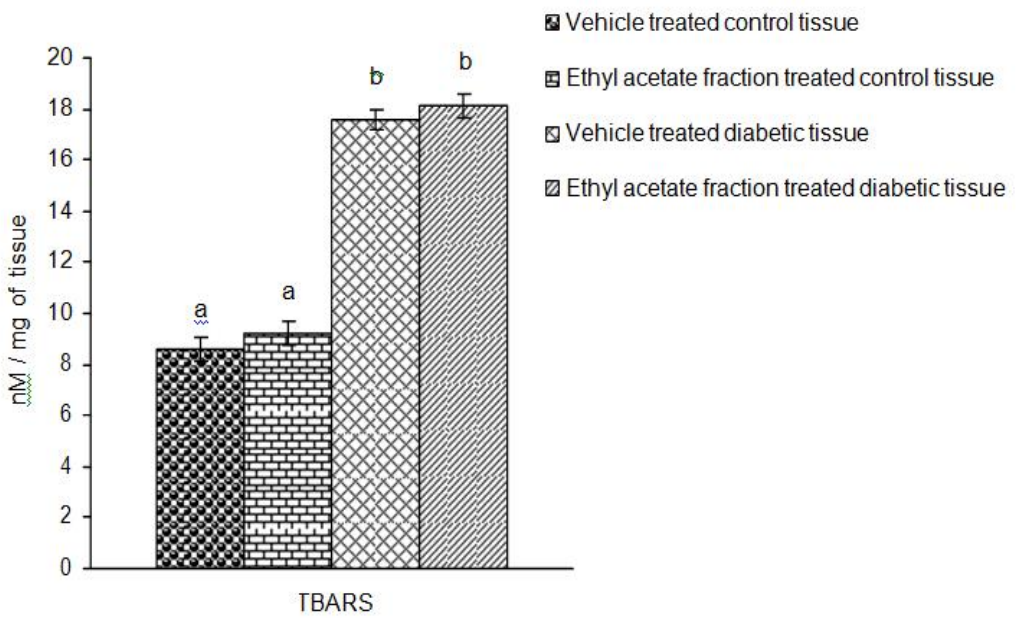

Fig 7.6 Variation in the levels of TBARS after in-vitro treatment of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* in control and diabetic liver tissue. Bars indicate Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Bars with different superscripts (a, b) differ from each other significantly at $p<0.05$.

Experiment No 08

Toxicity study of effective dose of ethyl acetate fraction prepared from aqueous-methanol :: 40:60 extract of seed of *Holarrhena antidysenterica* in male albino rat

Aim of the study

This study has been conducted to search out whether there is any toxic effect of the ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* on normoglycemic adult healthy male albino rat.

Experimental design

To fulfill the aim of the study eighteen normoglycemic adult male Wistar strain albino rats, 3 months of age, weighing 150±10gm were used. Animals were acclimated for 15 days in our hygienic, well ventilated animal house prior to start the experiment and all other conditions were maintained as per the guidelines of Institutional Animal Ethics Committee (IAEC). Animals were free access to standard feed and water *ad libitum*.

Animals were divided into three groups equally having six animals in each group and the process of treatment as represented below.

Untreated control:

Normoglycemic animals of this group were treated with 0.5ml of distilled water / 100gm body weight / day for 28 days.

Vehicle treated control:

Normoglycemic healthy animals of this group were treated with 0.5ml of 3% tween 80 / 100gm body weight / day for 28 days.

Fraction treated control:

Normoglycemic animals of this group were treated with ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* at the dose of 20mg / 0.5ml of 3% tween 80 / 100gm body weight / day for 28 days.

Treatment of all the groups was conducted at 8 A.M. and at fasting condition such as previous experiments. After completion of the treatment, animals were subjected to light ether anesthesia at 7 A.M. after recording the final body weight of all the animals. Animals were sacrificed by decapitation and both the liver and kidney tissues of each animal were dissected out and wet weights of these organs were recorded and the tissues were preserved at -20°C for biochemical studies. Blood was collected from dorsal aorta by heparinized syringe and plasma was separated by centrifugation for the measurement of serum biochemical parameters in this concern.

Parameters and methods

Assessment in the activities of glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), alkaline phosphatase (ALP) and acid phosphatase (ACP) in hepatic and renal tissues were performed as per standard methods **(Henry et al., 1960; Vanha–Pertula and Nikkanen, 1973; Malamy and Horecker, 1996)**. Levels of urea, uric acid, creatinine and blood urea nitrogen in serum were measured following standard methods **(Tiffany et al., 1972; Kabasakalian et al., 1973; Junge et al., 2001)**.

Haematological parameters like hemoglobin (Hb), red blood cell count (RBC), packed cell volume (PCV) and erythrocyte sedimentation rate (ESR) were studied as per standard protocol **(Willie and Cartwright, 2005)**.

Results

Feeding habit:

There was no significant change ($p<0.05$) in feeding habit of the animals in vehicle treated control and fraction treated control in compare with untreated control. No significant difference ($p<0.05$) was observed between vehicle treated control and untreated control.

Body weight and metabolic organo-somatic indices:

Body weight and hepato-somatic as well as reno-somatic indices did not differ significantly ($p<0.05$) among vehicle treated control, fraction treated control and untreated control groups **(Table 8.1)**.

Activities of GOT, GPT in hepatic and renal tissues:

Normoglycemic animals were treated with ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* at the dose of 20mg / 0.5ml tween 80% / 100gm body weight for 28 days resulted no significant difference ($p<0.05$) in the activities of GOT, GPT in hepatic and renal tissues when comparison was made among vehicle treated control, untreated control and fraction treated control groups **(Fig. 8.1 and 8.2)**.

Activities of ACP, ALP in hepatic and renal tissues:

There was no significant change ($p<0.05$) in ACP, ALP activities in hepatic and renal tissues of fraction treated control animals in respect to the vehicle treated control or untreated control. There was insignificant variation ($p<0.05$) in the activities of these enzymes among the said three groups **(Fig. 8.3 and 8.4)**.

Levels of serum urea, uric acid, creatinine and blood urea nitrogen:

No significant alteration ($p<0.05$) was observed in the levels of serum urea, uric acid, creatinine and blood urea nitrogen in fraction treated control rats in respect to the vehicle treated control or untreated control rats **(Table 8.2)**.

Haematological biomarkers:

Normoglycemic healthy rats were treated with ethyl acetate fraction of aqueous-methanol extract (3:2) of seed of *H. antidysenterica* at the dose of 20mg / 0.5ml tween 80% / 100 gm body weight / day for 28 days resulted no significant difference ($p<0.05$) in the levels of haematological biomarkers like hemoglobin (Hb), red blood cell count (RBC), packed cell volume (PCV) and erythrocyte sedimentation rate (ESR) when comparison was made with vehicle treated control or untreated control animals. From the comparative analysis of the data, it was observed that no significant difference ($p<0.05$) between vehicle treated control and fraction treated control in the levels of these parameters **(Table 8.3)**.

Discussion

From the previous experiments it has been indicated that the ethyl acetate fraction of aqueous-methanol :: 40:60 extract of the seed of *H. antidysenterica* has a significant antidiabetic effect. The present experiment was conducted to find out whether the ethyl acetate fraction prepared from aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* has any toxic effect in general or any hepatotoxicity or renotoxicity or haematotoxicity.

Toxicological evaluation was carried out in experimental rats to predict the toxicity and safety of the plant products. A survey conducted by World Health Organization indicated that 70%–80% of the world's population relies on alternative medicines, mainly of herbal sources, for the primary health care **(Sateesh and Addepalli, 2009)**. In this purpose, we studied GOT, GPT, ACP and ALP activities in hepatic and renal tissues as these are the important biomarkers in this concern **(Akther et al., 1990; McAnuff et al., 2003; Ali et al., 2009c)**. Ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* treatment resulted no significant changes in the levels of any of the said parameters.

Urea, uric acid, creatinine and blood urea nitrogen are the variable nitrogenous constituent of blood. These are chiefly filtered out of the blood by the kidneys (glomerular filtration and proximal tubular secretion). The increased blood content of these markers has been reported in renal injury, subsequent to trauma or anuria, in traumatic injuries to the muscles and in the muscular dystrophy **(Khedkar et al., 2011)**. In the present study, there was no significant alteration in the level of these parameters in fraction treated rats in respect to untreated or vehicle treated rats. This observation clearly indicates that the kidneys were working efficiently in fraction treated animal and hence, *H. antidysenterica* seeds has no toxic effect in kidney in general.

Analysis of blood parameters is important for risk evaluation because changes in the hematological system are predictive of toxicity. We also studied the hemoglobin concentration (Hb), red blood cell count (RBC), packed cell volume (PCV) and erythrocyte sedimentation rate (ESR) in untreated control animals, vehicle treated control animals and fraction treated control animals. Treatment of ethyl acetate fraction of said plant part resulted insignificant changes in the levels of Hb, RBC, PCV and ESR. Thus, the said fraction is non-toxic to circulating red cells, and does not interfere with their production. Haematopoiesis remain unaffected, even though the hematopoietic system is one of the most sensitive targets for toxic compounds and is an important index for physiological and pathological status assessment in humans and animals **(Adeneye et al., 2006; Essawy et al., 2010)**.

Conclusion

The active phytoingredient(s) present in ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* has no evidence of hepato or reno or hematotoxicity. Results of this study would be useful for the planning of future pre- clinical and clinical studies for antidiabetic remedy using the said plant part.

Table 8.1 Effect of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* on body weight and metabolic organo-somatic indices in male albino rat.

Group	Body Weight (gm)		Hepato-somatic index (gm %)	Reno-somatic index (gm %)
	Initial	Final		
Untreated control	143.42 ± 4.73[a]	156.74 ± 5.62[a]	3.90±0.36[a]	1.31±0.03[a]
Vehicle treated control	141.34 ± 5.58[a]	157.65 ± 4.88[a]	3.78±0.27[a]	1.32±0.04[a]
Fraction treated control	142.35 ± 3.89[a]	155.57 ± 3.78[a]	3.86±0.27[a]	1.29±0.06[a]

All the values denote Mean ± SEM (n = 6). ANOVA followed by 'Multiple comparison two tail-t-test'. Values in each vertical column did not differ from others significantly having same superscript (a), p>0.05.

Table 8.2 Levels of urea, uric acid (UA), blood urea nitrogen (BUN) and creatinine in serum of untreated control, vehicle treated control and fraction treated control rat.

Group	Urea (mg/dl)	Uric acid (mg/dl)	Blood urea nitrogen (mg/dl)	Creatinine (mg/dl)
Untreated control	40.82 ± 3.65[a]	3.44 ± 0.03[a]	19.06 ± 2.31[a]	0.65 ± 0.006[a]
Vehicle treated control	39.06 ± 3.74[a]	3.47 ± 0.04[a]	19.17 ± 1.97[a]	0.67 ± 0.005[a]
Fraction treated control	41.02 ± 2.84[a]	3.51 ± 0.07[a]	18.68 ± 1.67[a]	0.63 ± 0.007[a]

All the values indicate Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Values in each vertical column with same superscript (a) did not differ from others significantly, p>0.05.

Table 8.3 Effect of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* on the levels of haematological biomarkers in untreated control, vehicle treated control and fraction treated control male albino rats.

Group	Hb (gm%)	RBC (10^6/mm^3)	Packed cell volume (%)	Erythrocyte sedimentation rate (mm/1st hr)
Untreated control	13.2 ± 0.46[a]	7.28 ± 0.31[a]	38.46 ± 3.4[a]	7.86 ± 0.25[a]
Vehicle treated control	13.4 ± 0.52[a]	7.31 ± 0.33[a]	36.51 ± 3.8[a]	7.67 ± 0.27[a]
Fraction treated control	12.8 ± 0.61[a]	6.93 ± 0.37[a]	37.46 ± 3.4[a]	7.71 ± 0.22[a]

All the values represent Mean ± SEM (n = 6). ANOVA followed by 'Multiple comparison two tail-t-test'. Values in each vertical column with same superscript (a) did not differ from others significantly, $p>0.05$.

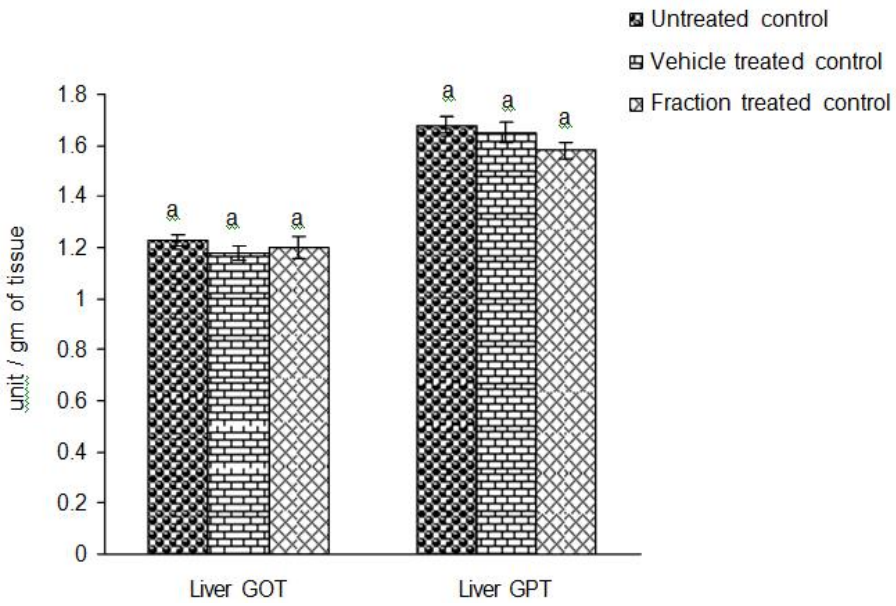

Fig. 8.1 Metabolic toxicity studies of effective dose of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* on liver GOT and GPT activities in male albino rat. Bars denote Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Bars having same superscript (a) did not differ from others significantly, p>0.05.

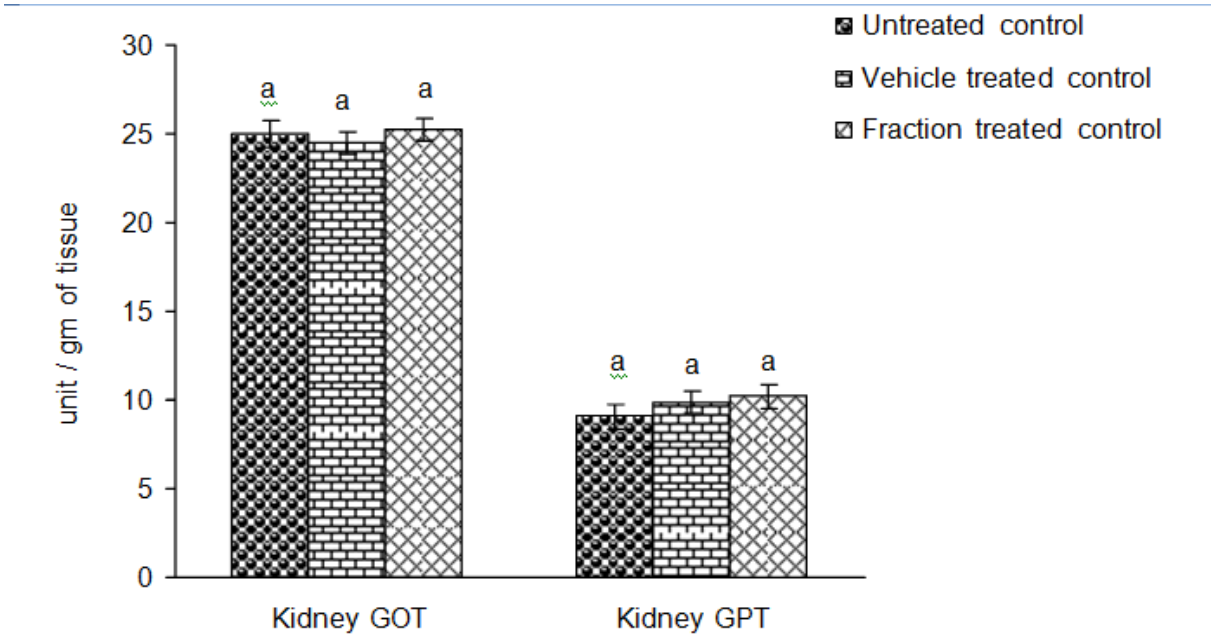

Fig. 8.2 Assessment of renal GOT and GPT activities after treatment with effective dose of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* in male albino rat. Bars denote Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail- t-test'. Bars having same superscript (a) did not differ from others significantly, p>0.05.

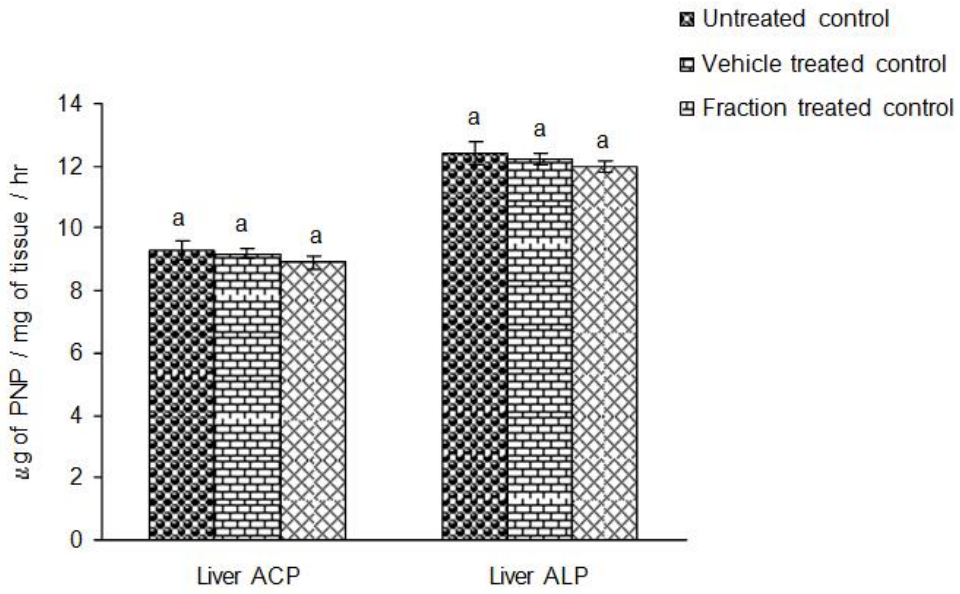

Fig. 8.3 Hepatic ACP and ALP activities in untreated control, vehicle treated control and fraction treated control groups. Bars showing the Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Bars having same superscript (a) did not differ from others significantly, p>0.05.

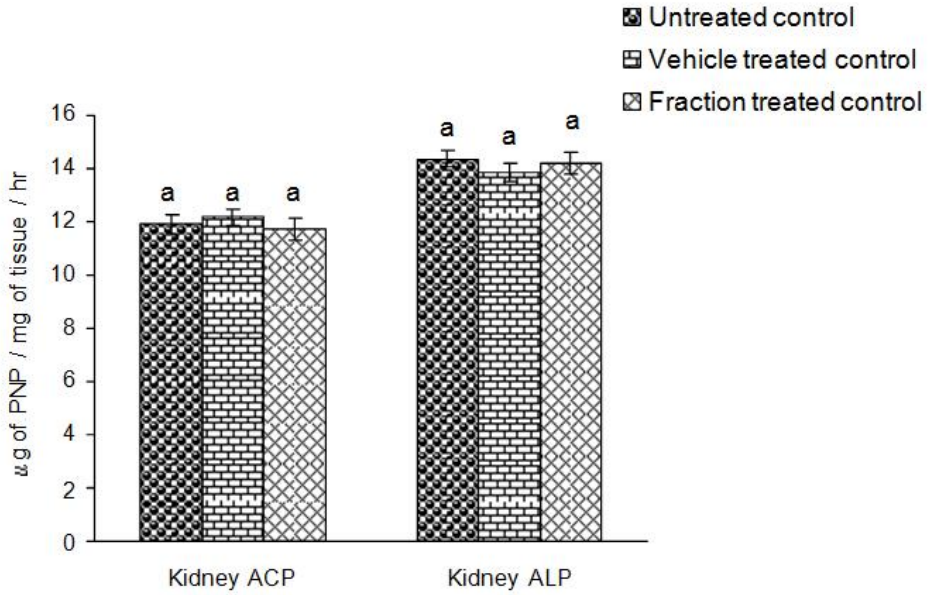

Fig. 8.4 Activities of kidney ACP and ALP after the treatment of effective dose of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* in male albino rat. Bars showing the Mean ± SEM (n=6). ANOVA followed by 'Multiple comparison two tail-t-test'. Bars having same superscript (a) did not differ significantly from other, p>0.05.

Experiment No 09A

Phytochemical analysis of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *Holarrhena antidysenterica*: A qualitative study

Aim of the study

This study has been conducted to find out the possible chemical nature of biologically active phytoingredient(s) present in ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* having antidiabetic effect.

Parameters and methods

Qualitative phytochemical analysis was done to find out the presence of the active chemical constituents such as alkaloids, flavonoids, terpenoids, steroids, glycosides, reducing sugar and tannin following the standard methods stated below –

Alkaloid

Alkaloids are basic nitrogenous compounds with definite physiological and pharmacological activity. Alkaloid solution produces white yellowish precipitate when a few drops of Mayer's reagents are added (Siddiqui and Ali, 1997). Most alkaloids are precipitated from neutral or slightly acidic solution by Mayer's reagent (Evans, 2002). The ethyl acetate fraction was evaporated to dryness and the residue was heated on a boiling water bath with 2% hydrochloric acid. After cooling, the mixture was filtered and treated with a few drops of Mayer's reagent. The samples were then observed for the presence of turbidity or yellow precipitation.

Terpenoid and steroid

Four milligrams of ethyl acetate fraction was treated with 0.5 ml of acetic anhydride and 0.5 ml of chloroform. Then concentrated solution of sulphuric acid was added slowly and red violet color was observed for terpenoid and green bluish color for steroids **(Siddiqui and Ali, 1997)**.

Flavonoid and flavones

Four milliliters of ethyl acetate fraction solution was treated with 1.5 ml of 50% methanol solution. The solution was warmed and metal magnesium was added. To this solution, 5- 6 drops of concentrated hydrochloric acid was added and red color was observed for flavonoids and orange color for flavones **(Siddiqui and Ali, 1997)**.

Tannins

In 0.5 ml of ethyl acetate fraction solution 1 ml of water and 1-2 drops of ferric chloride solution was added. Blue color was observed for gallic tannins and green black for catecholic tannins **(Iyengar, 1995)**.

Saponins

Amount about 0.5 gm of the ethyl acetate fraction was dissolved in distilled water in a test tube. Frothing which persisted on warming was taken as preliminary evidence for saponins (**Evans, 2002**).

Glycoside

Glycosides are compounds which upon hydrolysis give rise to one or more sugars (glycones) and a compound which is not a sugar (aglycone or genine). To the solution of the ethyl acetate fraction in glacial acetic acid, few drops of ferric chloride and concentrated sulphuric acid are added, and observed for a reddish brown coloration at the junction of two layers and the bluish green color in the upper layer **(Siddiqui and Ali, 1997)**.

Results of phytochemical analysis

Phytochemical analysis of the ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* revealed that alkaloids, flavonoids and tannins are present in ethyl acetate fraction but saponins, terpenoids, steroids and glycosides are absent in the ethyl acetate fraction of seed of *H. antidysenterica* **(Table 9.1)**.

Discussion

The present study was performed to find out the possible chemical nature of the phytoconstituent(s) present in the ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica*. The phytochemical studies of ethyl acetate fraction of seed of *H. antidysenterica* resulted the presence of alkaloids, flavonoids and tannins. Since various flavonoids and alkaloids and tannins have been reported to possess antidiabetic as well as antioxidative efficacies **(Singh and Dhawan, 1997; Ljubuncic et al., 2006)** so, the ethyl acetate fraction of *H. antidysenterica* executed the remedial effect on diabetic complications due to presence of above said phytoingredient (s) which is consistent with our previous reports in this line **(De et al., 2010; Ali et al., 2011)** as well as supported by other reports **(Loew and Kaszkin, 2002; Hazra et al., 2008)**.

Conclusion

Results of this study indicated that the ethyl acetate fraction of seed of *H. antidysenterica* is rich in alkaloids, flavonoids and tannins which may be responsible for the correction of diabetic complications in STZ induced diabetic rats.

Table 9.1 Qualitative phytochemical analysis of ethyl acetate fraction of seed of *H. antidysenteria* ('+' = present; '-' = absent).

Qualitative tests	Ethyl acetate fraction
Alkaloids	+
Flavonoids	+
Saponins	_
Terpinoids	_
Tanins	+
Steroids	_
Glycosides	_

Experiment No 09A

Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) of phytoingredient (s) present in ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *Holarrhena antidysenterica*

The analytical RP-HPLC analysis for ingredients isolation was performed on BLC-20 Liquid chromatography system, BUCK Scientific, equipped with Buck Separation and management Module, BUCK UV-VIS Detector, manual injector with binary pump system. Haisil 100 C18 (4.6 x 250 mm, 5 µm) column was used for RP-HPLC analysis (Higgins Analytical Inc, S/N-151856) using a premixed solvents (n-Hexane: Ethyl acetate: THF acid :: 60:40:0.5) as mobile phase with a flow rate of 1.0 mL/min and isocratic elution technique. The column temperature was maintained at 30 ºC and detection was performed at 336 nm.

For the HPLC analysis, fractionate was dissolved with the mobile phase to prepare the solutions of 1 mg/mL. The sample solutions were sonicated for 15 min and then filtered through Millipore Millex syringe filter unit (0.45 µm). 25µl of sample solution was injected through manual injector port with 200µL syringe. All the data of HPLC system was acquired and processed using Buck HPLC management software.

Chromatographic reflection indicated that three compounds are present in ethyl acetate fraction of aqueous-methanol (40:60) extract of seed of *H. antidysenterica* **(Fig. 1)**.

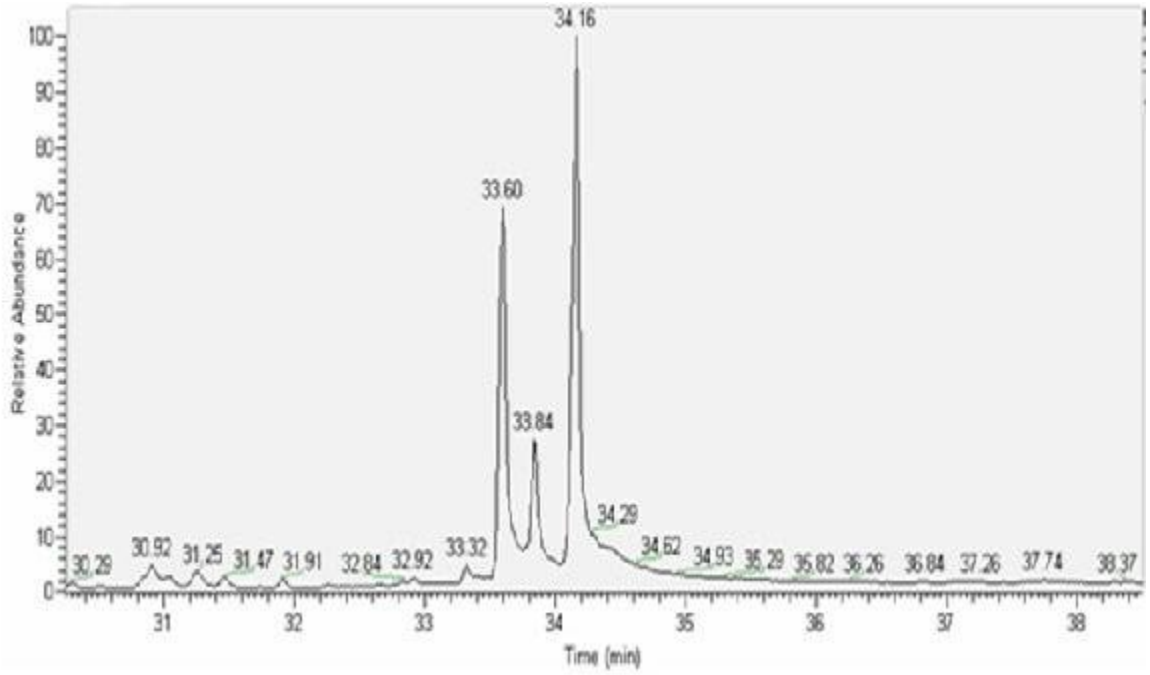

Figure 9.1 Reverse Phase-High Performance Liquid Chromatography of ethyl acetate fraction of aqueous-methanol :: 40:60 extract of seed of *H. antidysenterica* on C_{18} column at 336 nm.

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