ASSESSMENT OF β-AESCIN EFFECT IN STREPTOZOTOCIN INDUCED DIABETIC MODEL: DIABETIC HEPATOTOXICITY STUDY

ABSTRACT

Objective - Diabetic hepatotoxicity involves complex events which include kupffer cell activation, formation of reactive oxygen species, cytokines release (TNF-α, IL-1β), and finally leads to hepatocyte death. "β- Aescin showed anti-inflammatory, anti-oxidant, gastroprotective and anti-oedema properties. The present study investigated the protective effect of β- Aescin in streptozotocin induced diabetic hepatotoxicity.

Method - Female mice were divided into six groups, the first group served as the control, the second to sixth group received single i.p. dose of 90 mg/kg of STZ, the second group served as the untreated diabetic group, the third, fourth and fifth group received β- aescin intra-peritoneally at the dose of 0.9 mg/kg, 1.8 mg/kg and 3.6 mg/kg body weight respectively. The last sixth group was treated with 10 mg/kg glibenclamide i.p. for 14 days. A significant decrease in the blood glucose level was showed in β-aescin group as compared to the control group.

Result - A significant increase of blood glucose level was observed in high and mid dose of β- aescin (3.6 mg/kg and 1.8 mg/kg respectively), standard drug (glibenclamide 10 mg/kg) groups as compared to control group. ROS generation was evaluated by using DCF-DA estimation method for the acute toxicity in liver tissue. Streptozotocin group showed more ROS generation in comparison to β- aescin group (3.6 mg/kg). Serum biochemical markers showed a significant decrease in β- aescin treated diabetic mice compared to untreated diabetic mice. Histopathological evaluation showed severe changes in untreated diabetic liver tissue marked by large number of inflammatory cells such as lymphocytes along with hepatic sinusoidal inflammation and hepatocyte necrosis whereas treated diabetic mice with β- aescin showed reduction in hepatotoxicity marked by regeneration changes of hepatocytes and mildly hepatocyte degeneration.

Conclusion - In the study, β- aescin showed beneficial effects on the efficient properties of the liver and microscopic improvements in diabetic hepatotoxicity.

Keywords: *β- aescin, Diabetic hepatotoxicity, hepatic sinusoidal inflammation, Streptozotocin.*

1. INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by chronic increase of blood glucose. Diabetes is accompanied with long-term complications such as neuropathy, retinopathy, nephropathy and cardiovascular diseases[22]. However, hepatotoxicity did not receive as much attention as other prevalent complications until hepatotoxicity of antidiabetic drugs emerged as a common clinical complication[13]. Diabetic hepatotoxicity involves complex events which include kupffer cell activation, formation of reactive oxygen species and infiltration of neutrophil, cytokines release (TNF-α, IL-1β), and finally leads to hepatocyte death [1]. Phytochemicals derived from natural plants have been used commonly for the prevention or treatment of different diseases due to the extended belief of their therapeutic properties and safety[7]. β-Aescin is the active principle obtained from *Aesculus hippocastanum* (Hippocastanaceae), has shown anti-inflammatory, anti-edematous, venotonic properties[2,3], chronic venous insufficiency and associated ulceration, post-operative oedema and hemorrhoids[4,5]. Recently it has been reported that it inhibiting the immigration of inflammatory cells and reduces liver damage by decreasing the ALT (Alanine transaminase), AST (Aspartate aminotransferase), TNF-α (Tumor necrosis factor), IL-1β (Interleukin-1), 11β-HSD2 (11 beta-hydroxysteroid dehydrogenase)[6]. In this study, we investigated the hepatoprotective effect of β-Aescin in mice model of Streptozotocin diabetes- induced liver damage.

2. MATERIALS AND METHODS

2.1 Drugs and Chemicals

Alpspure Life Sciences Private Limited, Delhi, provided beta aescin (95 percent) oral organization (India). Streptozotocin, Ethanol, Carboxy methylcellulose sodium (CMC) was procured from Central Drug House Pvt. ltd, Delhi (India), DPX mountant was procured from Sisco Research Laboratories Pvt. Ltd, and Glibenclamide was Central Drug House Pvt. Ltd., Delhi, supplied the isopropyl alcohol (India). Dr. Morepen glucoone glucometer was used to check the instant glucose (Morepen Laboratories Ltd). All of the chemicals and biochemical agents is analytical grade and used in freshly formulated solutions.

2.2 Animals

Animal studies were conducted in accordance with the international guidelines and protocol approved by institutional animal ethical committee 1362/PO/Re/S/18/CPCSEA. Adult Female Swiss albino mice (n=) [(Mus musculus), 20-25g (~5 weeks)] were utilized for the examination. All animals were housed in experimental rooms with controlled temperature (23±2°C), humidity (60-70%) and standard 12 hours day/night cycle. Mice were maintained on a standard pellet diet (Hindustan lever cow pellet) and water ad libitum.

2.3 Induction of diabetes

Diabetes was induced in mice by a single intra-peritoneal injection of streptozotocin (90 mg/kg bwt.) with prior a dose of nicotinamide (120 mg/kg) in normal saline while control group were received only normal saline[15,23,24]. Diabetic mice that are induced by STZ-NA are useful to study molecular characteristics of diabetic complications and evaluation of the anti-diabetic prospective of natural compounds as it partially damages pancreatic β-cells[15,23]. Animals showed hyperglycemia were selected and grouped (n=6, uniformly distributed over glucose levels ~250 mg/dl) to start the experiments.

2.4 Experimental Design

Following four weeks of diabetes induction, diabetic mice were split into six classes (G1 to G6), along of five to six species. The mice in Group 1 (G1) obtained normal saline as a control. Group 2 (G2) diabetic mice were not given any medications and acted as streptozotocin-induced diabetic mice. β-aescin was administered intraperitoneally to mice in groups 3, 4, and 5 (G3, G4, and G5) at doses of 0.9 mg/kg, 1.8 mg/kg, and 3.6 mg/kg body weight, respectively. G6 mice were given 10 mg/kg glibenclamide intraperitoneally for 14 days. A 0.5 percent CMC solution was provided to mice in the non-diabetic and diabetic control classes. β-aescin was dissolved in a CMC (Carboxy Methyl Cellulose) solution comprising 1 percent w/v CMC[8]. An i.p. injection of ketamine was used to anesthetize the mice at the conclusion of the trial.

2.5 Blood glucose level estimation

A Dr. Morepen Glucoone glucometer was used to test blood glucose levels in tail vein blood prior to diabetes induction (Morpen laboratories ltd.). The value >600 mg/dL registers as ‘HI’ in the range 20-600 mg/dL. The concentration of ‘HI’ was measured at 600 mg/dL. Blood glucose and animal wellbeing were tested three times a week after the streptozotocin injection. As blood sugar levels approached 250 mg/dL, diabetes was diagnosed. To minimize fluctuations in blood glucose levels, all blood glucose tests were taken in the fed state early in the afternoon.

2.6 Assessment of serum biochemical parameters

Swiss mice were distributed in different groups of five animals each. Behavior, clinical signs and mortality were monitored at 30 min, 1h, 2h, 4h and 8h intervals post dosing on the first day and at least once a day for the next 14 days. On 15th day, blood samples were taken by cardiac puncture and serum was collected by centrifuging blood samples at 3000 rpm for 5 min and stored samples at -20°C for the evaluation of biochemical changes and mortality at 15th day was recorded. Serum levels of Alkaline phosphatase (ALP), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Total bilirubin (Tbil) and albumin (ALB) were assessed using Fujifilm biochemical instrument.

2.7 Histopathological examination of liver tissue

A histopathological analysis of liver tissue was carried out to determine inflammatory lesions. On day 15, the animals were anesthetized (Ketamine 0.2 mg/kg) and the liver was checked for any gross pathological shifts. The liver tissue was established and then extracted from the body of the animal; their weight was registered. The liver tissue was held at room temperature for 72 hours in a 10% formal saline solution. The liver tissue was formalin fixed and dehydrated in an ethanol gradient before being deposited in paraffin blocks. These blocks were deparaffinized, rehydrated, and stained with hematoxylin and eosin after being sliced into 4 m thin pieces (H & E). The stained slide pieces were then analyzed using a light microscope.

2.8 ROS (Reactive Oxygen Species) generation

To obtain a homogeneous cell suspension, liver tissue samples were homogenized in lysis buffer on ice. The composition of the tissue samples was then standardized using BCA protein estimation[14]. DCF-DA is oxidized by ROS, resulting in a fluorescent DCF compound. The degree of ROS is shown by the fluorescent strength of DCF. For 30 minutes at 37°C in the dark, an equivalent number of tissue samples were incubated with 25M of DCF-DA. At excitation: 485 nm and emission: 520 nm, DCF fluorescence was measured using a plate reader (Fluostar Omega, BMG).

2.9 Statistical analysis

Statistical analysis in all the above studies was performed using Graph Pad Prism software version 5.1 (GraphPad Software, La Jolla, CA, USA). All results were expressed as mean ± S.D. Data were analyzed using one-way analysis variance (ANOVA), and followed by Dennet's multiple comparison tests. Value $P<0.05$ and below were considered significant.

3. RESULT

3.1 Induction of diabetes in mice

The result indicated the successful induction of type-2 diabetes in mice[24]. The blood glucose was recorded after 7 to 14 days after the last treatment. The significant increase in glucose level was found 150 to 210 mg/dl, whereas decreases in body weight were also found in type-2 diabetes as shown in figure1.

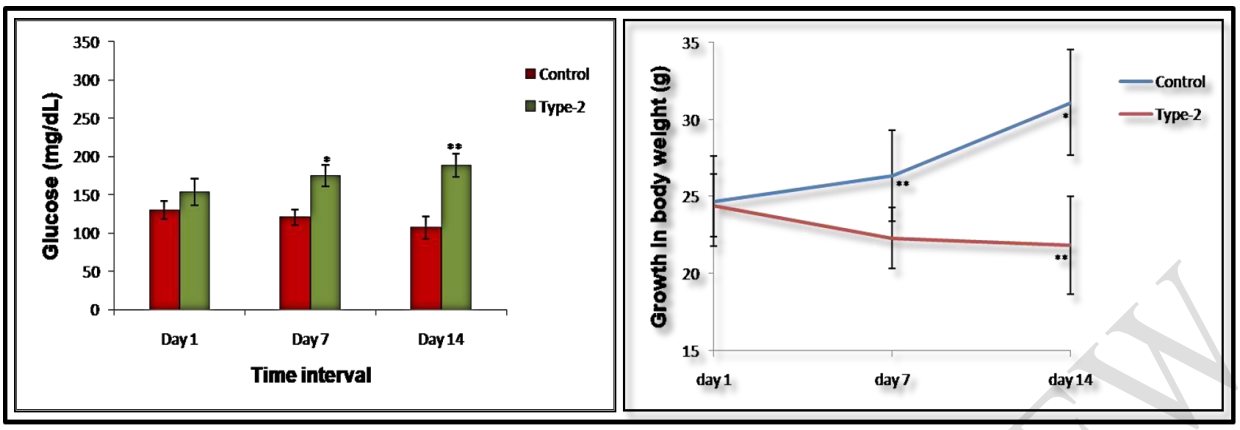

Figure 1: Induce blood glucose and reduce in body weight growth were found in type-2 diabetic mice group with compare to control group. Data were represented in mean ± SD;, ** denotes statistically significant difference at $p < 0.05$, $p < 0.01$ as compared to control respectively.*

3.2 Blood glucose level estimation

A significant increase in blood glucose level showed in fasted mice due to the induction of streptozotocin in all groups as estimated before the treatment of β- aescin group. A significant decrease in the β- aescin was showed as compared to the control group, as shown in the figure 2. A significant increase of blood glucose level was observed in high and mid dose of β- aescin (3.6 mg/kg and 1.8 mg/kg respectively), standard drug (glibenclamide 10 mg/kg) groups.

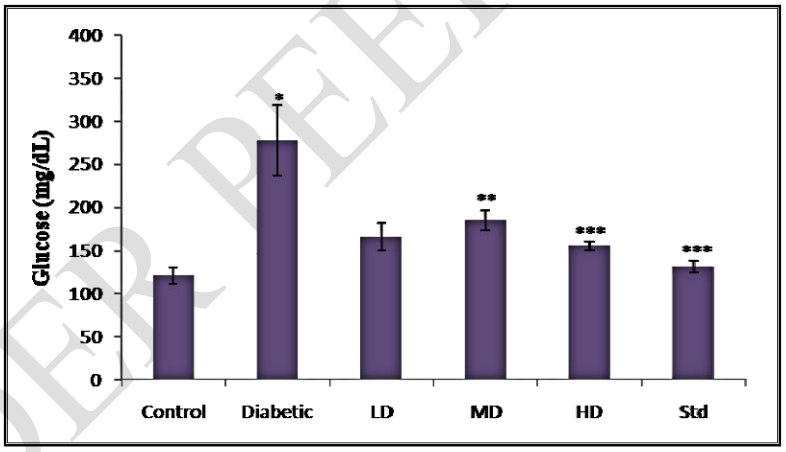

*Figure 2. Blood glucose level in female swiss mice. LD- Low Dose; MD-Mid Dose; HD- High Dose; Data were represented in mean ± SD; n= 6 animals per each group; *, **, *** denotes statistically significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$ as compared to control respectively.*

3.3 ROS (Reactive Oxygen Species) Generation

ROS generation was evaluated for the acute toxicity in liver tissue. The treatment of β- aescin and Glibenclamide after the induction of diabetes was compared to positive control (Strepozotocin) group and control group. Streptozotocin group showed more ROS generation in comparison to β- aescin as shown in figure 3. An astonishing high generation of free radicals was found in liver tissue at the low dose ($p < 0.05$) and high dose ($p < 0.01$) of β- aescin as compared to control group.

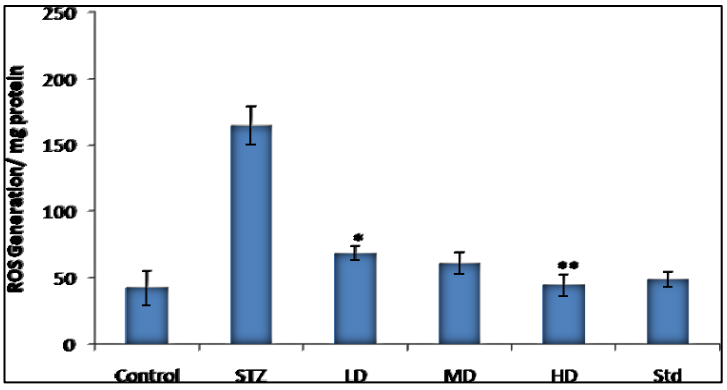

*Figure. 3. ROS generation in liver tissue. STZ- Streptozotocin, LD- Low Dose, MD- Mid Dose, HD- High Dose, Std- Standard Drug. Data were represented in mean ± SD; n=6 animals per each group; *, ** denotes statistically significant differences at p < 0.05, p<0.01 as compared to control respectively.*

3.4 Alteration in serum biochemical parameters

The Biochemical analysis of serum showed an increased ALP, ALT, AST, Albumin and Total bilirubin level in diabetic group (G2) as compared to control group (G1). In previous literatures it was showed that mice were received single dose of STZ showed an increase in glucose, AST and ALT levels [12]. It was also revealed in the present study. In high dose of β- aescin (G5) showed decrease effect in the level of ALP, ALT, AST and Total bilirubin. The mid dose of β- aescin (G4) showed an elevated level of ALP and ALT.

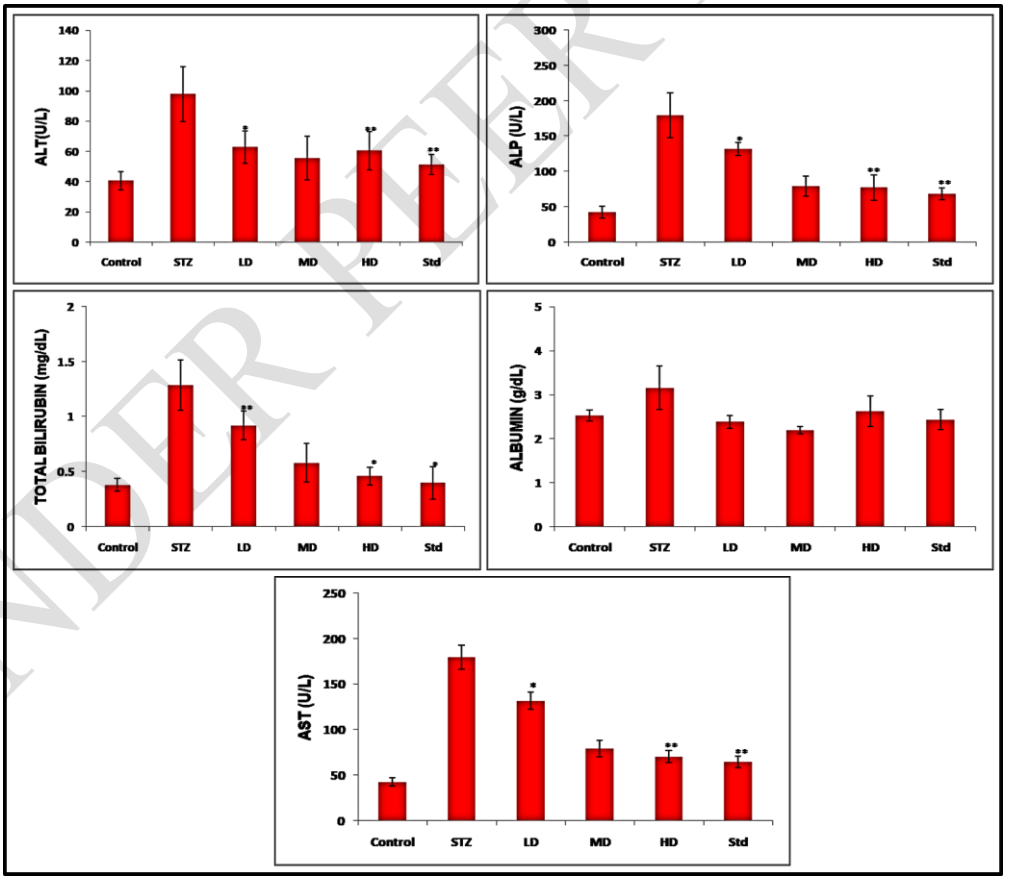

*Fig. 4. Alteration in serum hepatotoxic biomarkers in swiss mice after repeated 14 days treatment. Data were represented as mean ± SD; n=6 animals per groups; *, ** denotesstatisticallysignificantdifferencesatp<0.05,p<0.01ascomparedtocontrol respectively. STZ-*

Streptozotocin, LD- Low Dose, MD- Mid Dose, HD- High Dose, Std- Standard Drug.

3.5 Histopathological evaluation

The expression of hepatotoxic markers, some histopathological changes were observed in animals treated with β- aescin, standard drug and streptozotocin. H & E staining of liver sections showed various inflammatory cells and hepatocyte necrosis in streptozotocin group, mild hepatocyte degeneration in mid dose of β- aescin and marked regeneration changes of hepatocytes in high dose of β- aescin and standard treated group.

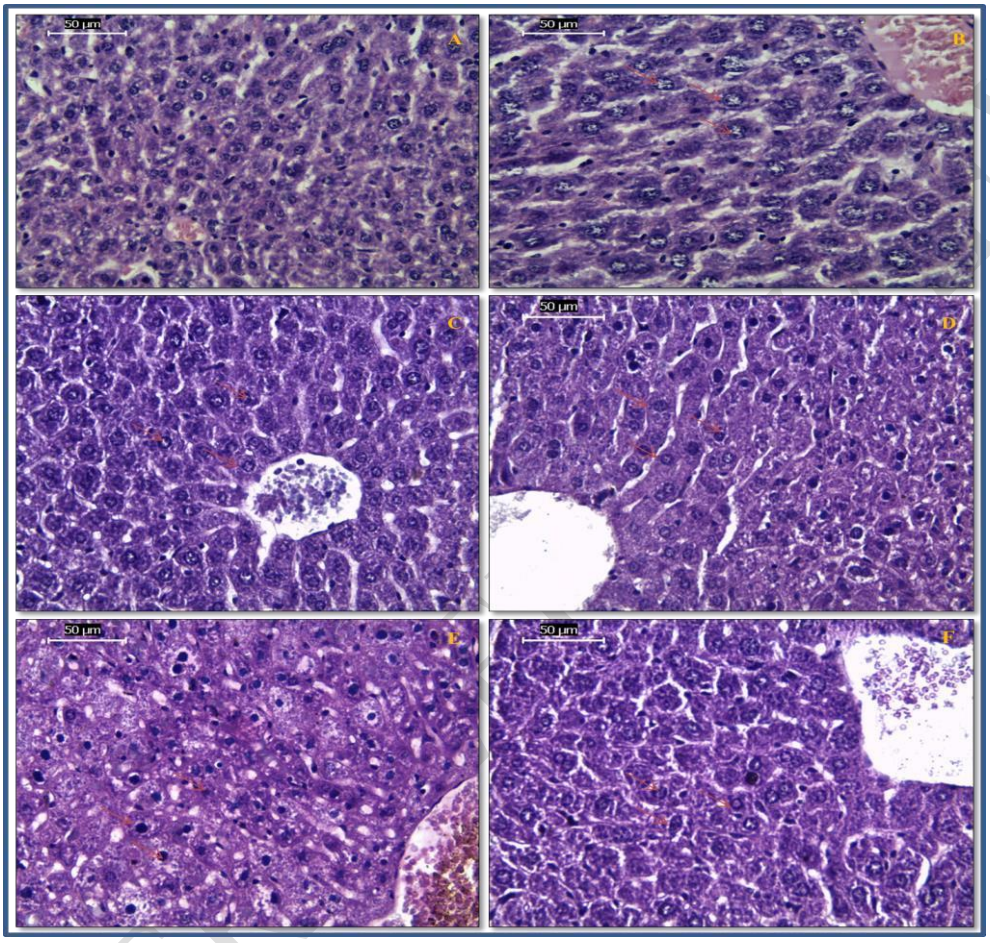

Fig. 5. Histopathological evaluation of liver tissue of female Swiss mice at (H & E, x100) (A) Normal control; (B) STZ control; (C) β- aescin 0.9 mg/kg treated; (D) β- aescin 1.8 mg/kg treated; (E) β- aescin 3.6 mg/kg treated; (F). Glibenclamide 10 mg/kg treated.

Table 1. Histopathological changes in liver of mice treated with β- aescin

Groups	Observations	Score
Normal control	No changes show in liver tissue	0
STZ control	Various number of inflammatory cells such as lymphocytes along with hepatic sinusoidal inflammation and hepatocyte necrosis	+++
β- aescin 0.9 mg/kg treated	Less congestion of inflammatory cells and hepatocyte necrosis	++
β- aescin 1.8 mg/kg treated	Mildly hepatocyte degeneration	+
β- aescin 3.6 mg/kg treated	Marked regenerative changes of hepatocytes	*
Glibeclamide 10 mg/kg treated	Area of regeneration and dark nucleus	*

0: indicates no score is evaluated.

+++ (Severe) indicates when ≥ 50% area of liver is affected by inflammatory cells and necrotic lesions.++ (moderate) indicates when 25-50% area of liver is affected by inflammatory cells and necrotic lesions.

+ (mild) indicates when ≤ 25% area of liver is affected by inflammatory cells and necrotic leaions.

* indicates when regenerating area and eminent nucleus has been observed.

4. DISCUSSION

Type-2 diabetes is a multi-organ, multi-factorial condition characterized primarily by insulin resistance, hyper insulinaemia and β-cell dysfunction, which ultimately leads to β- cell failure[13]. A well-known diabetogenic agent i.e. Streptozotocin is exerting cytotoxic action on pancreatic beta cells and liver tissue[21]. Streptozotocin produced rapid and irreversible necrosis of cells, partially damage islets, triggering an inflammatory process causes macrophages and lymphocyte infiltration followed by insulin deficiency[21,23]. Diabetic mice that are induced by STZ-NA are useful to study molecular characteristics of diabetic complications and evaluation of the anti-diabetic prospective of natural compounds as it partially damages pancreatic β-cells[15,23]. Aescin showed positive effects on blood glucose homeostasis and histology of β- cell of pancreatic islets (Islet of Langerhans)[20]. Many studies revealed that mice that received single dose of STZ showed an increase in glucose, AST and ALT level[12]. The serum ALP, AST and ALT were selected for monitoring liver damages as they are good indicators of hepatic dysfunction[18]. In our study revealed that on administration of STZ there was an increase in serum hepatotoxic biomarkers and the high dose of β-aescin (3.6 mg/kg) showed decrease effect in the level of ALP, ALT, AST and Total bilirubin. Aldehydes generated endogenously during lipid peroxidation contribute to the pathologic effects associated with the oxidative stress in cells and tissue[12]. In present study we assessed that serum glucose level was attenuated after the administration of β- aescin in streptozotocin induced hepatotoxicity in mice. Results of the β- aescin group were comparable with that of the glibenclamide administered group for further validating the beneficial effect of β- aescin. In previously literatures it was reported that β- aescin showed hepatoprotective effect in CCl4 induced hepatotoxicity in rats [8]. Aescin also showed anti-inflammatory, anti-oxidant, gastroprotective and anti-oedema properties [9]. Escin also showed protective effect on acetaminophen induced hepatotoxicity via inhibition of ERK signaling pathway [10]. β- aescin is a triterpenoid saponin obtained from the seeds of horse chestnut [11]. β- aescin is principally made up of aescin Ia and aescin

Ib[19]. In the present study, administration of single dose of STZ caused severe histopathological changes represented by various number of inflammatory cells such as lymphocytes along with hepatic sinusoidal inflammation and hepatocyte necrosis, whereas few literatures revealed that STZ also played an important role in dilation of veins, loss of usual arrangement of hepatocytes and accumulation of lipid droplets in the cytoplasm of hepatocyte[22]. In our study administration of 3.6 mg/kg β-aescin for 14 days revealed the reduction of hepatotoxicity in diabetic mice STZ induced marked by regeneration of hepatocyte cells and no inflammatory cells were presented.

5. CONCLUSION

The present study aimed to investigate the effect of β- aescin in STZ induced diabetic mice. It may be concluded that a dose dependent treatment with β- aescin shows hepatoprotective effect against STZ-induced hepatic damage by inhibiting oxidative stress, histopathological changes and improve injured liver. Hence, the finding suggests that β- aescin has protective effect on STZ induced diabetic hepatotoxicity by reducing glucose level and reduce oxidative stress in liver.

COMPETING INTERESTS DISCLAIMER

None of the authors has a conflict of interest to declare.

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