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ORIGINAL ARTICLE

Antihyperglycemic Activity of *Achyranthes aspera* Linn. Leaves Extract by Modulation of β-cell Functioning in Streptozotocin-Induced Diabetic Rats

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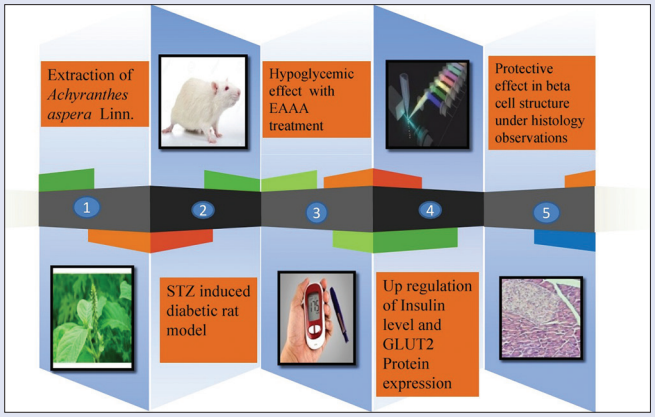
ABSTRACT

Objective: The objective of the study is to study the anti-hyperglycemic potential of Ethyl Acetate dissolved fraction of methanolic extract of *Achyranthes aspera* Linn. (EAAA) leaves on STZ induced diabetic rat model. **Materials and Methods:** Safety study of EAAA was carried out with different doses up to 2000 mg/kg. Hyperglycemia was developed in Sprague Dawley male (SD) rats by only one dose of streptozotocin 55 mg/kg in 0.1 M Citrate buffer by intraperitoneal route. Hyperglycemic rats were treated with EAAA 50,100mg/kg and Metformin 120 mg/kg body wt. Biological specifications such as glucose level in blood, insulin level in serum and glucose transporter (GLUT-2) protein expression were measured. In addition, histopathological study of pancreatic tissue was performed to check changes occurred in β-cell complex. **Results:** No mortality was observed during safety study. Weight of treated rats was found to be increased whereas glucose level in blood samples of EAAA treated rats were showed improvement as compare to control groups. The result shows up-regulation of serum insulin levels and small changes in the expression of GLUT-2 after 4-week treatment with the EAAA treatment. The presence of polyphenols in EAAA may have provoked ingestion of glucose by changing the glucose transporter. It has been identified by many researchers that herbal extracts which are rich in polyphenols are responsible for debilitating insulin resistance and thus restrain glucose uptake by expressing GLUT-2 in pancreatic cells of diabetic rats. Histological analysis demonstrated improvement in the structural decay of β-cells of pancreatic tissue. **Conclusion:** The results indicate the thinkable effect of EAAA in STZ-induced diabetic Rats.

Key words: *Achyranthes aspera,* beta cells, GLUT2, insulin, streptozotocin

SUMMARY

- The current research reveals attenuating effects of ethyl acetate dissolved fraction of methanolic extract of *Achyranthes aspera* (EAAA) Linn. on hyperglycemia and the same clarifies that flavonoids are responsible for its protective action toward beta cells. Thus, EAAA can be a potential herb for patients suffering from diabetes and its complications.



Abbreviations used: EAAA: Ethyl acetate fraction of *Achyranthes aspera* Linn.; STZ: Streptozotocin; GLUT: Glucose transporter; OECD: Organization of economic cooperation and development; ELISA: Enzyme-linked immunosorbent assay.

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INTRODUCTION

There is estimated 4% of people in the world suffered from diabetes, The percentage is quiet high as that was estimated a decade ago.[1] This count may increase up to 5.4% again after a decade. The World Health Organization pinpoints that diabetes mellitus is deadly killer nowadays. Different hypoglycemic agents such as biguanides additionally sulfonyl urea are currently available to control blood glucose level in diabetes mellitus patients. These agents show side effects and thus seeking for a different compound is essential to nullify these drawbacks.[2]

Half of the world's people who are suffering from such deadly diseases and disorders are using traditionally used medicines which are also mentioned in Ayurveda.[1,2] There are many formulations for the treatment of these chronic illnesses but traditional medicines are always preferred just because its low cost and bearable side effects.[3-5]

The information which is available in literature help us to mention that *Achyranthes aspera* is a significant herb for enormous medicinal values. It is just because of it contains large number of chemicals which are having medicinal properties.[5-7] Leaves of *Achyranthes aspera*

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contains various compounds like alkali like compounds, bioflavonoids, proanthocyanidins, sapogenin, and many more.[8] *Achyranthes aspera* leaves are used for the treating eye disorders, autoimmune diseases, fertility-related problems and kidney disorders. It has been proved that female rats showed antifertility effect after getting treated with methanolic fraction of *Achyrathes aspera* leaves. Antihyperglycemic effect, change in lipid levels, diuretic effects, fever control, pain relief, inflammation control, antioxidant, and antimicrobial properties have also claimed in literature before. The leaves of the same plant show curative effect on tumor cells particularly ductal carcinoma and cancer of cervix via P pathway. The Pentose Phosphate Pathway (PPP), it is a step of glucose metabolism, it synthesizes ribonucleotides and produce NADPH. NADPH is required for the scavenging of reactive oxygen species (ROS). Therefore, the P Pathway helps glycolytic cancer cells to fulfill their anabolic demands and fight oxidative stress. In case of diabetes oxidative stress plays major role for damaging pancreatic cells. Hence, leaves of *Achyranthes aspera* may be beneficial. Moreover, leaves are also used for the treatment of sore throat, bowel defects, hemorrhoids, stomach pain, rash on skin, dysentery, and respiratory problems as well.[3,8-10]

MATERIALS AND METHODS

Ethical considerations

Experimental protocol was presented in front of the members of Institutional Animal Ethics Committee (IAEC) of the PRADO Pvt. Ltd. (Preclinical Research and Development Organization), Pune and got approved by all members.

(Approval No.-1723/PO/RcBiBt/13/CPCSEA).

Extraction plant material

Collection and authentication of plant material *Achyrantes aspera* Linn. were carried out from Sri Venkateswara University Tirupati, India. A Voucher number is (2120).

Shade dried and powdered leaves of *Achyranthes aspera* were macerated with petroleum ether for 24 h [Figure 1]. The extraction of residue was carried out by a scientific method, i.e., soxhlet extraction and the solvent used was methanol. The obtained solvent was evaporated to one-third and residue was extracted with the help of ethyl acetate and water (1:8:2, i.e., 1 ml residue +8 ml ethyl acetate +2 ml water) with separating funnel. Filtrate was evaporated to get dry mass of ethyl acetate dissolved fraction of methanolic extract of *Achyranthes aspera* Linn. (EAAA).[5] This method is specifically for flavonoid extraction. In diabetes the damage to tissue is because of oxidative stress and flavonoids are good antioxidants so we used this fraction for further study.

Experimental animals

Sprague Dawley (SD) male rats weighing between 180 and 250 g were selected. Temperature and relative humidity was 25°C ± 2°C and 45%–55% respectively under normal environmental conditions. The rats had easy access to standard rat food and water *at libitum*. The activities were performed between 9 and 18 h a day.

Induction of diabetes

Hyperglycemia was produced by injecting only one dose of newly prepared STZ (55 mg/kg body weight, in 0.1M citrate buffer of pH 4.5) in intraperitoneal cavity of fasting SD rats.[7,11] Supply of 5% glucose solution throughout the night was helpful to control STZ-induced sudden hypoglycemic crises.[11,12] Hyperglycemia was confirmed at the 3rd day of STZ injection by estimating glucose level estimated by Glucometer (One touch). The rats having blood sugar level near or above 300 mg/dl were selected for the research.[6]

Experimental designs

Safety study reports indicated that 2000 mg/kg dose is safe in rats no mortality and no toxic effects were observed. In the literature, water extract of *Achyranthes aspera* was proved to be safe and effective below 500 mg/kg also. We extracted flavonoids in maximum amount by ethyl acetate fraction so we decided to select two doses 50 mg/kg and 100 mg/kg. Rats were divided in five groups each group contains a count of six rats. Group I termed as control as it contains healthy rats. Group II was diabetic control, Group III and IV treated with oral dose of EAAA 50 mg/kg and 100 mg/kg, respectively. Group V was served as standard control group which received 120 mg/kg of metformin (oral route). Fasting blood glucose was taken initially and after 4 weeks of treatment of extract animals were checked for antihyperglycemic effect. Blood serum was tested for insulin level and pancreatic tissue underwent flowcytometry analysis for finding out protein expressions of glucose transporter (GLUT-2). This was carried out in all groups. In support to this, histopathological studies were carried out for pancreatic tissue to figure out the changes in structure of β-cell.

Collection of blood and determination of blood glucose

Blood samples from the rats of experimental groups were collected from tail vein. The samples so collected were analyzed for glucose by using glucometer.

Biochemical parameter estimation

Serum insulin

ELISA enzyme-linked immunosorbent assay Krishgen insulin ELISA, Catalog No. SE120086 of Sigma Aldrich was used for the estimation

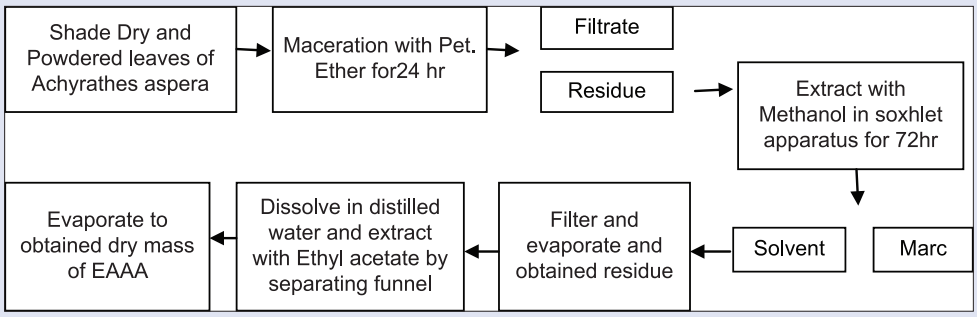

Figure 1: Schematic Representation of Scientific Extraction Method

of Insulin in serum samples. The Rat Insulin ELISA Kit was intended for the quantitative measurement of insulin in rat serum. It was a solid phase two-site enzyme immunoassay. It was based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample reacted with enzyme (HRP)-conjugated anti-insulin antibody and anti-insulin antibody bound to microplate well. A simple washing step removed unbound enzyme labeled antibody. The bound HRP complex was detected by reaction with TMB substrate. The colorimetric endpoint detected using ELISA reader at 450 nm.

Sample preparation

Blood samples were collected and serum was separated immediately. Specimens refrigerated at 2°C–8°C. Frozen sera were mixed well before starting with assay. Enzyme conjugate and Buffer were prepared according to protocol.

Procedure

Coated strips were placed into the holder. Insulin standards 25 ml control and sera were poured into wells. About 100 ml of working insulin enzyme conjugate was added to all wells followed by complete mixing and Incubated for 60 min at room temperature (18°C–26°C). Wells were cleaned with buffer for three times. TMB Substrate were added to all well and then incubated for 15 minutes at room temperature. Stop solution 50 ml was added to all wells and mixed properly. Absorbance was recorded on ELISA Reader at 450 nm within 15 min after adding the Stopping Solution. Standard curve constructed as per the protocol.

GLUT2 receptor

Tissue preparation

Mince tissue into 3 to 4 mm pieces with a sterile scalpel or scissors and washed several times with Hanks' Balanced Salt Solution (HBSS). The collagenase (50 to 200 U/ml in HBSS) was then added to it. It was incubated at 37°C for 4 to 18 hr and 3 M $CaCl_2$ was added to increase the efficiency of dissociation. The cell suspension was filtered through a sterile nylon mesh to separate the dispersed cells and the tissue fragments from the larger pieces. The fresh collagenase was added to the fragments for further disaggregation of the suspension. The suspension was washed several times by centrifugation in HBSS. The pellets were suspended in the culture medium, counted and seeded the cells for culture and further treatment.

Measurement of mean fluorescent intensity for GLUT2 by flowcytometry

Single cell suspension was prepared with collagenase treatment and single cells were filters through cell strainer. Single cells were washed 3 times with Phosphate Buffer Solution (PBS), Cells were counted and 50000 cells were stained with GLUT2 polyclonal antibody (Catalog # PA5-11567) (4 µl per sample, incubated on icepack for half an hour. Cells were cleaned thrice with PBS. Cells were incubated with FITC-secondary antibody (4 µl per sample, incubated on ice for 30 min.). Analysis of pancreatic cells were performed by using Attune NXT flow cytometer from Thermo fisher for mean florescent intensity.[7]

Histopathological examination of the pancreas

The rats were sacrificed after 4 weeks; the pancreatic tissues were trimmed properly and processed by alcohol-xylene as per standard protocol. The processed tissues then embedded in paraffin wax and sections were cut of 3–5 µm and slides were prepared. The slides were stained by hematoxylin-eosin staining method and mounted with DPX mount ant and observed under microscope. Interpretations of the slides with the help of computer fitted microscope with camera.[7]

Statistical analysis

The results are denoted as mean ± standard error of the mean. GraphPad Prism was used for analysis of complete data. Analysis includes One Way Analysis of Variance along with the Bonferroni test for *post hoc* multiple comparison between treatment groups. Statistical significance was set at $P < 0.05$.

RESULTS

Preliminary phytochemical evaluation of powder of *Achyranthes aspera* Linn. leaves and EAAA is indicated in Table 1.

Safety study

Safety study was carried out according to the guidelines of the Organization for Economic Co-Operation and Development (OECD 425) on normal rats with three different doses of EAAA. The rats were fasted overnight and the next morning fed with single dose of 500, 1000, and 2000 mg/kg body weight to three different groups respectively ($n = 3$). EAAA was administered in a single dose by oral feeding needle. All the rats were continually examined for 2 h to check any abnormalities in behavior of the animals and further continued to monitor and examine the rats for 24 and 72 h and no mortality were found. Table 2 represents the observed parameters during the safety study.

Effect of EAAA on body weight and blood glucose of hyperglycemic rats

Single-dose administration of STZ produced stable hyperglycemia. Figure 2 indicates hyperglycemic rats indicate sudden decline in body weight in correlation to Normal control group. EAAA 50 mg/kg, 100 mg/kg and Metformin 120 mg/kg treated animals showed minor changes in

Table 1: Phytochemical tests

Phytochemical test	Inference for powder	Inference for EAAA
Test for alkaloids		
Hager's test	+	+
Mayer's test	+	+
Dragendroff's test	+	+
Wagner's test	+	+
Test for carbohydrate		
Molisch's test	+	+
Fehling's test	+	+
Barfoed's test	+	+
Benedict's test	+	+
Test for phytosterols and triterpenoids		
Liebermann-Burchard test	+	−
Salkowski test	+	+
Test for glycosides		
Libermann's reaction	+	+
Test for saponins		
Froth test	+	+
Test for tannins		
Ferric chloride test	+	−
Lead acetate test	+	+
Congo red test	+	+
Test for flavonoids		
Lead acetate test	+	+
Shinoda test	+	+

+: Presence; −: Absence of particular constituent; EAAA: Extract of *Achyranthes aspera* Linn.

Table 2: Safety study for extract of *Achyranthes aspera* Linn. at 2000 mg/kg

Drug	Code	Toxicity		Number of death	Additional observation ANS/CNS								
		On set	Stop		Skin and fur	Eyes lacrimation	Salivation	Diarrhea	Respiration	Straub tail	Piloerection	Convulsions	
EAAA	Head	Nil	Nil	Nil	No	No	No	No	No	Yes	Yes	No	
	Back	Nil	Nil	Nil	No	No	No	No	No	Yes	Yes	No	
	Tail	Nil	Nil	Nil	No	No	No	No	No	Yes	Yes	No	
Drug		**Behavioral observation**								**Analgesia**	**Arching and rolling**	**Writhing**	
		Motor activity	**Stereotypy**	**Tremors**	**Catalepsy**	**Sedation**	**Hypnosis**	**Writhing**	**Muscle spasm**				
EAAA		No	No	No	No	No	No	No	No	No	No	No	
		Yes	No	No	No	No	No	No	No	No	No	No	
		Yes	No	No	No	No	No	No	No	No	No	No	

EAAA: Ethyl Acetate dissolved fraction of methanolic extract of *Achyranthes aspera* Linn.; ANS: Autonomic nervous system; CNS: Central nervous system

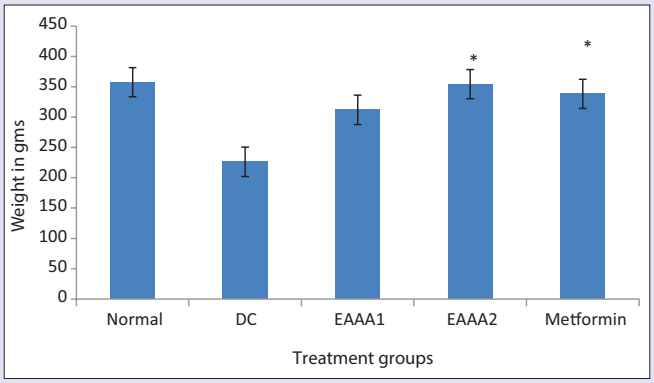

Figure 2: Effect of extract of *Achyranthes aspera* Linn. on the body weight level of diabetic rats during 28 days' treatment. All the values are expressed as the mean SE. * Indicates the significant difference when compared to the respective group ($P < 0.05$)

body weight as compare to normal control group. Hyperglycemic rats in each groups showed significant rise in serum glucose level as compared to Normal group I illustrated in Figure 3. Rats treated with EAAA 50 mg/kg, EAAA 100 mg/kg, and Metformin 120 mg/kg showed a highly significant ($P < 0.001$) fall in blood glucose with respect to control group.

Effect of EAAA on insulin and GLUT2 protein expression

Figure 4 indicates that the insulin level changes after treatment with EAAA in hyperglycemic rats. The level of Insulin was extremely reduced in case of diabetic group but it very significantly ($P < 0.0001$) gets restored with the treatment.

The expression of GLUT2 levels changes in Figure 5. Diabetic control group shows reduction in this protein but get regain its level (highly significant, $P < 0.0001$) near to normal after treatment of EAAA reflecting protective nature.

Histopathology of pancreas

Figure 6a-e emphasizes the effect of EAAA on structure of pancreatic beta cells. After 4 weeks of experiment, the sections of pancreatic beta cells collected from all groups were observed by the pathologist. It was observed that control group showed normal histopathology of pancreas. Diabetic control group showed severe destruction of islets Langerhans and β cells. In Group 3 and 4 (treatments group of EAAA) showed minimal, destruction of islets Langerhans and improved β cells. Metformin treated group showed moderate destruction of islets Langerhans and β cells.

DISCUSSION

Oral hypoglycemic agents which are available for treatment are having adverse effects such as weight gain, cardiovascular risks, and gastrointestinal effects, hence to overcome these drawbacks agents of herbal origin are truly important.[7,13] There is always a bright side for all times for traditional medicine systems specially medicinal plants and herbs. *Achyranthes aspera* is always revealing newer medicinal properties.[9] The useful properties are curative effects on allergies, nephron related problems, CVS diseases, inflammation, diabetes, pain, hepatitis and microbial infection.[5-8,14] It has been explained in literature that wound healing property of the *Achyranthes aspera* is may be because of its compounds of phenolic nature. They contain hydrogen donor group i.e., –OH group which

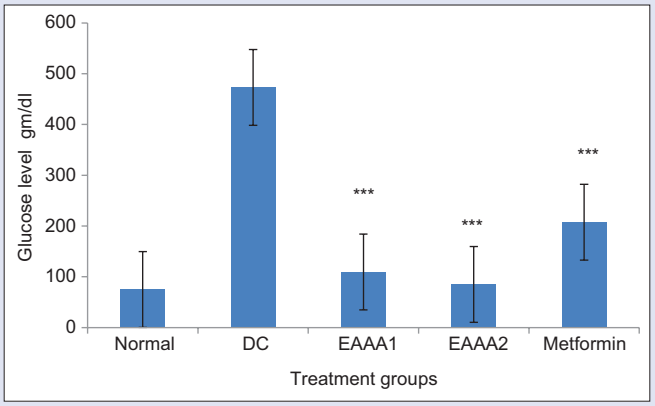

Figure 3: Effect of extract of *Achyranthes aspera* Linn. on the blood glucose level during 28 days treatment. All the values are expressed as the mean SE. *** Indicates the significant difference when compared to the DC group ($P < 0.001$)

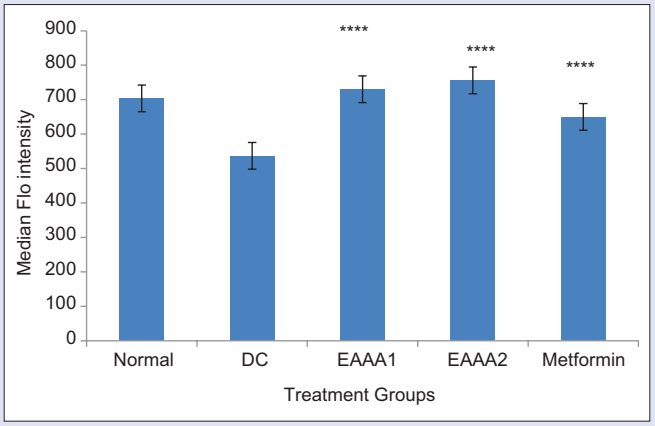

Figure 5: Effect of extract of *Achyranthes aspera* Linn. on the GLUT2 receptor level of normal and diabetic rats during 28 days' treatment. All the values are expressed as the mean SE. ****Indicates the highly significant difference when compared to the DC group ($P < 0.0001$)

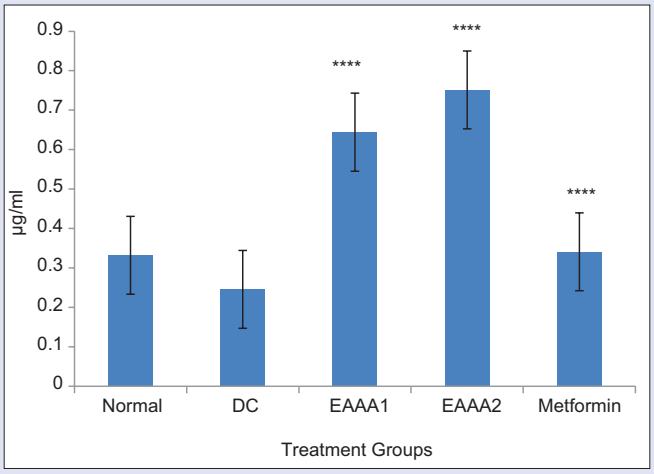

Figure 4: Effect of extract of *Achyranthes aspera* Linn. on the Insulin level of normal and diabetic rats during 28 days' treatment. All the values are expressed as the mean SE. #Indicates the significant difference when compared to the NC group. ****Indicates the highly significant difference when compared to the DC group ($P < 0.0001$)

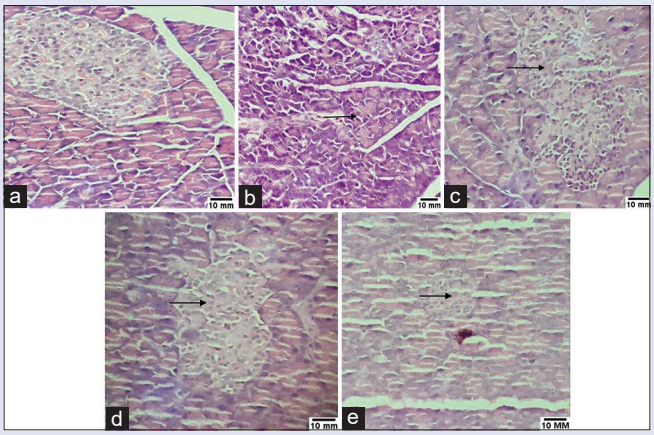

Figure 6: (a) The sections of pancreas of rats showed normal histopathology. (b) The sections of pancreas showed severe destruction of islets of Langerhans and β cells. (c) The sections of pancreas showed minimal destruction of islets of Langerhans. (d) The sections of pancreas showed minimal destruction of islets Langerhans and β cells. (e) The sections of pancreas showed moderately destruction of islets Langerhans and β cells

further responsible for strong hydrogen bonding with –COO group of protein, This ultimately results in forming protective layer which will be helpful in healing the wound.[8] Ameliorates oxidative stress.[13] Hence, *Achyranthes aspera* is found to be promising one and included in the present study. STZ induce rat model is one the useful animal model for studying antihyperglycemic activity.[7] The intraperitoneal injection of STZ is responsible for rapid production and release of proinflammatory cytokine including TNF-α which was responsible for tissue necrosis.[12] Degeneration of beta cells occurs because of oxidative stress.[7,11] Ethyl acetate fraction of *Achyranthes aspera* contains maximum amount of flavonoids as compare to water extract.[15] It has been proved that flavonoids possess hypoglycemic potential. Quercetin can improve glucose uptake in peripheral insulin, providing reduction in the blood glucose level, Kaempferol may improve generation of ATP in β-cells and provide an increase in the transcriptional activation of insulin mediated by cyclic adenosine monophosphate (cAMP) signaling.[16] Hyperglycemia induces oxidative stress via different mechanisms plays the important role in the development and progression of diabetes which may leads to further complications.[17] The oral administrations of EAAA 50 mg/kg and 100 mg/kg have shown beneficial effects not only on blood glucose but also on body weight.

The correlation between release of insulin and uptake of glucose was further revealed by Insulin ELISA and GLUT2 level estimation. There is significant increase in serum Insulin level as well as higher expression of GLUT2 in pancreatic tissue collected from the rats of EAAA groups. It has been proved that improvement in uptake of glucose at cellular level was relate with up-regulation of GLUT2 expressions in pancreas.[18] Evidences of literature clearly state that compounds having polyphenols plays convincible role in mitigating hyperglycemia-induced resistance of insulin. This also helps to tune glucose uptake mechanism by increasing protein expressions for GLUT2 in pancreatic beta cells of the diabetic rats. The current and previous evidences commend that the EAAA possesses the property of enhancing uptake of glucose at cellular

level was associated with the up-regulation of protein expressions for GLUT2 in the beta cells of pancreas. This may be because of rebuilding and recovering structure of beta cells which was observed in histopathology reports.[8] GLUT2 present in pancreatic β-cells, liver and the hypothalamus. It is involved in glucose transporter and glucose stimulated insulin release is. Recently, GLUT-2 has drawn attentions because of its involvement in the progression of diabetes mellitus. Researchers have claimed that the protein expression for GLUT-2 in diabetes is decreased in β-cells of pancreas. Multiple studies have been commended that the enhancement of GLUT2 is related with flavonoid content of the plant.[19] EAAA is rich in flavonoids and that might be the reason in up-regulation of GLUT2 protein expression. The damaged pancreatic cells seen in the current histopathological analysis [Figure 6] shows the effects of high sugar level induced oxidative stress. Antioxidant property of a plant material includes quenching of free radicals thereby protecting the tissue from oxidative damage. Antioxidants are responsible for inhibiting the peroxidation chain reaction which leads in prevention of destruction of cells and thus they may provide protective mechanism towards the progression of diabetes. Oral administration of EAAA decreased blood glucose level significantly. It was observed from the literature that the *Achyranthes aspera* extract could raises the levels of SOD and CAT activities in the hepatocytes of diabetic rats.[7] The role played by these enzymes is well known. They are responsible for converting reactive oxygen and nitrogen species in to stable molecule which will ultimately results in antioxidant effect. This shows that *Achyranthes aspera* Linn. extract may declining the oxidative stress in diabetes and its complications.[20-22] The anti-oxidative safeguard accomplished by EAAA which protects the pancreatic system. The renovated uptake of glucose in the insulin-signaling is covered by glucose transporter (GLUT) proteins which will have the effect in complete body glucose homeostasis.[7,11,12,15,18]

CONCLUSION

EAAA administration produces hypoglycemic effect by increasing serum Insulin level and GLUT2 level and hence improves β-cell function in streptozotocin induced diabetic rats. In conclusion data from the present study suggest *Achyranthes aspera* has potential effect against STZ-induced hyperglycemia.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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