ORIGINAL ARTICLE

Ameliorative effect of ethanolic *Gymnema sylvestre* extract on diabetic cardiomyopathy against streptozotocin-induced diabetes in Wistar rats

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Background: Diabetes leads to a cardiomyopathy characterized by myocyte loss. Streptozotocin (STZ)-induced diabetic cardiomyopathy is characterized by decreased left ventricular contractility and diminished ventricular compliance with marked abnormal systolic and diastolic function. **Aim:** The ameliorative effect of ethanolic *Gymnema sylvestre* extract (GSE) was evaluated in diabetic cardiomyopathy against STZ-induced diabetes. **Materials and Methods:** Diabetes was induced by a single intravenous injection of (STZ, 45 mg/kg) in male Wistar rats. Blood pressure, serum lactate dehydrogenase (LDH), glucose apolipoprotein B and lipids as well as heart weight, caspase-3, sodium potassium adenosine triphosphatase Na^+ K^+ ATPase, and DNA laddering were determined. **Results and Conclusions:** Administration of GSE (120 mg/kg/p.o.) treatment significantly ($P < 0.01$) reduced myocyte loss by suppressing the levels of cardiac caspase-3, DNA laddering; mean arterial blood pressure and heart rate as well as serum LDH, glucose, apolipoprotein B, and lipids levels. Further, it increased the heart weight and cardiac Na^+ K^+ ATPase activity in diabetic rats. The cardiomyopathy suppression is accompanied by decrease in cardiac caspase-3 levels, DNA laddering, blood pressures, serum LDH, apolipoprotein-B and glucose. Thus, this present study reports the anti-apoptotic potential of GSE in STZ-induced diabetic cardiomyopathy.

Key words: Apolipoprotein-B, caspase-3, diabetic cardiomyopathy, DNA fragmentation, *Gymnema sylvestre*

INTRODUCTION

The world is facing an explosive increase in the incidence of diabetes mellitus, affecting a total population of 220 million world-wide in the year 2020 based on a report presented by World Health Organisation (WHO).[1] Streptozotocin (STZ)-induced diabetic animals develop cardiomyopathy, which is characterized by decreased left ventricular (LV) contractility and by diminished ventricular compliance with markedly abnormal systolic and diastolic function.[2] Diabetic cardiomyopathy involves several cellular and molecular mechanisms, such as upregulation of rennin-angiotensin system, cardiac myocyte apoptosis and cell necrosis.[3] Studies in patients and animals have demonstrated a direct correlation between hyperglycemia and the production of reactive oxygen species.[4]

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Caspases are evolutionarily conserved cysteine-aspartyl specific proteases that play a key role in apoptosis. Apoptosis is generally believed to be mediated by two distinct pathways, caspase dependent and caspase-independent pathways. Caspase-3 is a key player involved in the caspase-dependent apoptotic pathway.[5] A recent study showed that apoptotic thymocytes had decreased protein levels of the sodium potassium adenosine triphosphatase Na^+ K^+ ATPase.[6] Administration of high-dose STZ has been shown to directly cause DNA damage, resulting in massive necrosis of β cells.[7] Apolipoprotein B (Apo-B) contains ligands for the receptor-mediated endocytosis of lipoproteins and is an essential component of chylomicrons and low-density lipoproteins (LDL). There is a direct relationship with Apo-B such that patients with coronary artery disease have generally increased levels of Apo-B.[8]

Currently available drugs for diabetes have a number of limitations, such as adverse effects and high rates of secondary failure. As a complementary/alternative approach, medicinal herbs with anti-hyperglycemic activities are increasingly sought after by diabetic patients and healthcare professionals. In this field, medicinal

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plants play a very important role.[9] *Gymnema sylvestre* R. Br. (family: Asclepidaceae), a plant native to the tropical forests of India, has long been used as a treatment for diabetes.[10] An extract of *G. sylvestre* leaves and purified Gymnemic acid inhibits glucose stimulated gastric inhibitory peptide secretion in rats.[11] Oral administration of *G. sylvestre* leaf extract has hypolipidemic and anti-atherosclerotic effect in Albino rats fed on high-fat diet.[12] Administration of *Gymnema sylvestre* extract (GSE) leaves modifies lipid metabolism in rats.[13] *G. sylvestre* leaves extract has an anti-inflammatory activity in rats.[14]

The aim of the present study, to evaluate the ameliorative effect and shed more light on the cellular and molecular mechanisms of water soluble fraction of ethanolic GSE on diabetic cardiomyopathy against STZ-induced diabetes in Wistar rats.

MATERIALS AND METHODS

Chemicals and Drugs

STZ was purchased from Sigma-Aldrich Co. (St. Louis, MD, USA). Other chemicals were of Analytical grade.

Preparation of Water Soluble Fraction of the Ethanolic GSE

The leaves of *G. sylvestre* were purchased locally, air dried and authenticated by Dr. H B Singh, Scientist 'F' and Head, Raw Materials Herbarium and Museum, National Institute of Science Communication and Information Resources NISCAIR, New Delhi, India. The specimen voucher (Ref. NISCAIR/RHMD/Consult/2008-09/980/11) was deposited at the Department of Pharmacology, Faculty of Pharmacy Jamia Hamdard, New Delhi-110062. One kilogram dried leaves of *G. sylvestre* extracted with ethanol (70%) in Soxhlet's apparatus. The ethanolic extract was standardized according to WHO guidelines.[15] The solvent was removed under reduced pressure using rotary evaporator. The ethanolic extract yield was 10.8% (w/w) in terms of starting material. The ethanolic extract was stirred in distilled water at room temperature and filtered to give water-soluble (W-S) fraction and water-insoluble (W-INS) fractions.[16] The yield obtained of W-S and W-INS were 60% and 40% in terms of the total ethanolic extract, respectively. According to WHO guidelines, phytochemical analysis of water soluble fraction of ethanolic GSE showed that it contains alkaloids, glycosides, proteins, carbohydrates, saponins and steroids.

Animals

Thirty Male Albino rats of Wistar strain, obtained from the Central Animal House Facility of Hamdard University, New Delhi, weighing 150-200 g, were used in the present study. All rats were housed in a room with controlled environment, at a constant temperature of 25°C ± 2°C, RH 50% ± 15% and (12 h light/dark cycle) for 7 days. The animals were housed in groups and fed with commercial pellet diet throughout the experimental period. The experimental protocol was approved by the Institutional Animal Ethics Committee of Hamdard University, New Delhi, which is registered with (Committee for the purpose of control and supervision of experiments on animals (CPCSEA), Registration No. 173/CPCSEA, Dated January 28, 2000), Government of India, India.

Experimental Design

After acclimatization, all rats were treated with STZ (45 mg/kg in 0.1 M citrate buffer, pH 4.5, i.v. once through tail vein) except rats of Group I (normal healthy control rats). Rats having hyperglycemia (fasting blood glucose level ≥200 mg/dl) after 72 h of single intravenous injection of STZ were included in this study and divided in following groups of 8 animals each and treated as follows: Group I (control group) – rats treated with citrate buffer (0.1 M) solution and standard diet for 28 days; Group II (diabetic group) – rats fed with rat pellet diet for 28 days; Group III (water soluble fraction of GSE treated diabetic group) – rats fed with rat pellet diet for 28 days + from 8th day 120 mg/kg/p.o. water soluble fraction of ethanolic GSE.

Measurement of Hemodynamic Parameters

Hemodynamic parameters (systolic, diastolic, mean arterial blood pressure, and heart rate) were measured by non-invasive blood pressure recorder using rat tail-cuff method (Kent Scientific Corporation, USA) on the 29th day.

Measurement of Biochemical Parameters

Blood was collected from the retro-orbital plexus of overnight fasted rats using microcapillary tubes on 29th day. Serum was separated by centrifugation (4,000 rpm for 10 min) and transferred to microcentrifuge tubes. Apolipoprotein-B (Apo-B) in the serum was measured by immunoturbidimetric immunoassay kit (Randox Laboratories Ltd., Antrim, UK). The concentrations of serum (lactate dehydrogenase (LDH); Reckon Diagnostics Pvt. Ltd, Baroda, Gujarat, India), glucose, total cholesterol (TC), (triglycerides (TGs); all the three from Span Diagnostics Ltd, Surat, Gujarat, India) and high-density lipoprotein-cholesterol (HDL-C; Reckon Diagnostics Pvt. Ltd., Baroda, Gujarat, India) were measured with commercial kits. Glycohemoglobin was estimated in blood with a commercial available kit (Asritha *In vitro* Diagnostic Reagents, Hyderabad, India). Atherogenic index was calculated by a formula, i.e., TC/HDL-cholesterol (TC/HDL-C).

Measurement of Cardiac Apoptosis

Caspase-3 activity was measured using Caspase-3/CPP 32

colorimetric assay kit (BioVision, USA). Fifty microliter of the supernatant from homogenized tissue with cooled lysis buffer was taken from each sample and 50 µl of 2 × reaction buffer (containing 10 mM Dithiothreitol DTT) was added to each sample. Then 5 µl of the 4 mM acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) DEVD-p-nitroanilide (pNA) substrate (200 µM final conc.) was added and incubated at 37°C for 1-2 h to allow a dissociation of pNA from the conjugate DEVD-pNA to form a blue colour. CPP-32 activity was measured spectrophotometrically at 405 nm. Caspase-3 activity was calculated as units/mg protein/hour.[17]

Sodium potassium ATPase activity was measured in heart tissue, method reported by Bonting.[18]

Heart was removed, washed with normal saline and then weighed.

DNA Gel Electrophoresis (BioVision, USA)

Apoptosis was evaluated by examining the characteristic pattern of DNA laddering generated in the apoptotic myocardium using gel electrophoresis. Myocardial samples were homogenized in solution containing 50 mmol/L Tris-HCl (pH 8.0), 100 mmol/L ethylenediaminetetraacetic acid EDTA, 100 mmol/L NaCl, and 1% Sodium Dodecyl Sulphate. The tissue homogenate was digested with 5 µl of proteinase K (stock solution 20 mg/ml) at 56°C for 2 h and incubated with ribonuclease RNAse A (1 µl/ml) at 37°C for 1 h. After that phenol/choloroform extraction was performed twice. The upper clear layer was transferred in to another fresh tube. To this clear layer, 600 µl isopropanol was added and kept at room temperature for 1 h and later centrifuged at 10,000 rpm for 10 min. Then the isopropanol layer was discarded by decantation and 100 µl 75% chilled ethanol was added and again centrifuged at 10,000 rpm for 10 min. The upper layer was discarded and DNA pellets were kept at room temperature overnight to dry. To the above test tubes, 100 µl TE solution (10 mmol/L Tris-HCl [pH 8.0], 1 mmol/L EDTA) was added and kept for 1 h dissolution. DNA samples (5 µl DNA + 1 µl gel loading dye) were subjected to electrophoresis on 2% agarose gel, stained with ethidium bromide. DNA laddering, an indicator of tissue apoptotic nucleosomal DNA fragmentation was visualized and photographed under ultraviolet transilluminator.[19]

Measurement of Superoxide Dismutase and Lipid Peroxidation

Superoxide dismutase activity was determined according to the method of Marklund and Marklund.[20] Lipid peroxidation was determined with spectrophotometric measurement of the amount of malondialdehyde equivalents with thiobarbituric acid and was expressed as (thiobarbituric acid-reactive substances; nmol malondialdehyde/mg protein), according to the method of Ohkawa *et al*.[21]

Statistical Analysis

The results are expressed as mean ± SEM Comparisons between the treatment groups and control were performed by analysis of variance (ANOVA) followed by Dunnett's-test. In all tests, the criteria for statistical significance were $P < 0.05$.

RESULTS

Effect on Hemodynamic Changes

Water soluble fraction of *G. sylvestre* ethanolic extract treatment significantly ($P < 0.01$) decreased the increase in the blood pressure (systolic, diastolic, and mean) and heart rate compared to the diabetic group, while these parameters were significantly increased in the diabetic group as compared to the control group [Table 1].

Effect on Biochemical Changes

TC (3-folds), low-density lipoprotein-cholesterol (C) (7-folds), very low-density lipoprotein-cholesterol (4.5-folds), TGs (4.5-folds), and atherogenic indexes were significantly ($P < 0.01$) increased, while HDL-C was significantly decreased in diabetic group as compared to the control group. These all parameters except HDL-C were significantly decreased in water soluble fraction of GSE treated diabetic group as compared to the diabetic group [Table 2].

Serum Apo-B (5-folds) and LDH (11-folds) levels were increased in diabetic group as compared to the control group, while these levels were significantly reduced in water soluble fraction of GSE treated diabetic group as compared to the diabetic group. Serum glucose (3.5-folds) and glycosylated haemoglobin (3-folds) were significantly increased in the diabetic group as compared to the control group, while levels of serum glucose and glycosylated haemoglobin were decreased in the water soluble fraction

Table 1: Effect of water soluble fraction of *Gymnema sylvestre* extract on heart rate beats per minute, systolic blood pressure (mmHg), diastolic blood pressure (mmHg) and mean blood pressure (mmHg) of Albino rats

Treatment	Heart rate	Systolic BP	Diastolic BP	Mean BP (mm Hg)
Vehicle control	421.83±15.77	127.83±6.86	96±4.65	106.16±5.00
Streptozotocin treated	641.05±7.32**	158.45±2.29**	136.90±1.71**	145.85±1.69**
Water soluble fraction of *Gymnema sylvestre* extract treated	494.64±12.48##	110.14±2.03##	91.78±2.00##	100±1.91##

$P < 0.01$ when compared with **vehicle control, ##Streptozotocin treated group, Values are mean±SEM from eight animals in each group, BP – Blood pressure

of GSE treated diabetic group as compared to the diabetic group [Table 3].

Effect on Apoptotic Parameters

Heart weight was increased in the water soluble fraction of *G. sylvestre* ethanolic extract group as compared to the diabetic group. The mean caspase-3 levels (3.5-folds) in the heart tissue were significantly increased in diabetic group as compared to the control group, while in water soluble fraction of GSE treated diabetic group, mean caspase-3 levels were significantly (2.1-folds) decreased as compared to the diabetic group. Na^+–K^+ ATPase levels in heart tissues were significantly decreased in diabetic group as compared to the control group, while Na^+–K^+ ATPase levels were significantly ($P < 0.01$) increased by *G. sylvstre* ethanolic extract as compared to the diabetic groups [Table 4].

Electrophoresis of DNA extracted from the left ventricle region of the heart of diabetic rats (i.e., group II) showed DNA laddering with the lowest band below 200 bps, indicating apoptotic inter-nucleosomal DNA fragmentation. Ladders were not detected in control group (i.e., group I), water soluble fraction of *G. sylvestre* ethanolic extract (120 mg/kg) treated diabetic group (i.e., group III), where genomic DNA band was preserved as compared to diabetic group (i.e., group II) [Figure 1].

Effect on Superoxide Dismutase and Lipid Peroxidation

Superoxide dismutase levels were significantly ($P < 0.01$) decreased in diabetic group as compared to the control group while these levels were significantly increased in water soluble fraction of GSE treated diabetic group as compared to the diabetic group. Lipid peroxide

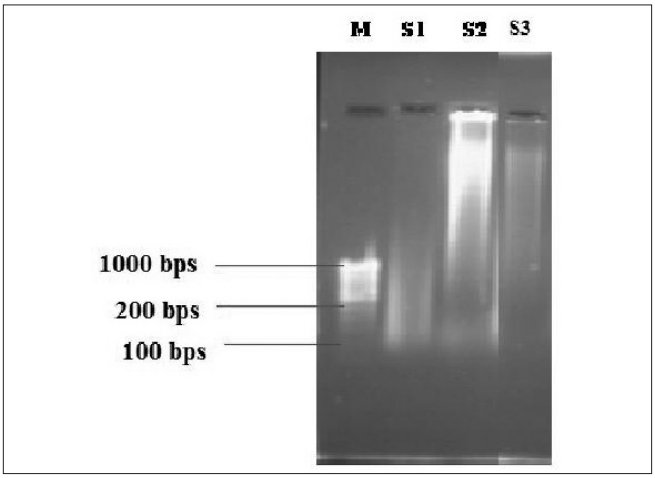

Figure 1: Effect of water soluble fraction of *Gymnema sylvestre* ethanolic extract on DNA fragmentation detected by agarose gel electrophoresis in streptozotocin (STZ)-induced diabetes in Wistar rats. Lane M – marker, S1 – normal control group, showed no DNA laddering, S2 – diabetic control group (STZ 45 mg/kg i.v. group), showed DNA laddering, (i.e., DNA fragmentation), S3 – water soluble fraction of *Gymnema sylvestre* extract treated diabetic group showed little DNA laddering

Table 2: Effect of water soluble fraction of *Gymnema sylvestre* extract on serum total cholesterol (mg/dl), HDL-C (mg/dl), triglycerides (mg/dl), LDL-C (mg/dl), VLDL-C (mg/dl) and atherogenic index of Albino rats

Treatment	Total cholesterol	HDL-C	Triglycerides	LDL-C	VLDL-C	Atherogenic index
Vehicle control	101.69±2.00	32.26±1.01	64.97±2.19	30.83±5.63	12.99±0.43	3.25±0.09
Streptozotocin treated	317.24±12.39**	24.48±0.92**	305.41±16.31**	231.67±14.45**	61.08±3.26**	13.03±0.61**
Water soluble fraction of *Gymnema sylvestre* extract treated	110.35±7.19##	41.74±1.62##	99.18±5.35##	56.64±0.31##	19.83±1.07##	2.68±0.25##

$P < 0.01$ when compared with **vehicle control, ##Streptozotocin treated, Values are mean±SE from eight animals in each group, HDL-C – High-density lipoprotein-cholesterol; LDL-C – Low density lipoprotein-cholesterol; VLDL-C – Very low density lipoprotein-cholesterol

Table 3: Effect of water soluble fraction of *Gymnema sylvestre* extract on serum apolipoprotein-B (mg/dl), LDH (IU/L), glucose (mg/dl) and glycosylated hemoglobin (in %) in Albino rats

Treatment	Apolipoprotein-B	LDH	Serum glucose	Glycosylated hemoglobin
Vehicle control	4.65±0.22	23.48±0.64	94.14±2.16	7.94±0.11
Streptozotocin treated	14.26±1.57**	275.14±21.99**	354.16±16.67**	24.61±0.73**
Water soluble fraction of *Gymnema sylvestre* extract treated	5.31±0.37##	97.89±9.05##	94.29±4.14##	8.91±0.28##

$P < 0.01$ when compared with **vehicle control, ##Streptozotocin treated group, Values are mean±SE from eight animals in each group, LDH – Lactate dehydrogenase

Table 4: Effect of water soluble fraction of *Gymnema sylvestre* extract on caspase-3 activity (nmole/hr/mg protein), Na-K ATPase activity (µmol of Pi liberated/min/mg protein), heart weight (gm), TBARS (nmol malondialdehyde MDA/mg protein) and SOD (IU/mg protein) of Albino rats

Treatment	Caspase-3 activity	Na-K ATPase activity	Heart weight	TBARS	SOD
Vehicle control	62.34±9.28	0.725±0.04	0.737±0.01	0.200±0.003	1.77±0.01
Streptozotocin treated	240.46±12.32**	0.565±0.01**	0.614±0.03*	0.822±0.02**	1.08±0.009**
Water soluble fraction of *Gymnema sylvestre* extract treated	105.62±2.37##	0.859±0.006##	0.712±0.01#	0.366±0.01##	1.21±0.008##

$P < 0.01$ when compared with **vehicle control, ##Streptozotocin treated group, Values are mean±SE from eight animals in each group, TBARS – Thiobarbituric acid-reactive substances; Sodium potassium adenosine triphosphatase Na-K ATPase; SOD - Superoxide dismutase

levels, (i.e., nmol malondialdehyde MDA/mg protein) were significantly (4-folds) increased in diabetic group as compared to the control group while the levels of lipid peroxides were significantly ($P < 0.01$) decreased in water soluble fraction of GSE treated diabetic group [Table 4].

DISCUSSION

This study provides the first suggestive evidence for the anti-apoptotic potential of water soluble fraction of *G. sylvestre* ethanolic extract on STZ-induced cardiac myopathy in rats. In this study, hemodynamic parameters (blood pressure and heart rate) were increased in the STZ treated group than normal control group, as Teuscher *et al*,[22] have reported that the prevalence of hypertension in diabetic individuals appears to be approximately 2-folds that in the non-diabetic population. Hyperglycemia results in glomerular hyperfiltration of glucose, which in turn, stimulates the proximal tubular glucose-Na^+ cotransporter.[23] STZ-induced diabetes was accompanied with hyperglycaemia and blood glycated Hb%. Increased hepatic glucose production plus decreased hepatic glycogen synthesis and glycolysis are the major mechanisms in diabetes mellitus that result in hyperglycaemia.[24]

Hyperglycaemia could be one of the risk-factor for apoptosis in myocytes as Laybutt *et al*,[25] reported that high glucose concentration impairs islet function by disturbing glucose metabolism in the mitochondria of β-cells and could induce apoptosis. In addition, it has been reported that high glucose could increase β-cell vulnerability to toxic damage by increasing the expression of potential auto-antigens on the cell membrane surface.[26]

Increased levels of Apo-B may contribute in cardiac apoptosis induced by STZ as Apo-B has an ability to induce apoptosis and plays a crucial role in the development of atherosclerotic lesions.[27] The leakage of cytoplasmic LDH caused by the damage of cell membrane integrity is also a good indicator of cell death and is used to estimate cytotoxicity.[28] In the present study, serum LDH levels were decreased in the water soluble fraction of GSE treated group.

The results of present study showed that the STZ-induced diabetes increased the levels of cardiac caspase-3. This enhances apoptotic cell death through activation of caspase-activated DNase, due to DNA degradation.[29] Treatment with water soluble fraction of GSE (120 mg/kg) (i.e., group III) significantly decreased the appearance of DNA laddering in the LV region of rat heart.

The present study has clearly demonstrated that the rate of cardiac myocyte apoptosis in diabetic rats is substantially higher than in the normal control group. In the diabetic control rats, the levels of Na^+ K^+ ATPase and oxygen free radical scavenger, superoxide dismutase, were decreased, while the levels of pro-apoptotic caspase-3 levels, DNA fragmentation of cardiac cell and free radical biomarker, malondialdehyde, were elevated. These changes may have contributed to the increased cardiac myocyte apoptosis in the diabetic control rats. The effect of diabetes on myocyte death by apoptosis was examined and the ameliorative effect of water soluble fraction of *G. sylvestre* ethanolic extract on development of cardiac myopathy induced by STZ in rats was established. Water soluble fraction of *G. sylvestre* ethanolic extract could be useful intervention in the treatment of diabetic cardiomyopathy and myocyte loss in STZ–induced diabetes.

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