WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 6, Issue 16, 1645-1657. Research Article ISSN 2277– 7105

PROTECTIVE EFFECT OF *GYMNEMA SYLVESTRE* IN STZ INDUCED DIABETIC NEPHROPATHY IN ALBINO RATS

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Article Received on
19 Oct. 2017,

Revised on 10 Nov. 2017,
Accepted on 30 Nov. 2017

DOI: 10.20959/wjpr201716-10698

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ABSTRACT

Diabetic Nephropathy is also known as nodular diabetic glomerulosclerosis and intercapillary glomerulonephritis. *Gymnema sylvestre* is used traditionally in diabetes and a few studies on animal models proved its usefulness in diabetes. So this study was carried out to find out the effectiveness of G*ymnema sylvestre* in albino rats in diabetic nephropathy. For the diabetic nephroprotective activity study in diabetic rats the rats were fasted for 16 h and classified into five groups of six each. Group I served as normal control, Group II as STZ-diabetic control, Groups III and IV STZ-diabetic rats treated with *Gymnema sylvestre* extracts orally at a dose of 200 mg/kg and 100 mg/kg, respectively and finally Group V STZ-diabetic rats with glibenclamide. The treatment was continued once daily for 14 days. Serum and urine renal parameters like creatinine, urea, uric acid, total proteins, glucose, albumin, antioxidant assays for lipid peroxidation, glutathione, superoxide dismutase, catalase and histopathology of kidney were assessed. For the statistical analysis one way ANOVA followed by Dunnette's multiple comparison test was used. The % yield of ethanolic extracts was found to be 8.7% for *Gymnema sylvestre*. The results of phytochemical screening of *Gymnema sylvestre* leaves showed the presence of phytosterols, like steroids, alkaloids, glycosides, saponins, flavonoids, tannins, carbohydrates. Ethanolic extracts of leaves of *Gymnema sylvestre*, reduced diabetic nephropathy incidence dose dependently, when compared to the control and the standard drug Glibenclamide.

KEYWORDS: *Gymnema sylvestre*, diabetic nephropathy.

INTRODUCTION

Diabetic Nephropathy is also known as nodular diabetic glomerulosclerosis and intercapillary glomerulonephritis.[1] It is a progressive renal disease caused by angiopathy of capillaries in

the glomeruli caused due to long standing diabetes mellitus. It is one of the most serious micro vascular complications of diabetes and therefore is a major cause of end stage renal disease (ESRD) in the general population.[2] In general it may develop in about 40% of all patients with diabetes, so prevention is critical for delaying the development and progression of diabetic kidney disease.[3,4,5]

Histopathologically, it is characterized by progressive remodeling of the glomerular structure, i.e. thickening of the glomerular basement membrane, activation of mesangial cells that produce increasing amounts of diffuse and nodular mesangial matrix deposits and podocyte damage altogether leading to glomerulosclerosis.[6] These structural alterations cause progressive hyperfiltration of the remaining glomeruli and albuminuria. As glomerulosclerosis progresses, glomerular filtration rate (GFR) declines and DN progresses to end-stage renal disease. Once overt DN is present, end stage renal disease (ESRD) can be postponed, but usually not prevented, even by effective antihypertensive treatment and careful glycemic control.[7,8]

Gymnema sylvestre is regarded as one of the plants with potent anti diabetic properties. The active compound of the plant is gymnemic acids. It has been observed that there could be a possible link between gymnemic acids and diabetes.[9]

The plant is popularly known as 'Gurmar'.G. sylvestre (Asclepiadaceae), a vulnerable species is a slow growing, perennial, medicinal woody climber found in central and peninsular India. It is a potent antidiabetic plant and used in folk, ayurvedic and homeopathic systems of medicine. It is also used in the treatment of asthma, eye complaints, inflammations, family planning and snakebite. In addition, it possesses antimicrobial, anti-hypercholesterolemic, hepatoprotective and sweet suppressing activities. It also acts as feeding deterrents to caterpillar, Prodeniaeridania: prevent dental caries caused by Streptococcus mutans and in skin cosmetics.[10]

G. sylvestre leaves have been found to cause hypoglycemia in laboratory animals and have found a use in herbal medicine to help treat adult onset diabetes mellitus (NIDDM). When gymnema leaf extract is administered to a diabetic patient, there is stimulation of the pancreas by virtue of which there is an increase in insulin release. Other uses for gymnema leaf extract are its ability to act as a laxative, diuretic and cough suppressant.[10,11]

Triterpenoidsaponins of gymnemic acid A, B, C and D with sugarresidues such as glucuronic, galacturonic acid, ferulic & angelic acids attached as carboxylic acids. Isopropylene derivative of gymnemagenin, a hexahydroterpene, gymnemagenin, gymnemic acid. The leaves also contain betaine, choline, gymnamine alkaloids, inositol, d-quercitol. Hydrocarbons such as nonacosane, hentriacontane, tritriacontane, pentatriacontane, phytin, resin, tartaric acid, formic acid, butyric acid, amino acids such as leucine, isoleucine, valine, alanine, γ-butyric acid.[11]

METHODS

Plant material & preparation of extract

The plants of *Gymnema sylvestre* were collected and shade dried, leaves of *Gymnema sylvestre* were separated and chopped into small pieces. The powder of was extracted with petroleum ether for defatting and then by ethanol (99.99%) by using Soxhlet apparatus for 72 hr.

The extract was concentrated until dryness under reduced pressure and controlled temperature (40-50ºC). Then preliminary phytochemical screening was performed and percentage yield of the extract was calculated.

Determination of acute oral Toxicity (LD_{50})

The acute oral toxicity study for the extract of *Gymnema sylvestre* was done by 'Up-and-Down' method in healthy adult male albino wistar rats according to CPCSEA recommended 'OECD' guideline 425.

Preparation of extract dose

Weighed quantity of ethanol extract of *Gymnema sylvestre* was suspended in water and administered orally to the experimental animals of different groups. The extract was administered at the constant volume of 200 mg /kg and 100 mg/kg of body weight for each animal according to the groups.

Experimental design

Induction of experimental diabetes

Rats were fasted for 16 h before the induction of diabetes with Streptozotocin (STZ). A freshly prepared solution of STZ (40 mg/ kg body weight) in 0.1 M cold citrate buffer, pH 4.5, were injected intraperitoneally in a volume of 1 ml/kg[12] and the control rats were injected with the citrate buffer alone. In order to control the hypoglycemia during the first day after the STZ administration, diabetic rats were given 5% glucose solution orally. Hyperglycemia was confirmed by the elevated fasting glucose levels in blood, determined at 48 hr and then on day 6 after injection. Rats with moderate diabetes exhibiting fasting blood glucose levels in the range of 250-280 mg/100 ml were selected for the studies.

Diabetic Nephroprotective activity study in diabetic rats (14 days)

Rats were fasted for 16 h and classified into five groups of six each.[13] Group I, normal control, were given normal saline orally at a dose of 5 ml/kg. Group II, STZ-diabetic control, received normal saline at a dose of 5 ml/kg orally. Groups III and IV STZ-diabetic rats were treated with *Gymnema sylvestre* extracts orally at a dose of 200 mg/kg and 100 mg/kg, respectively. Group V, STZ-diabetic rats, were administered with glibenclamide at a dose of 0.5 mg/kg orally. The treatment was continued once daily for 14 days.

Effect of *Gymnema sylvestre* extracts on serum and urine renal parameters

On the 15th day, blood was collected from the overnight-fasted rats by retro-orbital bleeding, using micro capillary technique. Fasting blood glucose level of each animal was determined. Serum was separated and used for the determination of biochemical parameters, such as creatinine, urea, uric acid and total proteins. Rats were accommodated in metabolic cages for urine collection for 2 days in order to become familiar with the environment of the cage. 24 hour urine samples were collected from all groups to determine urine total protein and albumin. After urine collection, all the rats were sacrificed by euthanasia. Kidneys were excised immediately, rinsed in ice-cold normal saline (pH 7.4), blotted dry and weighed.

Estimation of antioxidant assays

A 10% w/v of kidney homogenate was prepared in 0.15 M Tris-HCl buffers (pH: 7.4). The homogenate was centrifuged at 2000 × g for 20 min at 4°C to remove the cell debris and then the supernatant was centrifuged (REMI C-24) at 12,000 × g for 1 h at 4°C. The supernatant obtained were used for the determination of lipid peroxidation[14], reduced glutathione content[15], Superoxide Dismutase (SOD)[16] and Catalase (CAT).[17]

Histopathological study of kidney

The fragments from the kidney tissues were fixed in 10% neutral formalin solution, embedded in paraffin, and then, stained with Hematoxylin (H) and Eosin (E). The sections were examined microscopically for the evaluation of histopathological changes.

Statistical analysis

The results were expressed as mean ± S.E.M. The statistical analysis was carried out by using Graph Pad Prism version 5.01. The statistical analysis of the data was carried out using appropriate statistical method: one way ANOVA followed by Dunnette's multiple comparison test and significant levels was $p< 0.05$, $p< 0.01$ and $p< 0.001$.

RESULTS

Phytochemical Screening

The % yield of ethanolic extracts was found to be 8.7% for *Gymnema sylvestre*. The results of phytochemical screening of *Gymnema sylvestre* leaves showed the presence of phytosterols, like steroids, alkaloids, glycosides, saponins, flavonoids, tannins, carbohydrates. As tabulated in table 1.

Table 1: Phytochemical screening of ethanolic extracts of Leaves *of Gymnema sylvestre.*

Chemical test	*Gymnema slyvestre* extract
Carbohydrates	
Molish test	+
Benedicts Test	+
Borntrager's test	+
Fehling test	+
Proteins and amino acids	
Xanthoproteic	+
Millions test	+
Ninhydrine test	+
Fixed oil and fats	
Spot test	-
Saponification test	-
Glycosides	
Legal's Test	+
Keller-Killiani Test	+
Borntrager's Test	+
Steroides	
Salkowski Test	-
Liebermann's Reaction	-
Alkaloids	
Hager's Test	+
Mayer	+
Tannic acid test	+
Flavonoids	
$FeCl_3$ Solution	+
Shinoda Test	+
Saponins	
Foam Test	+
Tannin & phenol	
FeCl3 Sol. Test	+
Lead Acetate Test	+
Dil. HNO_3 Test	-

(+) = present: (-) = absent.

Acute oral toxicity study

Administration of ethanolic leaf extract of *Gymnema sylvestre* to Wistar rats did not show any mortality and gross behavioral changes up to 2000 mg/kg body weight. According to the OECD 425 guidelines for the acute toxicity, an LD50 dose of 2000 mg/kg and above is categorized as unclassified and hence the drug is found to be safe. Based on the acute toxicity studies, the dose 200 mg/kg and 100 mg/kg were selected as the therapeutic dose.

Effect on serum and urine renal function parameters

Repeated oral administration of both the extracts at a dose level of 200 mg/kg and 100 mg/kg in STZ induced diabetic rats for 14 days significantly ($P<0.05$, $p<0.001$) reduced the elevated fasting blood glucose levels when compared to diabetic control rats. In STZ induced diabetic control rats there was a significant ($p<0.001$) increase in serum creatinine, urea and uric acid, but were reduced by GSEE at 200 mg/kg and 100mg/kg. In addition, there was a significant ($p<0.001$) decrease in total proteins levels of diabetic control rats compared with normal control.

Oral administration of GSEE significantly ($p<0.001$) increased the serum total protein. Compared to the non-diabetic control rats, urine albumin and total proteins levels increased significantly ($p<0.001$), in STZ-induced diabetic control rats. Treatment of STZ-induced diabetic rats with GSEE resulted in marked decrease in urine albumin and total proteins ($p<0.001$) as compared to diabetic control rats (Table 2).

Table 2: Effect of oral administration of ethanolic extract of *Gymnema sylvestre* on serum and urine biomarkers by sub-acute treatment in STZ-induced diabetic rats.

Groups	Serum						Urine	
	Creatinine (mg/dl)	Urea (mg/dl)	Uric acid (g/dl)	Total proteins (g/dl)	Glucose (mg/dl)		Albumin (mg/dl)	Total proteins (mg/dl)
					1st day	15th day		
Group I (Normal Control)	0.97 ± 0.06	27.23 ± 1.02	3.17 ± 0.12	7.92 ± 0.11	91.47 ± 0.13	93.21 ± 1.60	2.62 ± 0.11	15.65 ± 0.97
Group II (DM Control)	2.67 ± 0.05**	52.77 ± 1.51**	5.71 ± 0.13**	4.52 ± 0.16**	285.25 ± 3.51	287.71 ± 2.95**	12.71 ± 0.43**	36.19 ± 1.15**
Group III (DM + GSEE 200mg/kg)	1.24 ± 0.06a	38.41 ± 1.24a	4.01 ± 0.61a	6.56 ± 0.11a	283.74 ± 1.96	181.41 ± 2.12a	5.13 ± 0.29a	22.21 ± 1.01a
Group IV (DM + GSEE 100mg/kg)	1.32 ± 0.07a	41.32 ± 1.31a	4.25 ± 0.73a	6.81 ± 0.41a	284.31 ± 1.62	191.15 ± 3.01a	6.22 ± 0.31a	24.52 ± 0.98a
Group V (DM+ GLIB)	1.08 ± 0.15a	31.10 ± 1.42a	3.42 ± 0.31a	7.02 ± 0.08a	284.15 ± 2.24	124.23 ± 2.18a	4.41 ± 0.26a	18.21 ± 1.69a

DM: Diabetes mellitus: GSSE: *Gymnema sylvestre* ethanolic extract, GLIB: Glibenclamide

Values are given as mean ± SEM, 6 rats in each group: **P<0.001 as compared to normal control group: a P<0.001 as compared to diabetic control group.

Effects on renal in vivo antioxidant activities

As oxidative stress is an important pathogenetic mechanism leading to chronic diabetic complications, we investigated oxidative stress using several parameters like lipid peroxidation, glutathione, superoxide dismutase, catalase. The amount of MDA, an end product of lipid peroxidation, in the rats kidney tissues, significantly increased in STZ-induced diabetic control rats, compared to the non-diabetic control rats. The treatment of rats with GSEE (200 mg/kg and 100mg/kg) and Glibenclamide resulted in a significant decrease in the concentration of MDA than in the diabetic control rats (Table 3).

Reduced glutathione content superoxide dismutase and catalase: The total GSH content, SOD and catalase (CAT) activities in the STZ-induced diabetic rats were significantly (p<0.001) decreased in kidney. However, the renal SOD, CAT activities and GSH levels were significantly elevated in the diabetic rats treated with GSEE (200 mg/kg and 100mg/kg) and Glibenclamide when compared with the diabetic control rats.

Table 2: Effect of oral administration of ethanolic extract of *Gymnema sylvestre* in vivo antioxidant system in STZ-induced diabetic rats.

Group	Lipid peroxidation (nmol/mg)	Glutathione (µM/gm)	Superoxide dismutase (IU/mg)	Catalase (nmol/mg)
Group I (Normal Control)	7.71 ± 0.51	62.31 ± 1.90	39.71 ± 0.67	57.54 ± 1.32
Group II (DM Control)	24.25 ± 0.81^{b**}	28.47 ± 0.48**	15.14 ± 0.17**	15.22 ± 0.85**
Group III (DM + GSEE 200mg/kg)	14.01 ± 0.61^{a**}	41.71 ± 1.61^{a**}	25.13 ± 0.37^{a**}	38.75 ± 1.43^{a**}
Group IV (DM + GSEE 100 mg/kg)	15.21 ± 1.01^{a**}	43.17 ± 0.98^{a**}	27.32 ± 0.71^{a**}	39.05 ± 1.12^{a**}
Group V (DM+ GLIB)	9.97 ± 0.45^{a**}	51.19 ± 1.21^{a**}	37.92 ± 0.48^{a**}	46.14 ± 0.98^{a**}

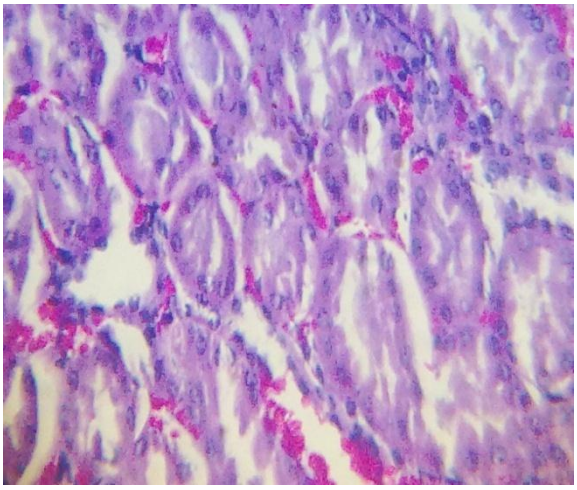
Values are given as mean ± SEM, 6 rats in each group: **P<0.001 as compared to normal control group: a P<0.001 as compared to diabetic control group.

Histopathology of kidney

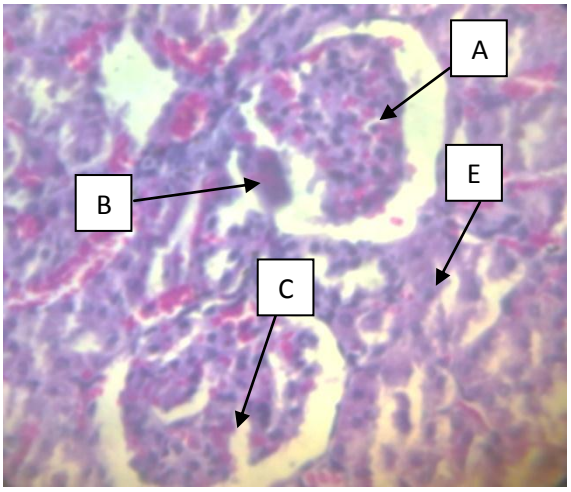
Histopathological changes in kidneys of normal, diabetic untreated, diabetic treated with ethanolic extracts and treated with Glibenclamide were studied. Diabetic rats showed mesangial expansion and other degrees of focal lesions in many sections, glomerular congestion, tubular cast, peritubular congestion and interstitial inflammation. Whereas animals treated with plant extract and Glibenclamide exhibited marked improvement in the abnormal histology of the kidney.

Table 4: Histopathological features of the rats kidneys of different treatment groups in STZ induced Diabetic Nephropathy.

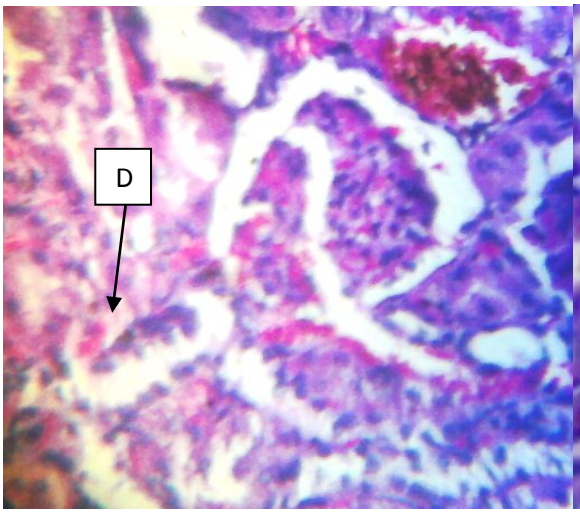
Group	Mesangial expansion	Tubular cast	Glomerular congestion	Peritubular congestion	Interstitial Inflammation	Mononuclear Cells
Group I (Normal Control)	-	-	-	-	-	-
Group II (DM Control)	+++	+++	++	+++	+++	++
Group III (DM + GSEE 200 mg/kg)	++	-	++	+	-	++
Group IV (DM + GSEE 100 mg/kg)	++	+	++	+	-	++
Group V (DM+ GLIB)	+	–	+	–	–	–

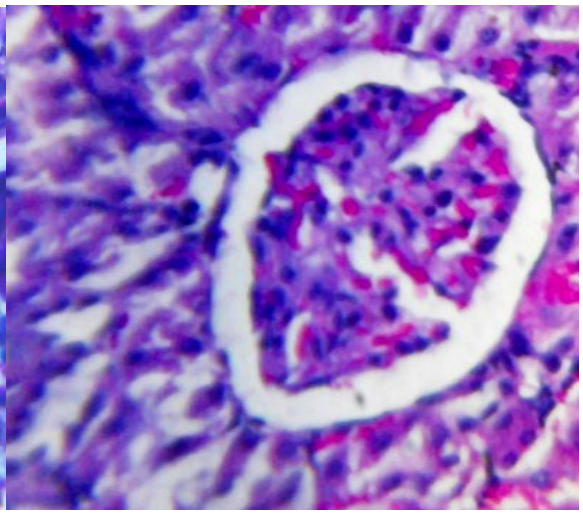


Group I: Normal control.

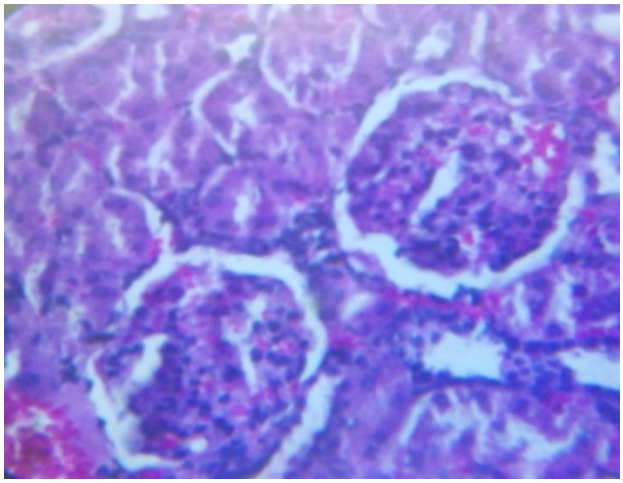


Group II: STZ induced DN.





Group III: Treated with GSEE (200 mg/kg) Group IV: Treated with GSEE (100mg/kg).



Group V: Treated with Standard (Glibenclamide).

Figure 1: Histopathological images of nephropathic kidney of different groups showing.

A) Mesangial expansion, B) tubular cast, C) glomerular congestion, D) peritubular congestion and E) interstitial edema.

DISCUSSION

Diabetes mellitus and Hyperlipidemia are recognized as few of the greatest risk factors for atherosclerosis, myocardial infarction. DM of long duration is associated with several complications such as atherosclerosis, neuropathy, nephropathy etc. These complications have long been assumed to be related to chronically elevated glucose levels and subsequent oxidative stress. Mechanisms that contribute to increased oxidative stress in diabetes include non-enzymatic glycosylation, autooxidative glycosylation and metabolic stress. Oxidative stress in diabetes may partially be reduced by antioxidants and as seen antioxidants have been prescribed to reduce the long term complications seen in diabetes.[18]

Earlier studies showed that extract of *Gymnema sylvestre* possesses anti-diabetic and antioxidant activities in STZ-induced diabetic rat model. Many reports are published related to these activities but none of them had worked on the late complications of the diabetes namely diabetic nephropathy.[10,11]

STZ is well known for its selective pancreatic islet β-cell cytotoxicity and has been extensively used to induce DM in animals. It interferes with cellular metabolic oxidative mechanisms. We have selected 45 mg/kg of STZ for induction of diabetes because higher dose of STZ could increase the mortality. *Gymnema sylvestre* extracts produced decrease in glucose level in diabetic rats in spite of counter regulatory factors avoiding reduction in blood glucose levels. Therefore anti-hyperglycemic activity of *Gymnema sylvestre* extracts could be

mediated by stimulation of surviving β-cells to release more insulin and may be by increasing the peripheral glucose uptake.

The present data indicates that *Gymnema sylvestre* extracts significantly reduced hyperglycemia in 8 weeks diabetic studies. The efficacy of the extract was better at higher dose level and also comparable to standard drug Glibenclamide. Several studies reported that STZ administration elevated serum renal markers in rats[19,20] which is the indicator of diabetic nephropathy with altered glomerular filtration rate. The current study also revealed that serum renal bio markers such as creatinine, urea, uric acid and urine renal markers as total proteins and albumin levels were increased in diabetic control rats. The daily administration of *Gymnema sylvestre* extract for 14 days caused a significant reduction in serum creatinine, serum urea, serum uric acid, urine total protein and urine albumin levels, and a significant elevation in serum total protein levels in diabetic rats when compared to diabetic control. This data indicates that the GSEE improved renal functions and reversed the damage in the kidney tissues of diabetic rats.

We also examined antioxidant capacity of the plant extract, since antioxidants have been reported to prevent oxidative stress and diabetic nephropathy. In diabetic nephropathy, oxidative stress has been found to be mainly due to an increased production of reactive oxygen species and a sharp reduction of antioxidant defenses. In diabetes, hyperinsulinemia increases the activity of the enzyme, fatty acyl coenzyme A oxidase, which initiates β-oxidation of fatty acids, resulting in lipid peroxidation.[21] The findings supported that the GSEE may exert antioxidant activities and protect the renal tissues from lipid peroxidation. The possible mechanism by which these may bring about their diabetic nephroprotective action in STZ-induced diabetic rats may be by inhibiting lipid peroxidation in kidney tissues.

Glutathione (GSH), a tripeptide present in all the cells is an important antioxidant. SOD and CAT are enzymes that destroy the peroxides and play a significant role in providing antioxidant defenses to an organism.[22,23] The functions of all three endogenous antioxidants are inter-connected and a lowering of their activities results in the accumulation of lipid peroxides and increased oxidative stress in diabetic rats. In our study, it was observed that the GSEE caused a significant increase in the renal SOD, CAT and GSH activities of the diabetic rats. This means that GSEE can reduce reactive oxygen free radicals and improve the activities of the renal antioxidant enzymes to protect the kidney cellular damage during diabetes nephropathy.

Histopathological estimations also support the protective effects of both the treatment. Mesangial expansion is due to increased extracellular matrix accumulation by the glucose, insulin and angiotensin II together stimulation, with altered composition and function. Other focal lesions are resulted from the nephrotoxicity due to the hyperglycemia.

Moreover, the significant antioxidant and nephroprotective activity of GSEE on STZ-induced diabetic rats may be attributed to the presence of biologically active compounds flavonoids, saponins, tannins and terpenoids. According to these results, these plant constituents could be a supplement, as an antioxidant therapy, and may be beneficial for correcting the hyperglycaemia and preventing diabetic nephropathy due to lipid peroxidation and free radicals. As the present study is a preclinical one, further proceedings with human volunteers may pave a way for the usage of the drug in human beings.

CONCLUSION

The study was taken up to comparatively evaluate the diabetic nephropathy activity of *Gymnema sylvestre* in albino rats. Ethanolic extract of leaves of *Gymnema sylvestre* exhibited significant anti- diabetic nephropathy activity in STZ induced model. Ethanolic extract of leaves of *Gymnema sylvestre*, reduced diabetic nephropathy incidence dose dependently, when compared to the control and the standard drug Glibenclamide.

Hence, it can be concluded that extract of leaves of *Gymnema sylvestre* have anti- diabetic nephropathy effect and a further research work is required to isolate the compound responsible for this activity and it may be a potential source for diabetic nephropathy.

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