International Journal of Herbal Medicine
Available online at www.florajournal.com

I J H M International Journal of Herbal Medicine

E-ISSN: 2321-2187
P-ISSN: 2394-0514
IJHM 2016; 4(4): 103-107
Received: 13-05-2016
Accepted: 14-05-2016

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Preventive effect of phloroglucinol against adverse protein alterations in streptozotocin induced diabetic rats

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Abstract
Diabetes mellitus is often associated with long term vascular complications including neuropathy and nephropathy. Long lasting diabetic complications are known to cause abnormalities in the synthesis and transport of protein with the increase the net protein loss in the body. The aim of the present study was to evaluate the protective effect of phloroglucinol (150,200,250 mg/kg; per oral) on the protein loss in diabetic rats. Diabetes was induced by a single dose of STZ (streptozotocin) (55mg/kg i.p.) and was treated with phloroglucinol for 8 weeks. Phloroglucinol treated animals showed a significant increase in protein level as evidence from increase serum albumin and total protein (kidney and sciatic nerve homogenate) along with the reduction in serum glucose and creatinine. Treatment with phloroglucinol for 8 weeks significantly improves the biochemical deficit in a dose dependent manner. The study demonstrated the effectiveness of phloroglucinol in attenuation of protein loss in diabetic complications.

Keywords: diabetes mellitus, neuropathy, nephropathy, protein loss

1. Introduction
Diabetes mellitus (DM) is one of the most common chronic disorder affecting over 100 million people worldwide. India is the developing country has the highest population suffering from DM. DM is associated with microvascular and macrovascular complications, including anatomic, structural and functional changes leading to multiorgan dysfunction. The debilitating consequences of DM are neuropathy and nephropathy which globally affect up to 50% of diabetic patients [1]. Both complications have similar etiologic characteristic. Long lasting chronic hyperglycemia is responsible for the initiation and progression of diabetic vascular complications through numerous metabolic and structural derangements which includes the elevation in the formation of reactive oxygen species (ROS), abnormal activation of signaling cascades, formation of advanced glycation end products (AGEs) and abnormal stimulation of hemodynamic regulation systems [2]. Elevated serum glucose level causes profound changes in people with type 1 DM during insulin deprivation resulting in a net protein loss [3].
Along with carbohydrate and fat, protein is one of the major energy providing macronutrients. Protein helps to build muscle, repair damage to the body and can also be broken down into glucose and used for energy [4]. Insulin exerts a major role in regulating the metabolism of protein and amino acids [5]. In type 1 diabetes insufficient amount of insulin causes breakdown of protein to obtain glucoseleading to the reduction in levels of protein in the body [3].
Abnormalities in the synthesis and transport of structural proteins and abnormal phosphorylation of protein, particularly myelin proteins have been observed in diabetic neuropathy [6]. On the other hand, in diabetic nephropathy is characterized by a progressive increase in proteinuria with urinary protein excretion higher than 300mg/day. In both conditions increase in protein breakdown is greater than the increase in protein synthesis resulting in a net protein loss [7]. Therefore, searching for effective drugs which can prevent the elevation of net protein loss in diabetes related complications. Especially the naturally occurring ones, has gained the extensive attention.
Phloroglucinol (1,3,5-trihydroxy benzene) is a naturally occurring secondary plant metabolite available as a treatment for gastrointestinal disorder in a number of countries worldwide [8]. Recent studies have shown that phloroglucinol exerts a vast array of pharmacological activities including anti-spasmodic activity [9], inhibitory effect on inflammation and oxidative stress [10], an antithrombotic and profibrinolytic activity [11] etc. Several published reports have explained the protective effect of phlororglucinol against oxidative stress *in vitro* and *in vivo* [12]. Recently Park *et al* found that phloroglucinol protected

pancreatic beta cells against glucotoxicity induced apoptosis using a rat insulinoma cell lines [13]. The LD_{50} value of phloroglucinol by oral administration is closed to 4000 mg/kg bw.

Hyperglycemia leads to glycation of proteins which can damage the protein throughout the body [14]. Thus the present study was designed to investigate the protective potential of phloroglucinol in the experimental diabetic neuropathy and nephropathy by assessing the protein content in serum, sciatic nerve and kidney.

2. Materials and Methods

2.1 Animals

Adult male Wistar rats (250-300g) bred in Central Animal House facility of Al-Ameen College of Pharmacy were used. The animal were kept separately in polypropylene cages (six per cage) randomly and paddy husk was used as bedding. The animal were housed under standard laboratory conditions at temperature 20-24ºC and a humidity of 40-70%, with 12:12 light: dark cycle and had free access to food. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of Al-Ameen College of Pharmacy and performed in accordance with the guidelines of Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India on animal experimentation. Animals were acclimatized to laboratory conditions before the tests.

2.2 Induction of diabetes mellitus and drug treatment schedule

Diabetes mellitus (DM) was induced by single intraperitoneal injection of streptozotocin (STZ) at the dose of 55mg/kg body weight dissolved in cold citric buffer (pH 4.5) [15]. Hyperglycemia was confirmed by the estimation of blood glucose after 72h after STZ administration. Rats with fasting blood glucose values more than 250 mg/dl were considered diabetic and used for further experiment. After confirmation of diabetes the rats were divided into four groups (n=6) matched for body weight. Group I serves as the normal control and was given normal saline throughout the study period. Group II serves as diabetic control group (STZ+ normal saline). The three test diabetic groups were orally administered 100,200 and 250 mg/kg of phloroglucinol daily. The doses of phloroglucinol employed in the present study were decided based on the previous published studies [16]. After 8 weeks the animals were anesthetized (ketamine: xylazine 80:5 mg/kg i.p.) and the blood was withdrawn from the retro-orbital plexus using capillary tubes. Blood was collected in clot activator vacutainer for estimation of various biochemical parameters. Tubes were left to clot at 37°C for 10 min, then centrifuged and serum was separated. At the end of the study animals were sacrificed by an overdose of anesthesia and tissues (sciatic nerve and kidney) was isolated carefully after dissection for protein estimation.

2.3 Sciatic nerve homogenate preparation

Sciatic nerve segments were weighed and rinsed with ice cold saline (0.9% sodium chloride). Sciatic nerve was cut into small pieces and then homogenized in chilled phosphate buffer (pH 7.4) and centrifuged at 12,000g for 30 min at 4ºC. The supernatant thus obtained was used for the protein estimation [17].

2.4 Kidney homogenate preparation

Kidney tissue was chopped into small pieces on ice. A 10% w/v homogenate was prepared in 10mM phosphate buffer (pH7.4) and centrifuged at 12,000g for 30 min at 4ºC. The supernatant thus obtained was use for the protein estimation [18].

2.5 Biochemical estimation

2.5.1Estimation of serum parameters

Serum levels of glucose and proteins i.e. albumin and creatinine were estimated using commercial diagnostic kits.

2.5.2Estimation of tissue homogenate parameters

The protein concentration in the sciatic nerve and kidney homogenate was estimated according to the method of Lowry *et al*., (1951) using bovine serum albumin as a standard. The protein content was determined spectrophotometrically at 660nm and expressed as µg/ml of 10% of the homogenate [19].

2.6 Statistical analysis

All data are expressed as the mean ± standard error of mean (SEM) for six rats in each group of rats. Statistical evaluation of the data was performed by Graph Pad Prism 5 using one way analysis of variance (ANOVA), followed by Tukey's multiple comparison test.

3. Results

3.1 The effect of oral administration of phloroglucinol on serum glucose levels in STZ induced diabetic rats

The levels of serum glucose were increased in diabetic control rats (372 ± 2.5 mg/dl). Oral administration of phloroglucinol 100, 200 & 250 mg/kg for 8 weeks significantly decreases levels of serum glucose to 195 ±1.3, 175±1.3, 161 ±1.5 mg/dl respectively (fig.1).

3.2 Effect of oral administration of phloroglucinol on serum creatinine levels in STZ induced diabetic rats

Serum creatinine levels in STZ diabetic rats was 2.5 ± 0.16 mg/dl, which was significantly higher than the serum creatinine levels in normal rats (0.5 ± 0.08 mg/dl). Serum creatinine levels in STZ diabetic rats administered phloroglucinol 100, 200 & 250 mg/kg, p.o was 1.7 ± 0.14 mg/dl, 0.83± 0.05 mg/dl and 0.48 ± 0.01 mg/dl, respectively which was significantly lower than that of diabetic control (2.5 ± 0.16mg/dl) (fig.2).

3.3 Effect of oral administration of phloroglucinol on serum albumin levels in STZ induced diabetic rats

Serum albumin levels were significantly decreased in STZ-treated animal (2.2 ± 0.08g/dl) as compared to the normal control (4.02 ± 0.06g/dl). Phloroglucinol (100,200 & 250 mg/kg) treatment significantly and dose dependently inhibited this increase in serum albumin levels in STZ-treated rats (fig.3).

3.4 Estimation of total protein (µg/ml) in sciatic nerve homogenate

Significant (p<0.05) decrease in the protein levels(55 ±1.25 µg/ml) in the sciatic nerve were observed in the diabetic control rats compared with normal control rats (89 ±1.25 µg/ml). The treatment with phloroglucinol (100,200,250 mg/kg) showed a significant increase in protein level of the sciatic nerve of STZ treated rats (64 ±0.84 µg/ml, 65.79±1.534 µg/ml 73 ±1.89 µg/ml)(fig. 4)

3.5 Estimation of total protein (µg/ml) in kidney homogenate

Protein levels were markedly decreased in the sciatic nerve of STZ treated rats (28 ±0.92 µg/ml) as compared to a normal

control group (71 ±2.45 µg/ml). Phloroglucinol treatment significantly and dose dependently elevates the protein level in STZ treated rats (50 ±1.36, 56 ±1.01, 65±0.56) (fig. 5).

4. Discussion

Type 1 diabetes mellitus causes metabolic alterations leading to failure of pancreas to produce insulin due to autoimmune destruction of the beta cells. Insulin deficiency in type I diabetes causes severe depletion of both energy stores and protein mass due to the net increase in the protein breakdown rather than a decline in protein synthesis causing increase in the catabolism [20]. Nair *et al* noted a reduction in whole-body protein synthesis in type I diabetic male patient off insulin therapy [21]. Because of the progressive nature of the disease it is very important to prevent the protein loss in diabetic complications. Numerous dietary plant polyphenols have been used to prevent the development and progression of long term diabetic complications [25].

Therefore we found it worthwhile to look into the potential of phloroglucinol a naturally occurring polyphenol in preventing the depletion of the protein level in STZ induced diabetic rats.

In the present pre-clinical study the experimental rat model for type I diabetes mellitus was created by administration of diabetogenic drug streptozotocin (STZ) which causes destruction of pancreatic beta cells. Decrease in beta cells leads to reduction in the release of insulin causing elevation in blood glucose levels i.e. hyperglycemia. The major complications associated with hyperglycemia are neuropathy and nephropathy which are known to develop and progresses within 4-6 weeks in animals injected with streptozotocin [22, 23]. Both complications have similar etiologic characteristic.

Hyperglycemia has been demonstrated as an important parameter for diabetes mellitus. Prophylactic treatment with antihyperglycemic reduces the serum glucose levels and have protective effect on the progression of diabetic complications. STZ-induced diabetic rats treated with phloroglucinol showed a significant reduction in blood sugar level compared to STZ treated group. The present results confirmed the anti-hyperglycemic activity of phloroglucinol in accordance with the previous studies that demonstrated anti-hyperglycemic properties of phloroglucinol derivative by inhibiting α-glucosidase and α-amylase [26].

Biochemical estimation of serum albumin provides a direct method to determine the protein metabolism and functional status of liver and kidney. Serum albumin is also known to associate with the severity of microvascular complications. Our results clearly demonstrated the depletion the serum level of diabetic rat which confirms increase in protein catabolism. We found that phloroglucinol treatment has beneficial effect in conserving the albumin level. This meant phloroglucinol is inhibiting excessive protein catabolism, might be due to its anti-hyperglycemic effect.

Several studies clearly demonstrated that hyperglycemia is a critical factor induces elevation of serum level of creatinine which is considered as significant marker of renal dysfunction [24]. Our results indicate the inhibition of elevated serum creatinine level in diabetic rats after 8 weeks of oral administration of phloroglucinol when compared with diabetic control rats. Phloroglucinol administration reduces serum creatinine level in the dose dependent manner, which indicate the decreased protein degradation and beneficial effect in kidney.

Diabetic neuropathy is characterized by myelin abnormalities in sciatic nerve. Myelin contain about 20-30% protein which decreases in diabetes due to demyelination [6]. To find the effect of phloroglucinol on myelin protein level, we have determined the total protein level in the sciatic nerve of diabetic rat. In the present study levels of total protein was significantly decreased in sciatic nerve of diabetic group. Treatment with phloroglucinol significantly increases protein content and protects against the demyelination.

Currently, drugs used for the treatment of diabetes can affect the kidney function. The reduction in the level of total protein in kidney is directly interrelated with likelihood of renal dysfunction. The results revealed the oral administration of phloroglucinol may improve the renal function by escalating the protein concentration in diabetic rats.

These results demonstrated that phloroglucinol treatment can preserve the biochemical deficit in animal model of diabetes, suggesting the treatment can be beneficial to prevent the diabetic related microvascular complications. Further studies can also be performed to determine various proteins like acidic protein, basic protein, heat shock proteins etc. and also the expression of the protein involved the breakdown. As such the phloroglucinol treatment may be of interest, it is then considered for further evaluation in a clinical studies.

5. Conclusion

The findings of the present study suggests the oral administration of phloroglucinol have protective potential against the STZ induced abnormal protein alterations in rats by its antihyperglycemic effect. In addition improved protein levels in the nerve and kidney homogenate also contribute significantly the preventive effect of phloroglucinol against the development of diabetes induced neuropathy and nephropathy.

6. Conflict of interest

The authors declare no competing conflict of interests

7. Acknowledgement

Authors are thankful to Indian Council of Medical Research (ICMR) New Delhi, India for providing senior research fellowship to the corresponding author.

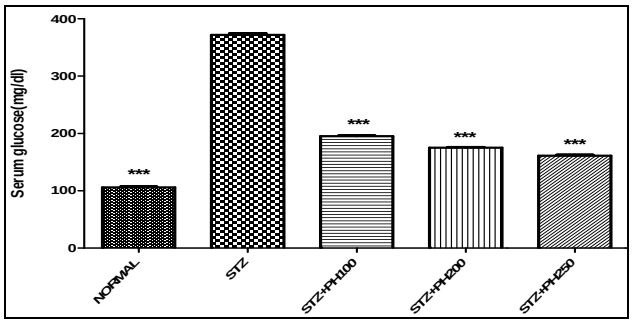

Fig 1: Effect of oral administration of phloroglucinol for 8 weeks on serum glucose levels in rats.

Values are expressed in mean ± SEM, n=6 *** (p<0.001) when compared with the diabetic control group by using one way ANOVA followed by Tukey's multiple comparison test using Graph Pad Prism 5.

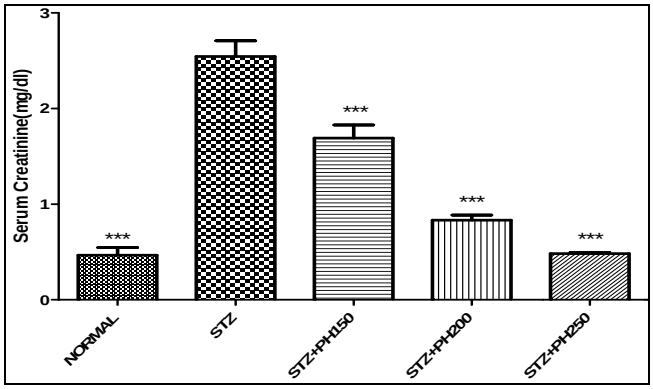

Fig 2: Effect of oral administration of phloroglucinol for 8 weeks on serum creatinine levels in rats.

Values are expressed in mean ± SEM, n=6 *** (p<0.001) when compared with the diabetic control group by using one way ANOVA followed by Tukey's multiple comparison test using Graph Pad Prism 5.

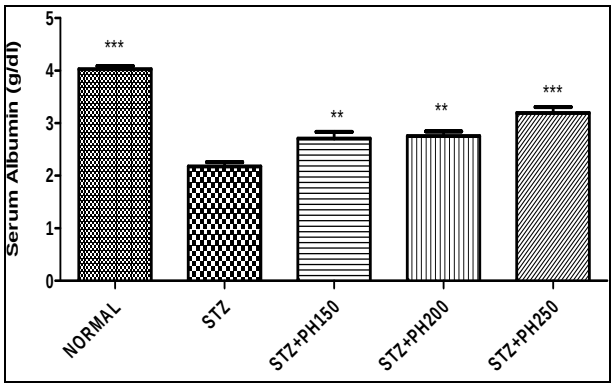

Fig 3: Effect of oral administration of phloroglucinol for 8 weeks on serum albumin levels in rats

Values are expressed in mean ± SEM, n=6 ** (p<0.01), *** (p<0.001) when compared with the diabetic control group by using one way ANOVA followed by Tukey's multiple comparison test using Graph Pad Prism 5.

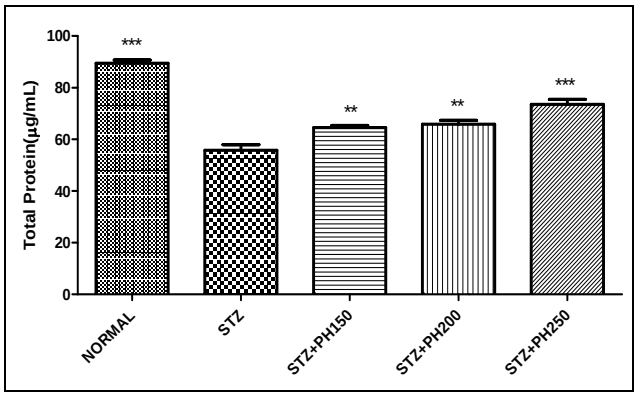

Fig 4: Effect of oral administration of phloroglucinol for 8 weeks on total protein levels in sciatic nerve homogenate in rats

Values are expressed in mean ± SEM, n=6, ** (p<0.01), *** (p<0.001) when compared with diabetic control group by using one way ANOVA followed by Tukey's multiple comparison test using Graph Pad Prism 5.

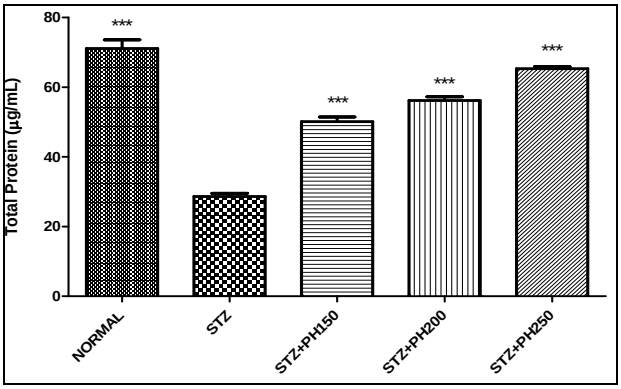

Fig 5: Effect of oral administration of phloroglucinol for 8 weeks on total protein levels in kidney homogenate in rats

Values are expressed in mean ± SEM, n=6 *** (p<0.001) when compared with the diabetic control group by using one way ANOVA followed by Tukey's multiple comparison test using Graph Pad Prism 5.

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