IMMUNOHISTOCHEMISTRY OF GLUT-2 BASED STUDIES ON ISLETS OF PANCREAS IN TYPE 2 DIABETIC MALE WISTAR ALBINO RAT AFTER THE ADMINISTRATION OF GYMNEMA SYLVESTER AND METFORMIN

Abstract

Diabetes mellitus is the generally known metabolic disorder affecting the individuals' not only the elderly population but impacts irrespective of all age groups. Amongst the diverse etiology of diabetes, reduction of glucose transporter GLUT-2 is accompanied by a loss in glucose-mediated insulin secretion causing diabetes. Though there are various treatment strategies practiced worldwide to treat diabetes, the present scenario is the demand of time to use the herbal drugs with minimal detrimental effects. Considering the benefits of *Gymnema sylvester* anti-diabetic herbal drug, our current study is designed to expose the plausible relationship between the anti-diabetic activity and GLUT-2 expression, and the efficacy is evaluated in a streptozotocin-induced diabetic model. **Materials and Methods:** Wistar albino rats (n=36) of weighing about 140g-160g were used for this study. Each group consisting of 6 animals and were divided such as **Group I,** a control animal, **Group II,** animals were fed a high-fat diet for 42 days**,** Group **III,** a streptozotocin-induced diabetic model **(** multiple-dose for 5 successive days**).** The **Group IV** and **Group V** animals were induced diabetic with Streptozotocin and treated using ethanolic extract of *Gymnema sylvestre* at low dosage (200 mg/kg b.w.) and high dosage (400 mg/kg b.w.) respectively**. Group VI** is positive control of animals induced diabetic with Streptozotocin and administered with Metformin **(25mg**/kg b.w. for 22 days). During the experimental period blood glucose level and the animal weight were carefully monitored. **Results**: Decreased body weight, pancreases weight, and increased blood glucose were observed Group III along with reducing level the GLUT-2 expression indicating the manifestation of diabetes. Conversely these abnormalities are significantly restored after the treatment with *Gymnema sylvestre in diabetic rats* interestingly in the higher dosage. The *Gymnema sylvester* displays the antidiabetic activity through the regeneration of β-cells maintaining GLUT-2 expression in diabetic induced animal model dose-dependently suggesting effective anti-diabetic herbal drug. Our histological of pancreatic tissue also confirm the regeneration of beta cells. The ameliorating effects of the *Gymnema sylvestre* could be attributed to the bioactive components present in this herbal drug the saponin.

Keywords: Type-2 diabetes, *Gymnema Sylvestre*, GLUT-2, Immunohistochemistry.

Comment [PERI1]: abstract is too long

Introduction:

Diabetes mellitus is the most common metabolic disorders. It is characterized by hyperglycemia that results from an insulin deficiency and it is associated with complications affecting the eye, heart, kidney, and nerves (Punthakee et al., 2018). Diabetes mellitus is classified into Type1 and Type2. Type1 diabetes is an autoimmune disease characterized by an inflammatory reaction around the islets of Langerhans followed by specific destruction of β-Cell (Tosone et al., 2013). Type2 diabetes is characterized by the occurrence of peripheral insulin resistance and impaired insulin secretion (Mahler and Adler, 1999). Diverse etiology intricate the cause of diabetes, amongst the GLUT is an important biochemical entity involved in the development of diabetes. GLUT-2 is a family of glucose transporter, restricted in the plasma membrane of insulin-positive beta cells of adults and other tissues such as liver, kidney, and intestinal mucosal epithelium (Jorns et al. 1995). GLUT-2 regulates the transportation of glucose into the pancreatic cell and thus promotes insulin secretion and the reduction of GLUT-2 is correlated with a loss in glucose-induced insulin secretion causing diabetes (Mueckler et al., 1994; Olli Laukkanen et al., 2005).

Comment [PERI2]: The magnitude of the incidence of DM has not been seen

Comment [PERI3]: Please use the latest library references

Various treatment strategies currently practiced worldwide to treat Type2 diabetes however leaving behind some side effects (Pritesh Patel et al., 2012). Therefore the present scenario time demands to use the herbal drugs from plant sources to treat such ailments with minimal detrimental effects. *Gymnema Sylvester* commonly known as **Sirukurunjan** (Tamil) is a very popular anti-diabetic plant traditionally used as a sugar destroyer (Najafi and Deokule, 2011) in India for centuries. This herbal drug is also used in many ailments such as jaundice (Di Fabio et al., 2013), cardiopathy (Kumar et al., 2012), asthma, bronchitis (Pragya Tiwari et al., (2014), appetite suppressant (Sujin RM. 2008) and conjunctivitis (Parijat Kanetkar et al., 2007) and other diseases (David and Sudarsanam 2013; Rongzi Li et al., 2019).

The *Gymnema sylvester* is widely used to treat diabetes and validated scientifically. However the molecular mechanism supporting the hypoglycemic and anti-diabetic properties remains unclear.

Furthermore many studies have reported antidiabetic properties *of Gymnema sylvester* (Prakash et al., 1986; Baskaran et al., 1990; Shanmugasundaram et al., (1990) but only a few reports are available *Gymnema Sylvester* and GLUT-2 on the diabetic model. Thus considering the benefits of *Gymnema Sylvester* the current study is designed to elucidate the plausible link between the anti-diabetic activity of *Gymnema Sylvester* and GLUT-2 expression and the efficacy is evaluated in streptozotocin-induced diabetic model.

Material and methods:

Adult Wister albino rats, weighted about 140 to 160g were segregated, kept in polypropylene cage, and maintained under standard housing condition of 12:12 hour's day/night cycle with temperature 22-25º with a relative humidity of 50-60 %. The animals were fed with standard rat pellets and drinking water ad libitum. The present study was conducted according to a protocol approved by the animal ethical committee, Sathyabama Institute of Science and Technology (No. SU/CLATR/IAEC/XI/100/2018) and CPCSEA (2003) guidelines.

Experimental Plan:

Animals were divided randomly into 6 groups namely Group I, control animals fed with normal pellet food. Group II, animals were fed with High-fat diet (67.5% lard oil, 31% cholesterol, 1% d1-methionine, 0.3% yeast powder and 0.1% NaCl) for 42 days, Group III animals were fed the high-fat diet for 14 days then streptozotocin (40mg/kg b.w., i.p) is administered for 5 consecutive days to establish Type 2 diabetic model. The Group IV and Group V were Type 2 diabetic induced animals and treated with ethanolic extract of *Gymnema sylvestre* by gavage at low dosage (200mg/kg of b.w. for 3 weeks) and high dosage (400mg/kg of b.w. for 3 weeks) respectively. Group VI is a positive control animal, induced Type 2 diabetes with Streptozotocin and administered with Metformin (25mg/kg b.w. for 22 days). During the experimental period, the weight of the animal and the level of blood glucose were carefully monitored and recorded. The glucose level was diagnosed by collecting the blood sample from the tail vein and determined by using a glucose analyzer with a glucose strip inserted in the glucometer.

Tissue Harvesting

At the end of the experiment organs such as heart, liver, kidney and pancreas, were dissected out and weighed immediately after the sacrifice (over dose of ketamine i.p.) to determine the change in the weight of organs with respect to their body weights. The harvested tissues were post-fixed in 10% formaldehyde and processed for standard histology and immunohistochemistry. Sections were taken at 5 µm thickness and stained using Harris hematoxylin and eosin (Bancroft 2012).

Immunohistochemistry

For detection of GLUT-2 expression, the sections were deparaffinized, rehydrated, and subjected to 1% H_2O_2 in PBS to quench the endogenous peroxidase activity, followed by blocking buffer (5% normal chicken serum in PBS and 0.3% Triton X-100) treatment at 4ºC to minimize non-specific expression. Then the sections were incubated with a 1:100 dilution of human anti-GLUT-2 monoclonal antibody (CUSABIO TECHNOLOGY LLC, USA) overnight at 4ºC. After washing with PBS, tissues were incubated with a 1:400 dilution of biotinylated goat anti-mouse secondary antibody for20 min at 42^{o}C. Subsequently sections were treated with an avidin-biotin-peroxidase complex for 2 hours. The peroxidase activity was visualized using a stable diaminobenzidine solution and counterstained with hematoxylin. The GLUT-2 expressions were examined using a compound light microscope (Olympus CX31) and these results were quantified using the Image analysis J 1.46 software. (National Institutes of Health, Bethesda, Maryland, USA).

Statistical Analysis:

Data were analyzed using Microsoft Excel (Version 2003) and SPSS (SPSS, Version 25 Inc., IBM) software. The values were expressed as the mean and standard error of the mean. One-way ANOVA was performed in SPSS, the level of significance was determined with a “Tukey’s post hoc” test and $P<0.05$ was considered as statistically significant.

Results:

Table 1. Animal Body weight

Groups	14th day	19th day	28th day	42nd day
Group I	169.50 ± 1.94	172.50 ± 1.82	182.67 ± 1.36	204.83 ± 2.01
Group II	178.17 ± 1.2	186.33 ± 1.31	209.17 ± 2.19 @*	232.50 ± 1.53 @*
Group III	176.33 ± 1.11	148.17 ± 1.28 @***	105.67 ± 1.58 @***	88.67 ± 1.14 @***
Group IV	175.33 ± 1.49	147.67 ± 1.57@***	164.67 ± 3.51 $***	176.50 ± 3.89 $***
Group IV	174.33 ± 1.62	149.83 ± 1.34@**	171.83 ± 2.00 $***	185.83 ± 2.38 $***
Group VI	175.33 ± 1.46	147.33 ± 1.75@***	177.50 ± 2.46 $***	194.67 ± 1.57 $***

Table 1 illustrates body weight of control and various groups during experimental period and values are presented as Mean ± SEM (n=6 animals each) with * P<0.05, ** P<0.01 and *** P<0.001. @ - compared with control; $ - compared with streptozotocin-induced diabetic animals (Group III).

Table 2 Weight of the pancreas, heart, liver and kidney immediate after the sacrifice

Groups	Pancreas	Heart	Liver	Kidney
Group I	0.423 ± 0.018	0.755 ± 0.007	5.31 ± 0.128	1.158 ± 0.019
Group II	0.566 ± 0.023	0.773 ± 0.008	6.54 ± 0.222	1.409 ± 0.020 @***
Group III	0.222 ± 0.008 @*	0.700 ± 0.012	4.86 ± 0.170	1.167 ± 0.022
Group IV	0.293 ± 0.022	0.752 ± 0.012	5.20 ± 0.208	1.248 ± 0.033
Group IV	0.332 ± 0.018	0.757 ± 0.016	5.23 ± 0.090	1.210 ± 0.018
Group VI	0.362 ± 0.020	0.746 ± 0.014	5.26 ± 0.156	1.409 ± 0.019 @*

Table 2 displays organ weight of pancreas, heart, liver and kidney of different experimental groups. The values are stated as Mean ± SEM of 6 animals each; Significance at * P<0.05, ** P<0.01 and *** P<0.001. @ - comparison of all experimental groups with control.

Table 3 Body weight and Organ weight of the animals immediate after the sacrifice

Groups	Animal Weight (grams)	Liver (grams)	Pancreas (grams)
Group I	204.83 ± 2.01	5.31 ± 0.128	0.423 ± 0.018
Group II	232.50 ± 1.53 @*	6.54 ± 0.222	0.566 ± 0.023
Group III	88.67 ± 1.14 @***	4.86 ± 0.170	0.222 ± 0.008 @*
Group IV	176.50 ± 3.89 $***	5.20 ± 0.208	0.293 ± 0.022
Group V	185.83 ± 2.38 $***	5.23 ± 0.090	0.332 ± 0.018
Group VI	194.67 ± 1.57 $***	5.26 ± 0.156	0.362 ± 0.020

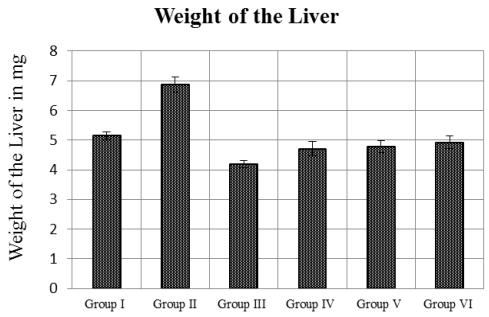
Table 3 exhibits the weight of the animals and the weight of the liver and pancreas of control and various groups during the experimental period. Data are illustrates as Mean ± SEM of 6 animals each; significant difference from control was *P<0.05; **P<0.01; ***P<0.001. @ - all experimental groups were compared with control; $ - Group IV and Group V and Group VI were compared with Group III.

Table 4 Ratios of organs weight contrast to animal weight.

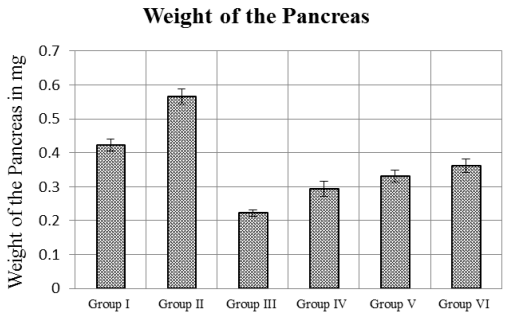
Groups	Liver gm/kg	Pancreas gm/kg
Group I	2.065	25.142
Group II	2.434	29.548
Group III	2.504	47.254
Group IV	1.660	26.629
Group V	1.787	25.776
Group VI	1.860	25.274

Table 4 shows comparison between ratios of the weight of organs in grams to the weight of the animal in kilograms.

Graph.1



Graph.2



Graph 1 and 2 Illustrate the weight of the liver and pancreas of experimental groups. The columns represents Mean, and error bars denoting SEM; with *P<0.05. @ - compared with control.

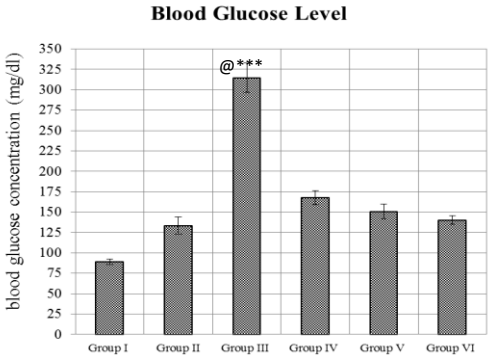
Table 5 Blood Glucose Level

Groups	14th day	19th day	28th day	42nd day
Group I	89.83 ± 2.79	91.33 ± 2.85	101.67 ± 4.3	89 ± 3.3
Group II	84.67 ± 2.5	129.50 ± 11.78	150.50 ± 9.72	133.67 ± 10.67
Group III	86.83 ± 2.45 @***	223 ± 19.88 @***	229 ± 14.93 @***	314.83 ± 18.31 @***
Group IV	85.33 ± 1.75 @***	221.67 ± 14.9 @** $***	180.17 ± 6.31 @*** $***	167.67 ± 8.27
Group V	87 ± 1.83 @***	230.67 ± 20.69 @** $***	175.50 ± 5.49 @** $***	150.83 ± 8.73
Group VI	84.17 ± 3.28 @***	227.17 ± 21.78 @* $***	169 ± 10.72 @* $***	140.17 ± 5.10

Table 5 shows blood glucose level weight of various groups in 14th, 19th , 28th and 42nd days of the experiment, and values are denoted as Mean ± SEM (n = 6 animals each) and significance at * P<0.05, **

P<0.01 and *** P<0.001. @ - compared with control; $ - compared with streptozotocin-induced diabetic animals (Group III).

Graph 3



Graph 3 demonstrates blood glucose level and each column represents Mean and error bar denotes Standard error of mean (n = 6 animals each) and significance at *P<0.05, **P<0.01 and ***P<0.001. @ - comparison with control.

Fig. 1 Histology of pancreas

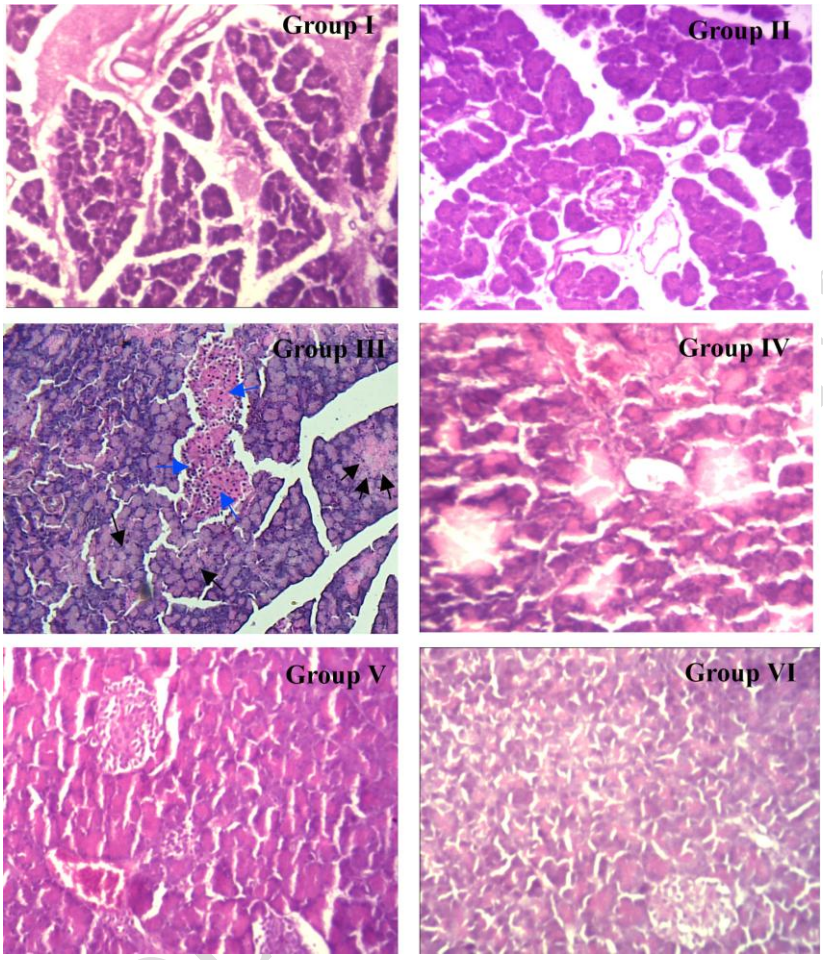


Fig.1 shows the histology of the pancreas of different experimental animals. Blue arrows indicating shrinkage of Islets of Langerhans and black arrows are pointing damage of acini in the diabetic animal.

Fig. 2 Immunohistochemistry of GLUT-2

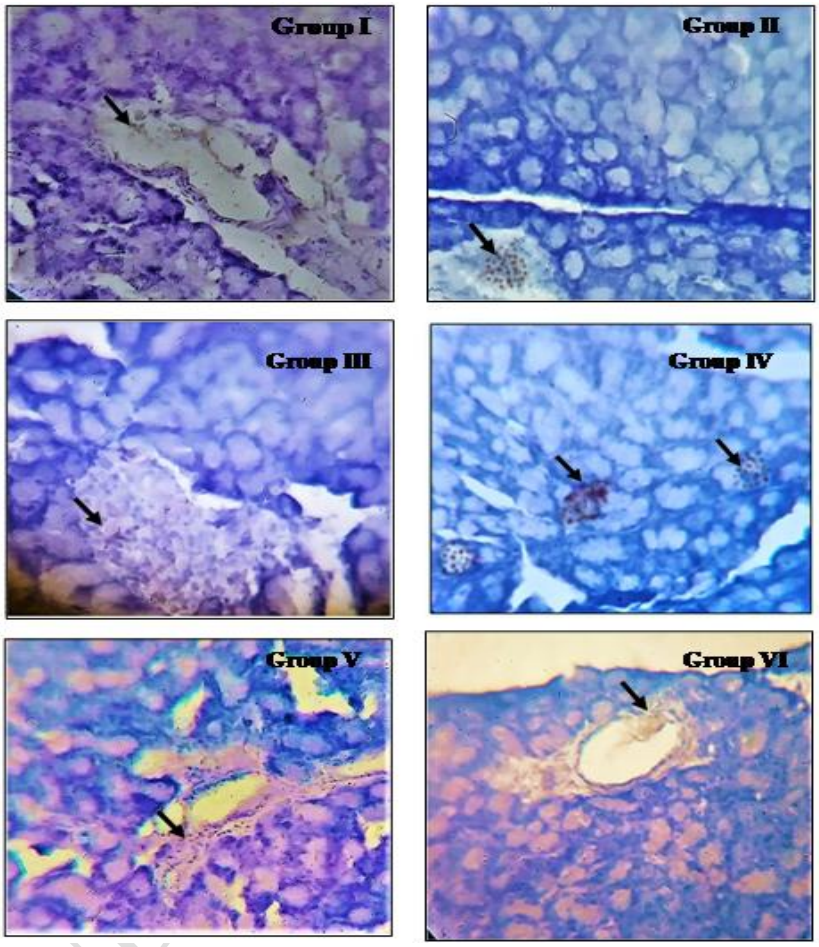


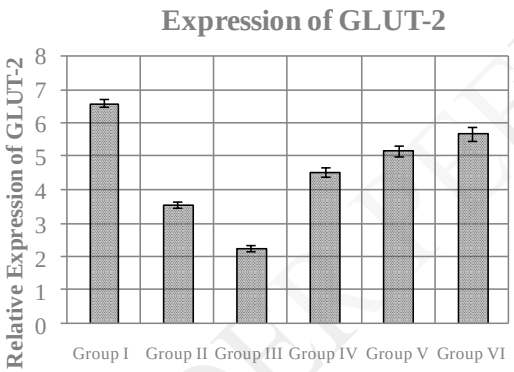
Fig.2 demonstrates immunohistochemistry of GLUT-2 in the pancreas of control and various experimental animals. Arrows indicating the darkly stained area with brown shows the expression of GLUT-2.

Table 6 Relative Expression of GLUT-2

Groups	Relative Expression of GLUT-2
Group I	6.58 ± 0.121
Group II	3.56 ± 0.103 @***
Group III	2.24 ± 0.096 @***
Group IV	4.51 ± 0.137 @* $***
Group V	5.16 ± 0.172 $***
Group VI	5.68 ± 0.195 $***

Table 6 Relative Expression of GLUT-2, quantified by the Image analysis J 1.46 software and the values represent Mean ± SEM with significance at *P<0.05; **P<0.01; ***P<0.001. @ - comparison with control; $ - comparison with streptozotocin-induced diabetic animals.

Graph.4



Graph 4 Relative Expression of GLUT-2 quantified by the Image analysis J 1.46 software. Each vertical column representing Mean, and error bar indicating SEM and significant difference from control at *P<0.05, **P<0.01 and ***P<0.001. @ - compared with control; $ - compared with streptozotocin-induced diabetic animals.

Results

Body Weight

In this present investigation the animals in group II showed increased body weight (P<0.05) after the supplementation of a high-fat diet. The group III animals administered with streptozotocin-induced diabetes showed much reduction in body weight (P<0.001) when compared to control animals (Group I).The Group IV and Group V animals were maintained their body weight after treatment

of *Gymnema sylvestre* at low and high dosages compare to the Group II. These observations are very close to streptozotocin-induced diabetic animals treated with Metformin [Table 3].

Liver and Pancreas Weight

The weight of the pancreas and liver in Group II was highly elevated after the supplementation of a high-fat diet [Table 3]. While in Group III i.e., streptozotocin-induced diabetic animals, the liver weight was slightly decreased whereas pancreas weight was considerably ($P<0.05$) reduced. Whereas *Gymnema sylvester* treatment in Group IV and Group V animals, pancreas weight [Graph 1] was considerably augmented at both dosages (200mg and 400 mg) but there was a marginal reduction of liver weight was observed as metformin-treated groups did not show statistical significance [Graph 2]. The metformin administered animals (Group VI) did not show any noticeable changes in liver and pancreas weight and these values were close to control animals.

Blood glucose levels

Blood glucose levels [Graph 3] were significantly elevated ($P<0.001$) in Groups III compared to control animals (Group I). Administration of *Gymnema sylvester* (400mg) and Metformin in streptozotocin-induced diabetic animals (Group V and Group VI) revealed a reduction of blood glucose level. Though this level was decreased at low dose *Gymnema sylvester* administration in group IV, the improvement was noticeable at high dosage. The Group II rats fed with high fat did not show any significant shifts in the blood glucose level. The data of Blood glucose level was shown in Table 5.

Histological study

The histological study [Fig.1] of the pancreas in group III animals induced type 2 diabetic showed shrinkage of islets of Langerhans, damage of acini and reduced number of beta cells whereas the animal treated with *Gymnema sylvestre* in both low and high dosages (Group IV and Group V) showed regeneration of islets of Langerhans and increased number of the beta-cell. This recovery is near to the rats in Group VI. A similar observation was reported after supplementation of *Gymnema sylvestre* in diabetic rat (Hafizur 2015).

Immunohistochemistry

The immunohistochemistry of GLUT-2 protein [Fig.2] in the pancreas appeared as the darkly stained area with brown (arrow mark). The GLUT-2 expression was noticeably down-regulated

(P<0.001) in high-fat diet and streptozotocin-induced diabetic animals (i.e., Group II and Group III) when compared to the control (Group I). Conversely, the pancreas of *Gymnema sylvester* and metformin-treated animals (Group IV, Group V, and Group VI) indicated the up-regulation of GLUT-2 activity (P<0.001) when compared to diabetic animals (Group III). Though the GLUT-2 expression was increased in a low dose of *Gymnema sylvester* administration in group IV, the improvement was remarkable at high dosage [Graph 4]. These changes in GLUT-2 expressions were measured using Image analysis J 1.46 software to substantiate immunohistochemistry of GLUT activity [Table 6].

Discussion

The weight of the body and the pancreas decreased unfailingly in diabetic animals (Table 3). The decrease in the pancreas weight in streptozotocin-induced diabetic animals could be the result of disruption of pancreatic islets and selective destruction of insulin-producing cells (Kim et al., 2006; Heidari et al., 2008; Zafar et al., 2010) The administration of *Gymnema sylvestre* (both low dosage and high dosage) were markedly maintained the body weight and pancreas weight towards the normal undoubtedly indicated the treatment effects of the herbal drug. These results were further correlated with the known drug metformin-treated diabetic animals. The significant increase in blood glucose levels was noted in the high dose of *Gymnema sylvester*. However both dosage groups were restoring blood glucose level proved to be the protective effect of *Gymnema sylvester* administration. On agreement of our present results a recent study demonstrated the protective effects of *Gymnema sylvester* against type2 diabetes mellitus and its associated abnormalities (Dhananjay Yadav et al., 2019).

The experiment in diabetic rodent model treated with *Gymnema sylvester* suggested that the anti-diabetic effect could be achieved by the improvement in glycogen synthesis, glycolysis, gluconeogenesis, and hepatic and muscle glucose uptake (Porchezhian and Dobriyal 2003) and facilitated the hinder of hemoglobin and protein glycosylation (Shanmugasundaram ERB et al., 1988). Another study stated that *Gymnema sylvestre* reduced the fasting glucose levels together with a considerable lowering of serum lipid levels and concomitantly ameliorating serum protein levels (Shanmugasundaram KR et al., 1981) substantiated protective effect of this herbal drug in diabetic animals. In vitro studies shown that the alcoholic extract of *Gymnema sylvestre* in HIT-T15, MIN-6, and RINm5F β-cells stimulates the release of insulin by increasing membrane permeability (Persaud SJ, et al., 1999; Sheoran et al., 2015) through a channel-independent influx of Ca++ into the β-cells

thereby maintains blood glucose level. The ameliorating effects of the Gymnema *sylvestre* could be attributed to the bioactive components present in this herbal drug chiefly the saponin. The gymnemosides and gymnemic acid are saponin components responsible for the antihyperglycemic effect of Gymnema sylvestre (Murakami et al., 1996).

The GLUT is the facilitated diffusion glucose transporters expressed in the plasma membrane of insulin-positive beta cells and required for glucose-stimulated insulin secretion (Benard 2015) and it is the major glucose transported in islets of beta cells (Piero marchetti et al., 2017; Rosa gasa 2016; Becca 2017). The down regulation of GLUT-2 expression in high-fat diet and type 2 diabetic animals' demonstrated impairment of beta-cell function. The study suggested that the risk of type 2 diabetes and obesity assumed to have the primary defect in insulin action probably by a single nucleotide polymorphism in genes that regulates insulin secretion (Olli Laukkanen et al., 2005). Another earlier study further supports our GLUT-2 results stated that the loss of GLUT-2 in pancreatic cells is an early indicator of beta-cell dysfunction (Bonny et al., (1997) commencing diabetes.

The treatment of *Gymnema sylvestre* at both dosages and metformin restored the GLUT 2 expression in diabetic induced animals. Interestingly high dosage of *Gymnema sylvestre* administration showed remarkable improvement. The findings revealed that *Gymnema sylvestre* enhance the expression of GLUT-2 dose-dependently. Ahmed et al, (2010) suggested *Gymnema sylvestre* exerts antidiabetic activities through the regeneration of beta cells in alloxan-induced diabetic rats. Likewise other studies proved a cluster of beta-cell regeneration in the diabetic rat (Hafizur 2015) after the treatment of *Gymnema sylvestre*. Our histological of pancreatic tissue also confirm the regeneration of beta cells. Supporting our GLUT-2 result, the methanolic leaf extract of *Gymnema sylvestre* has shown increased glucose uptake and facilitated the enhanced GLUT-4, an isoform of glucose transporters (Mahesh Kumar et al., 2016). The same mechanism could be orchestrated in the expression of GLUT-2. Thus it could be suggested that the effectiveness of *Gymnema sylvestre* could be attributed to the augmentation of glucose transporters and glucose absorption. However the possible link between the antidiabetic activity of *Gymnema sylvestre* and GLUT-2 expressions remain to be explored. More studies would be warranted to elucidate the underlying mechanism. In agreement with earlier reports we proposed that *Gymnema sylvestre* regenerate and/or restore the pancreatic beta cells via increasing glucose uptake enabled the improved GLUT-2 expression.

On the whole of our study we suggested that the *Gymnema sylvestre* could be a potential herbal drug that prevents the deteriorating effect of diabetes via the regeneration of β-cells and regularize blood glucose level.

Comment [PERI4]: focus on explaining the findings

Conclusion

In the essence of our current investigation we concluded that the *Gymnema sylvestre* treatment improved the body weight, maintain pancreas weight and blood glucose level, the high dosage of *Gymnema sylvester* were more effective. The *Gymnema sylvester* protects the pancreatic beta cells to sustain glucose transporter GLUT-2 level and thereby maintaining the glucose level in the diabetic induced animal model dose-dependently. The bioactive components, the saponin present in this herbal drug could be responsible for its ameliorating effects. Thus the herbal drug *Gymnema sylvester* could be a potential herbal drug, would be a better choice for the treatment of type2 diabetes. However further studies are required before clinical implication.

Comment [PERI5]: conclusion too long

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors

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