Original Research Article

# An experimental study to evaluate preventive role of Aspirin and Gymnema sylvestre (Meshashringi) in type 2 diabetes mellitus in rats

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## Abstract

**Background:**Type 2 diabetes mellitus (T2DM) results from relative insulin deficiency, which may either be due to decreased insulin secretion, or increased insulin resistance. Obesity, and the resulting oxidative stress, is intricately involved in pathogenesis of insulin resistance. A chronic subclinical inflammation has also been sought to be involved in development of T2DM.**Objective(s):**This study was aimed at finding out the preventive role of aspirin, a well-known drug for its anti-inflammatory effects, and Gymnema sylvestre (GS), a herb having antioxidant properties, in T2DM. **Material & Methods:** Adult female Wistar rats were procured & randomly divided into nine groups with six rats in each group. Group I animals were kept on standard chow diet for 21 days and, were administered citrate buffer injection on Day 15.Remaining animals were kept on high fat diet (HFD) for two weeks and, on day 15, were given (after overnight fasting) a single intra-peritoneal injection of streptozotocin (STZ) and HFD was continued for a further week. These rats were treated with the test drugs (aspirin, GS; alone and in combinations) and the standard drug (metformin), from day 1 through day 21.**Results and Conclusions:** Both Aspirin and GS prevented rise in RBS levels, but did not show any significant effect on prevention of weight gain in T2DM rat models. Aspirin exerted either no effect or a negative effect on the anti-oxidant status, while GS exerted a protective effect. These findings are suggestive of their potential role in prevention of T2DM in humans.

**Keywords:** Type 2 diabetes mellitus, insulin resistance, oxidative stress, anti-inflammatory, anti-oxidants



## Introduction

Diabetes mellitus imposes a substantial social and economic burden. The effects and complications of diabetes mellitus include various organs damages such as renal failure, blindness, leg amputation, as well as also increases the risk of stroke, myocardial ischemia/ infarction and peripheral artery disease. Insulin resistance and islet beta-cell exhaustion results in relative insulin deficiency. Sedentary lifestyle has led to a steep increase in the incidence of diabetes globally[1-3]. Insulin resistance progresses to T2D Munder the influence of a various factors like genetic make-up of the individual, lifestyle, calorie intake and the environment.Now, a state of subclinical chronic inflammation is also thought to be the pathogenesis of T2DM[5-7].Inflammation is a protective response of body secondary to stress, injuries and infections. Low grade systemic inflammation is characterized by 2-3 folds increase in plasma concentration of cytokines like TNF-α, interleukin (IL-6), chemokines, C-reactive protein (CRP), etc[6-8].Aspirin exerts its anti-inflammatory action by inhibition of cyclooxygenase enzymes (COX1 and COX2). A COX independent mechanism of aspirin action in reducing inflammation has also been identified to act through inhibition of NF-κB pathway.As inflammation has been implicated in causation of diabetes, aspirin being an anti-inflammatory drug, may be expected to have some beneficial effects in diabetes. Also, seeing the widespread use of aspirin in various cardiovascular disorders, it seems prudent to explore its scope in prevention of diabetes and comorbid conditions. Obesity is characterized by an increase in adipose tissue mass which results in chronic activation of immune system. In obesity, monocytes are activated by chemokines which are released from adipose tissue. They in turn initiate a cascade of inflammation, which leads to development of insulin resistance and consequent risk for T2DM [8].Lipid toxicity and low-grade inflammation appear to decrease insulin sensitivity[9].Use of complementary and alternative medicines (CAM) for the management of chronic diseases is a rapidly developing field and has the potential for other health benefits. Many studies have shown the beneficial effects of medicinal plants in the treatment as well as prevention of chronic metabolic disorders like diabetes, obesity, hypertension, etc. Gymnema sylvestre is an indigenous herb of India and Africa, has been known to mankind for many of its therapeutic potentials and has been used for its anti-oxidant properties for ages[10].The plant extract is being used in Ayurvedic medicine as an anti-diabetic agent. The present study was planned to evaluate the role of aspirin and G. sylvestre in prevention of T2DM in high fat diet and low dose streptozotocin induced diabetes in rats, separately as well as in combination. Further, these effects were compared with those of standard drug (Metformin). We also evaluated the anti-oxidant effects of aspirin and G. sylvestre in high fat diet and low dose streptozotocin induced diabetes in rats.

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## Materials &methods

**Chemicals:**The following drugs & chemicals were used: Aspirin, Metformin, Phosphate buffer saline (PBS) - HiMedia Laboratories Pvt. Ltd., Mumbai, India

Gymnema sylvestre (Meshashringi) - The Himalaya Drug Company, Bangalore, India

Streptozotocin (STZ), 5,5-Dithiobis(2-Nitro Benzoic Acid) (DTNB), Glutathione reduced (GSH) – Sisco Research Laboratories Pvt. Ltd.

Citrate buffer, Hydrogen peroxide ($H_2O_2$), Glacial acetic acid, Sodium dodecyl sulfate (SDS) – Thermo Fisher Scientific.

Sodium azide, Trichloroacetic acid (TCA) – Fisher Bioreagents

2-Thiobarbituric acid (TBA) - Merk

Malonaldehyde (MDA) – TCI Chemicals (India) Pvt. Ltd.

All the drugs, chemicals and reagents were procured from M/s. Delta Biosciences, Lucknow.

**Animals**

The present study was conducted in the Department of Pharmacology & Therapeutics, King George's Medical University, Lucknow,only after obtaining clearance from Institutional Animal Ethics Committee (IAEC) vide ethical clearance No. 96/IAEC/2018 dated 20/08/2018 and all the procedures done were in accordance with institutional guidelines. A total of 54 adult female Wistar rats weighing 180-250 g were procured from IITR, Lucknow, India.The animals were kept in Institutional animal house under standard housing conditions (room temperature 24-27°C and humidity 60-65%) with 12 hour light and dark cycle. The food was given in the form of dry pellets and water was available ad libitum.

**Rat model of type 2 diabetes mellitus**

In this study, the animals were divided into 9 groups with 6 animals in each. Group I animals were kept on standard chow diet for 21 days and were administered citrate buffer injection on Day 15, this group served as control. Rest of the 48 experimental animals were kept on (HFD) for two weeks and to induce diabetes, on day 15, rats were (after overnight fasting) given a single intra-peritoneal injection of streptozotocin 35mg/kg (STZ in citrate buffer, pH 4) and the high-fat diet (HFD) feeding was continued for a further week[11]

**Group 1- Control:** Animals were administered with standard chow diet and on 15$^{th}$ day, i.p. injection of citrate buffer (vehicle).

**Group 2- Negative control:** Animals were administered with HFD for 21 days and streptozotocin (35mg/kg/i.p) on 15$^{th}$ day.

**Group 3-** Animals were administered with HFD + aspirin (2mg/kg) for 21 days and streptozotocin (35mg/kg/i.p) on 15$^{th}$ day[12]

**Group 4-** Animals were administered with HFD + aspirin (10mg/kg) for 21 days and streptozotocin (35mg/kg/i.p) on 15$^{th}$ day[12]

**Group 5-** Animals were administered with HFD + G. sylvestre extract (60mg/kg) for 21 days and Streptozotocin (35mg/kg/i.p) on 15$^{th}$ day.

**Group 6-** Animals were administered with HFD + G. sylvestre extract (120mg/kg) for 21 days and Streptozotocin (35mg/kg/i.p) on 15$^{th}$ day[13]

**Group 7-** Animals were administered with HFD + G. sylvestre extract (240mg/kg) for 21 days and Streptozotocin (35mg/kg/i.p) on 15$^{th}$ day.

**Group 8-** Animals were administered with HFD + aspirin (10mg/kg) + G. sylvestre extract (240mg/kg) for 21 days and Streptozotocin (35mg/kg/i.p) on 15$^{th}$ day.

**Group 9-** Animals were administered with HFD + metformin (100mg/kg) for 21 days and streptozotocin (35mg/kg/i.p) on 15$^{th}$ day[14]

**Measurement of Random Blood Sugar**

Random blood glucose was measured with the help of a portable blood sugar monitoring device (Accu-Chek Instant-S glucometer, Batch no. 91400009) from a drop of blood from the tail vein.

**Measurement of Body Weight**

The body weight of rats was measured with the help of a digital weighing machine available in Institutional Animal House. All the rats were properly marked and weighed.

**Blood Sampling**

Blood samples were collected from retro-orbital bleeding in EDTA vials, for measurement of biochemical parameters (Catalase, Glutathione peroxidase, LPO activity). The samples were centrifuged at 3000 rpm for 15 minutes; after that, the separated plasma was stored at -80°C until biochemical analysis.

**Blood Lysate Preparation**

Blood lysate was prepared according to a previous method[15]After separating plasma, the pellet (RBC) remaining at the bottom of the tube was washed three times with cold normal saline at 2000 rpm for 15 minutes at 4°C. Finally, to the RBC pellet, chilled triple distilled water (TDW) was added and centrifuged at 10000 rpm for 15 minutes at 4°C and the supernatant (lysate) was collected in separate Eppendorf tube and the pellet (cell debris) was discarded.

**Estimation of Catalase activity**

Catalase activity of blood lysate samples were determined according to a previously reported methods[16].Catalase activity was measured by UV spectrophotometric method. 2.0 ml of phosphate buffer and 1 ml. of diluted (0.2M) $H_2O_2$ were taken in a cuvette, in this 0.02 ml enzyme source was added and mix thoroughly. The decrease in absorbance at 240 nm was recorded on spectrophotometer after every 30 seconds for 3 minutes against reagent blank. Protein content in enzyme source was determined by NanoDrop method. Amount of $H_2O_2$ consumed was determined by recording absorbance of solution at λ 240 nm and the activity was expressed as μ mole/ min / mg protein.

**Estimation of Glutathione Peroxidase (GPx) Activity**

GPx activity of blood lysate samples were determined according to a previously reported method[17]. An incubation mixture containing of 0.4 ml buffer, 0.2 ml of GSH, 0.2 ml EDTA, 0.2 ml Sodium azide and 0.2 ml hydrogen peroxide was pre-incubated at 37ºC for 10 min. 0.1 ml of blood lysate was added and incubated at 37ºC for 10 min. The reaction was terminated by the addition of the 0.1 ml of 10% TCA. Supernatant was taken and 3 ml of phosphate buffer and 1 ml of DTNB were added. The color developed was read immediately at 412 nm in a spectrophotometer. Protein content was estimated in enzyme source by NanoDrop method. Enzyme unit was defined as nmole of NADPH oxidized per minute per mg protein. The results were expressed as unit/min/mg protein.

**Estimation of lipid peroxide (LPO) Activity**

LPO activity of blood lysate samples were determined according to a previously reported method[18]. A volume of 0.2 ml of blood plasma was mixed with 1 ml of 20% acetic acid followed by the addition of 0.5 ml of aqueous SDS, 0.5 ml glacial acetic acid and 1.5 ml TBA solution and sufficient amount of TDW, to achieve a final volume of 4.0 ml. The reaction mixture was heated in a boiling water bath for one hour. After cooling at room temperature, 3.0 ml of n-butanol was added into it and mixed well. The reaction mixture was centrifuged at 10,000 rpm for 15 minutes. A clear butanol fraction containing LPO was obtained after centrifugation, the absorbance of which was measured at 532 nm. Protein content was estimated in enzyme source by NanoDrop method. Lipid peroxide content in the sample was expressed as nanomoles of MDA/mg protein.

**Histological Examination**

The histological examination of pancreatic tissue was done according to a previously reported method[19]. Pancreas of sacrificed animals, preserved in 10% buffered formalin (pH 7.4) were removed and fixed. The fixed specimens were sliced, processed, and embedded into paraffin blocks. Blocks were cut into 4 μm paraffin sections by a rotator microtome. The sections were stained with Hematoxylin and Eosin (H&E) and examined under light microscope.

**Statistical Analysis**

The results are presented as mean ± SD. The statistical analysis was done using SPSS 21.0. All data were analyzed by one-way ANOVA followed by Tukey's test. Paired "t" test was used for within group comparison. The results were considered significant at $p < 0.05$.

## Results

### Random blood sugar

Fig. 1 shows an increase in RBS levels in groups II-VIII ($p<0.001$), though the change was not found to be statistically significant in Group I ($p=0.526$) and Group IX ($p=0.055$). Maximum change in RBS levels was observed in Group II (195.57%) followed by Group III and Group IV (195.57% & 166.16%) while minimum change was observed in Group I (2.93%) followed by Group IX (26.09%) and Group VIII (59.55%).

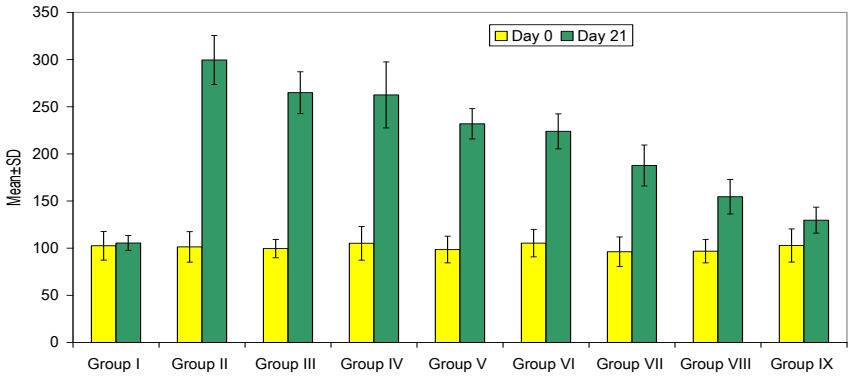


**Fig 1: Mean changes in Random blood sugar levels in different study groups**

### Body weight

An increase in baseline body weight (Day 0) was observed on Day 21 in all the groups (Fig. 2). In all the groups except Group I, change in body weight was found to be statistically significant. Minimum change was observed in Group I (11.22%) followed by Group VII (39.91%) while maximum change in baseline body weight was observed in Group II (71.70%) followed by in Group VI (56.06%).

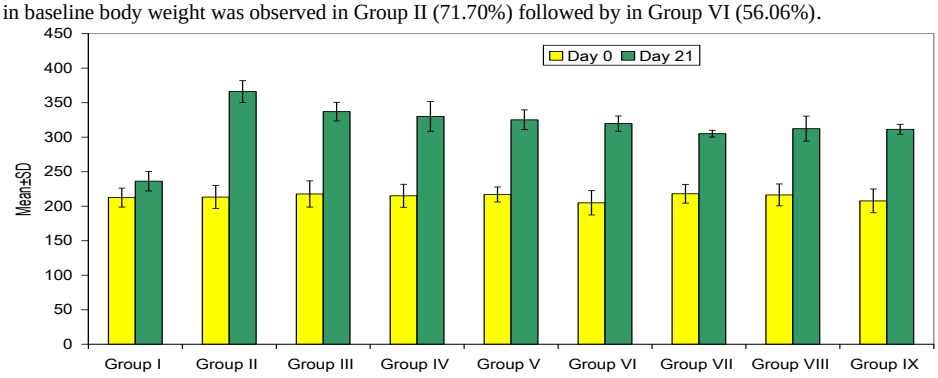


**Fig 2: Mean changes in Body weights in different study groups**

### Catalase Activity

On applying ANOVA, difference in enzyme activity of catalase in above groups was found to be statistically significant ($p<0.001$).Tukey's test showed the differences in mean catalase levels of all the groups II-VIII were statistically significant, when compared to group I ($p<0.05$). The difference in mean catalase levels of group I vs IX was statistically not significant ($p=0.086$). The differences in mean catalase levels of group III-VI were not significant ($p>0.05$), while the difference in mean catalase values of groups VII-IX were significant ($p<0.001$) when compared to group II (Fig. 3).

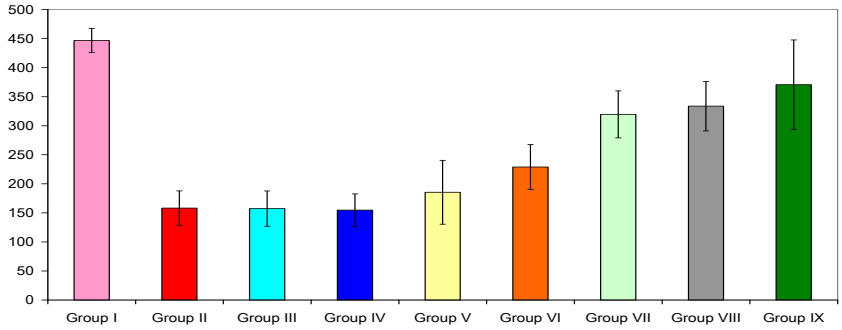


**Fig 3:Mean Catalase levels in different study groups**

**Glutathione peroxidase**

On applying ANOVA, difference in enzyme activity of glutathione peroxidase of above groups was found to be statistically significant ($p<0.001$). Tukey's test showed differences in mean GPx values for group II-IV were significant ($p<0.001$) while those for group V-IX were not significant ($p>0.05$) when compared to group I. The differences in mean GPx values for group III-V were not significant ($p>0.05$) while those for group VI-IX were significant ($p<0.001$) when compared to group II (Fig. 4).

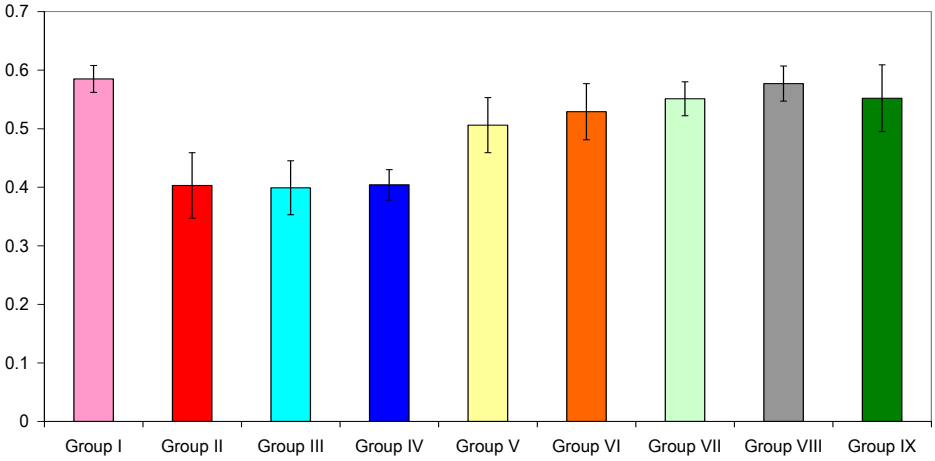


**Fig 4: Mean Glutathione peroxidase levels in different study groups**

**LPO Activity**

On applying ANOVA, difference in LPO activity of above nine groups was found to be statistically significant ($p<0.001$).Tukey's test showed differences in mean MDA values for group II-VI were significant ($p<0.001$) while those for group VII-IX were not significant ($p>0.05$) when compared to group I. The differences in mean MDA values for group III& IV were not significant ($p>0.05$) while those for group V-IX were significant ($p<0.001$) when compared to group II (Fig. 5).

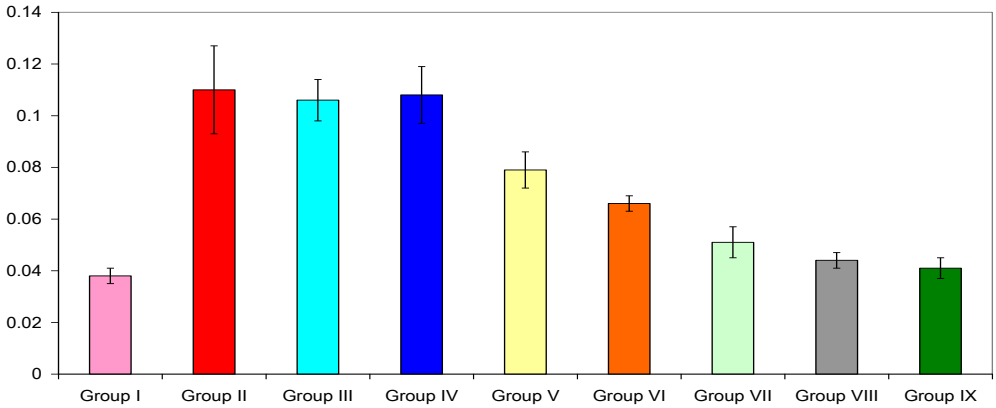


**Fig 5: Mean LPO activity (MDA levels) in different study groups**

**Histological findings in pancreatic tissue of experimental rats**

The pancreatic tissue of diabetic rats showed a marked decrease in size and the shape of the islets of Langerhans. The islets became irregular in their architecture showing capsular degeneration. There was also a decrease in number of islets. The cells of islets decreased in size and number and also showed signs of degeneration. In addition to the above findings, inflammation and congestion was also seen in the pancreatic parenchyma (Fig. 6,7,8).

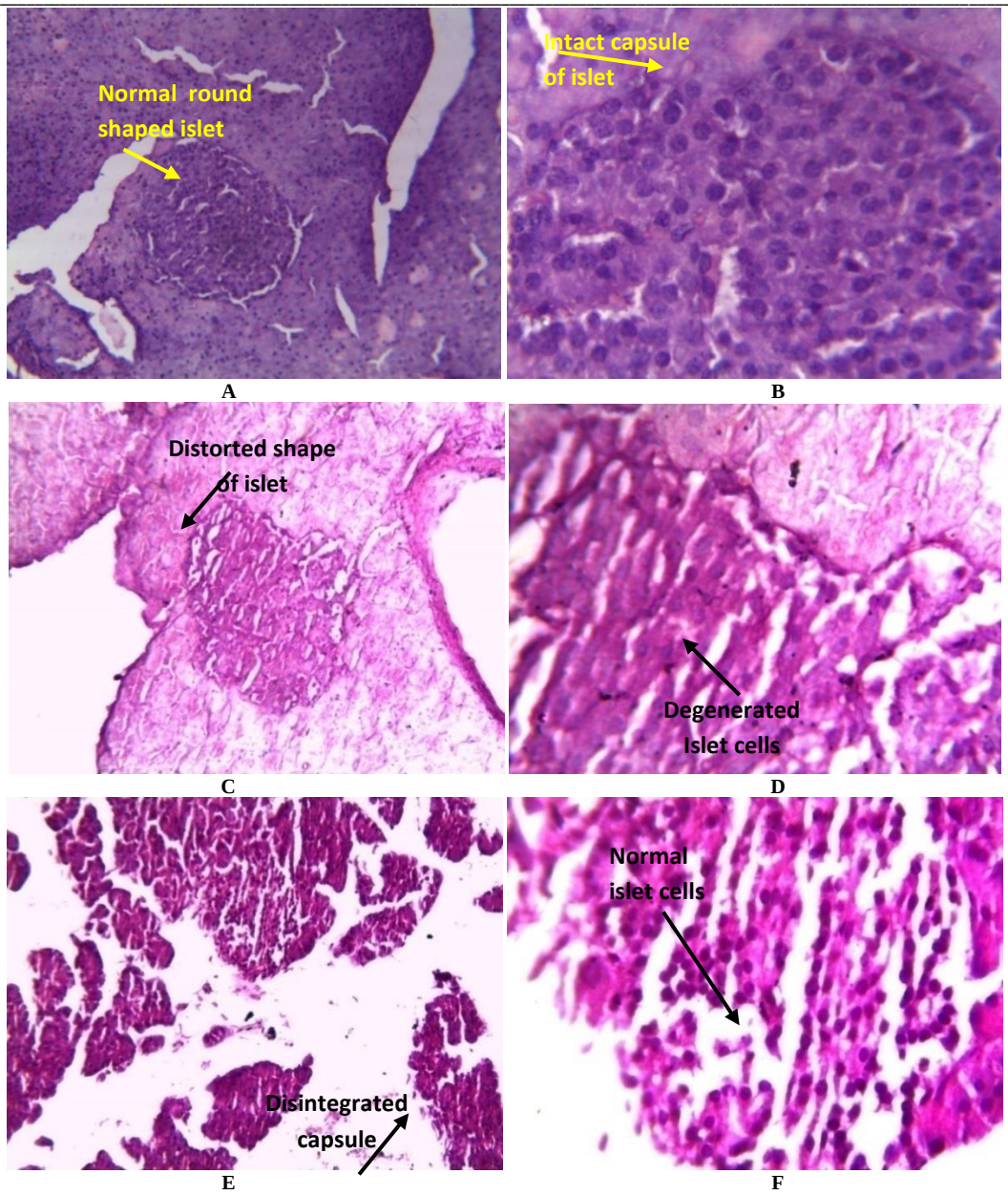


**Fig. 6 A, B:- Histology slide of the pancreatic tissue of a normal control rat showing normal shape & structure of Islets of Langerhans with intact capsule at low (100X) & high (200X) power respectively**
**C, D- Histology slide of the pancreatic tissue of a T2DM model in rats showing degenerative changes at low (100X) & high (200X) power respectively**
**E, F- Histology slide of the pancreatic tissue of a T2DM model in rats treated with aspirin 2 mg/kg, showing mild reduction in degenerative changes at low (100X) & high (200X) power respectively**

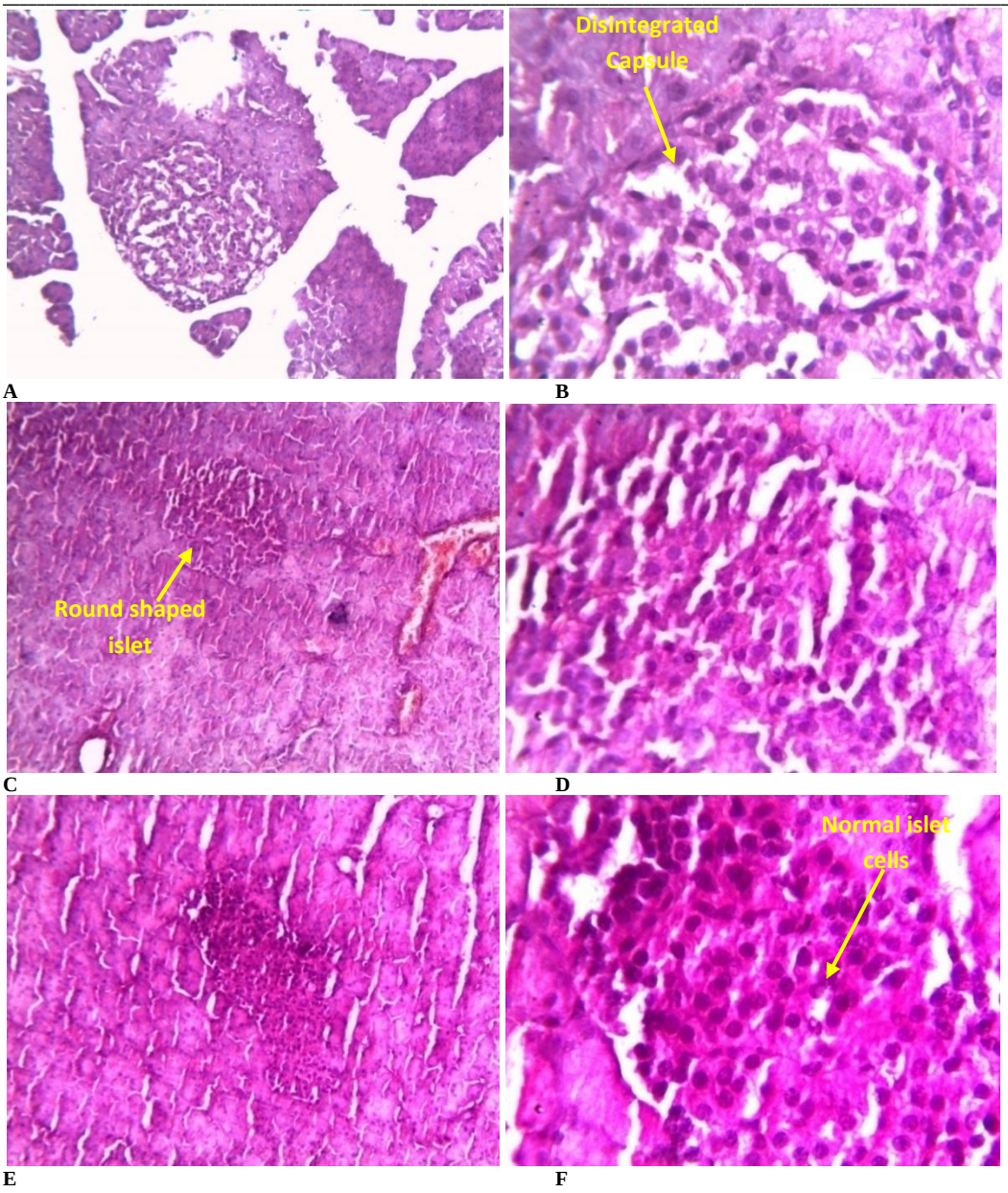


**Fig 7 A, B:Histology slide of the pancreatic tissue of a T2DM model in rats treated with aspirin 10 mg/kg, showing mild reduction in degenerative changes at low (100X) & high (200X) power respectively**
**C, D- Histology slide of the pancreatic tissue of a T2DM model in rats treated with G. sylvestre 60 mg /kg, showing mild reduction in degenerative changes at low (100X) & high (200X) power respectively**
**E, F- Histology slide of the pancreatic tissue of a T2DM model in rats treated with G. sylvestre 120 mg /kg, showing moderate reduction in degenerative changes at low (100X) & high (200X) power respectively**

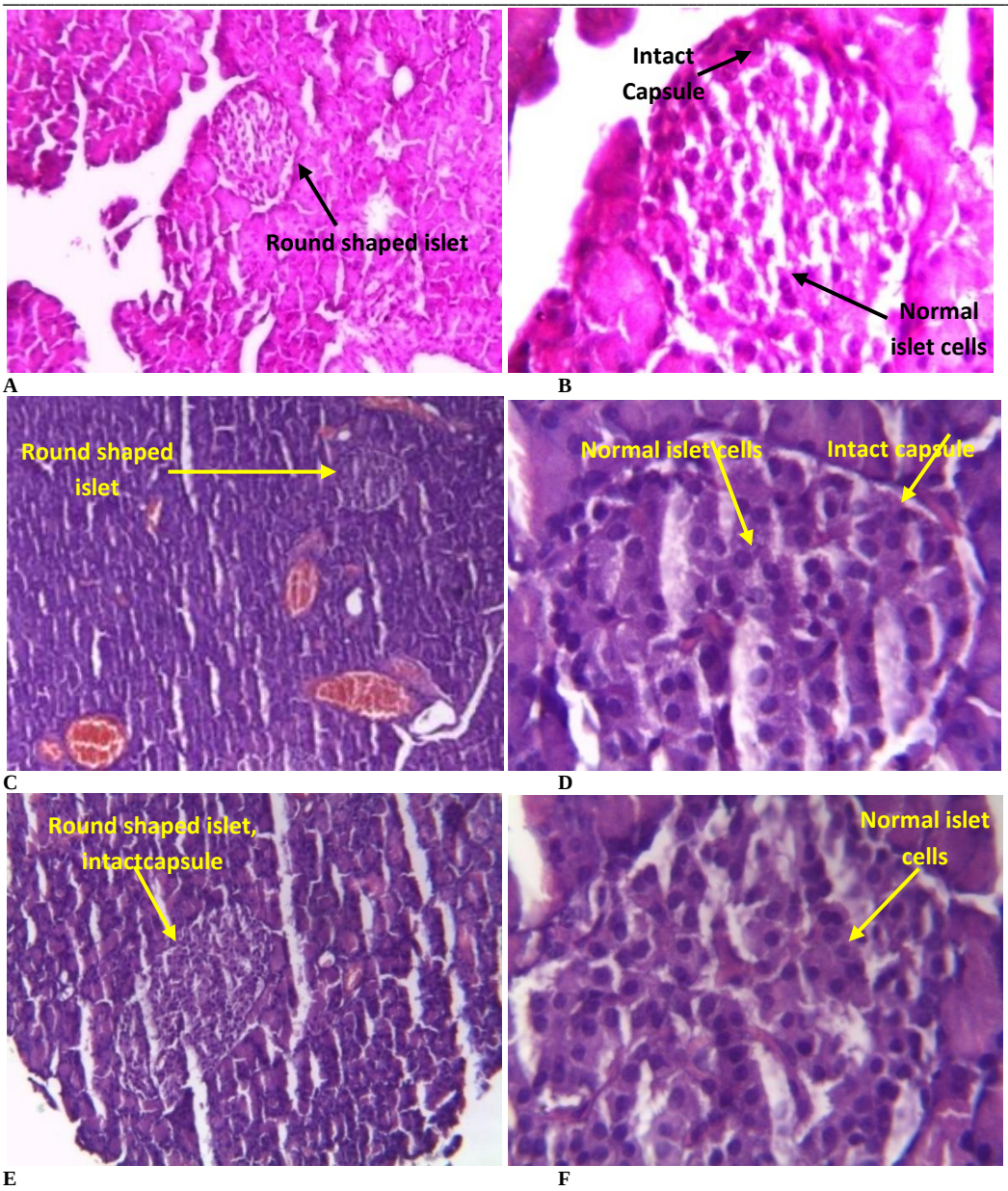


**Fig 8 A,B:Histology slide of the pancreatic tissue of a T2DM model in rats treated with G. sylvestre 240 mg /kg, showing moderate reduction in degenerative changes at low (100X) & high (200X) power respectively**
**C,D- Histology slide of the pancreatic tissue of a T2DM model in rats treated with aspirin 10 mg/kg and G. sylvestre 240 mg /kg combination, showing moderate reduction in degenerative changes at low (100X) & high (200X) power respectively**
**E,F- Histology slide of the pancreatic tissue of a T2DM model in rats treated with Metformin 100 mg /kg, showing moderate reduction in degenerative changes at low (100X) & high (200X) power respectivel**

**Discussion**

Aspirin decreased the mean RBS values in the treatment groups, but this reduction in RBS was only marginal. Similar findings were observed in a study conducted in the past[20].Blood glucose levels were significantly lower in T2DM rat models treated with G. sylvestre extract than in untreated ones, although they remained diabetic. While mean RBS values of T2DM rat models treated with G. sylvestre high dose, and G. sylvestre & aspirin combination, were near normal. These findings point out the diabetes preventing effect of G. sylvestre and are similar to the findings of previous studies which showed the blood glucose lowering effect of G. sylvestre in diabetic rats[21,22].Metformin caused maximum reduction in RBS values followed by the combination of G. sylvestre and aspirin.A significant increase in baseline body weight was observed in all the groups except the normal control group. Our finding that HFD led to weight gain in the rats is similar to the findings of a previous study conducted to find out the relationship between increased caloric intake and weight gain in wistar rats[23].Also, the different

treatments across the groups did not result in significant change (reduction) in body weight when compared to the untreated T2DM rat models. Significant reduction in weight gain was not observed with any dose of G. sylvestre (60, 120, 240 mg/kg). A similar study to see the effect of G. sylvestre on body weight in rats showed a marginal but not significant reduction in weight[24].Aspirin also was found ineffective in causing significant weight reduction. Metformin did not show significant reduction in weight gain when compared to untreated group, different from the findings of a study which found metformin effective in reducing weight. Though pooled data of other studies demonstrate no effect of metformin on weight reduction[25]. The shorter duration of our study might not be able to pick up the reduction in weight gain across the treatment groups.As chronic inflammation may ultimately contribute in the pathogenesis of diabetes mellitus by generation of oxidative stress at cellular levels, the anti-oxidant effects of our test and standard drugs may be enquired for their protective role in diabetes mellitus. Aspirin was not found to be effective in increasing catalase activity in T2DM rat models when compared to the untreated ones. The finding was similar to the finding of a study conducted in the past to enquire its effect on anti-oxidant enzymes, which revealed that aspirin had deteriorating effect on anti-oxidant enzymes[26].But few studies have shown that aspirin has beneficial effect in improving the anti-oxidant status[27].Further studies are warranted to establish the effect of aspirin on the anti-oxidant status.G. sylvestre (60, 120 mg/kg) was not found to be effective in improving the anti-oxidant status in T2DM rat models when compared to the untreated ones. But, G. sylvestre (240 mg/kg) and G. sylvestre and aspirin combination significantly increased the catalase activity in T2DM rat models when compared to the untreated ones. Protective role of G. sylvestre is further supported by a similar study done in the past[28]. We also found that metformin has the greatest effect in increasing the catalase activity in T2DM rat models compared to untreated group ($p<0.001$). The anti-oxidant effect of metformin is supported by a similar study conducted in the past to evaluate the effect of metformin on anti-oxidant status in aging rats. They found metformin to be beneficial in improving the anti-oxidant profile of the aging rats[29]. G. sylvestre in all doses, and in combination with aspirin, was effective in increasing the mean glutathione peroxidase levels in all treated groups. This finding is supported by a similar study conducted in past which demonstrated the protective role of G. sylvestre as an anti-oxidant[21].Aspirin alone was not effective in improving the anti-oxidant status of the diabetic rats. This finding is further supported by a study done to evaluate the effect of aspirin on anti-oxidant status, which reported that aspirin actually deteriorated the anti-oxidant status[26].Metformin treated rats, were having GPx values comparable to the normal controls, ($p=0.092$) which means that metformin is effective in restoring the anti-oxidant status of the body towards normal. This finding is further supported by a similar study done in the past to enquire about the anti-oxidant role of metformin [30].The mean lipid per-oxidation (LPO) activity (measured in terms of MDA level) of aspirin treated groups were not statistically different from that of the untreated group ($p>0.05$). It indicates that aspirin was ineffective in preventing lipid peroxidation in diabetic rats. This finding is further supported by a similar study conducted in the past[26].Mean LPO activity of G. sylvestre groups (all doses) were significantly lower than that of the untreated group ($p<0.001$). It indicates that G. sylvestre was effective in preventing lipid peroxidation in T2DM rat models. The results of the present study are consistent with those of earlier studies[21]. Metformin was found to cause maximum decrease in LPO activity in T2DM rat models and this finding is supported by a study done in the past to enquire the antioxidant potential of metformin[30].The analysis of histology slides revealed mild reduction in degenerative changes in the rat pancreas treated with aspirin (2 &10 mg/kg). This signifies that aspirin in both doses, has some protective role against the inflammation caused in the pancreas due to STZ and HFD induced diabetes.Also, it was found that G. sylvestre in doses 60 mg/kg showed mild improvement in size and shape as well as morphology of the islets of Langerhans, when compared to the untreated diabetic group. G. sylvestre in dose of 120 mg/kg, 240 mg/kg and G. sylvestre 240 mg/kg + aspirin 10 mg/kg combination caused more reduction in degenerative changes of the pancreatic tissue of rats. Metformin 100 mg/kg showed moderate improvement in size, shape and cellularity of the islets of Langerhans under low power. Under high power, it showed improvement in islet cells and capsular morphology. It means that G. sylvestre in higher doses (120, 240 mg/kg), in combination, as well as the standard therapy (Metformin) showed noticeable protective effect on the rat pancreas.

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