

Amelioration of Neurobehavioral and Biochemical Changes by *Withania somnifera* Root Extract During Type 2 Diabetes Mellitus Induced Cognitive Deficit in Rats

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Abstract

Objective: To investigate the effects of *Withania somnifera* root extract (WS) on type 2 diabetes mellitus induced cognitive deficit.

Methods: Type 2 diabetes mellitus (T2DM) was induced in rats by feeding them with high fat diet (HFD) for 8 weeks followed by intraperitoneal administration of Streptozotocin (STZ; 35 mg/kg). After four weeks, the fasting blood glucose, serum triglycerides, serum cholesterol and serum insulin levels were analyzed. After the confirmation of diabetes, WS was administered (two doses) for 8 weeks to the diabetic rats. After completion of the treatment animals were tested for memory deficits by Morris water maze (MWM) test and blood sample were collected for biochemical analysis. Glucose tolerance was determined by oral glucose tolerance test (OGTT) and insulin resistance by HOMA-IR models and all the results were compared with standard drug Pioglitazone (PZ).

Results: Significant elevation in fasting blood glucose, triglyceride, cholesterol and insulin levels were observed in HFD-STZ induced diabetic rats as compared to control animals and treatment with WS for 8 weeks significantly lowered these levels. In MWM test, diabetic rats spent significantly less time in target quadrant (TSTQ), an indicator of cognitive deficit for spatial learning, as compared to control animals. The animals treated with WS spent significantly more time in TSTQ when compared with diabetic group.

Conclusion: The data suggested that 8 weeks of treatment with *Withania somnifera* lowered the metabolic parameters of type 2 diabetes when compared with diabetic group. The reversal of biochemical markers was accompanied with significant improvement in memory retention and reduction in insulin resistance in rats with T2DM induced cognitive deficit. Thus, it can be concluded that *Withania somnifera* has therapeutic potential and can be explored in the management of T2DM induced Alzheimer's disease.

Keywords: Metabolic Syndrome; Type 2 Diabetes; Insulin Resistance; Cognition; Neurobehavioral Studies; High-fat Diet; *Withania somnifera*

Abbreviations

T2DM: Type 2 Diabetes Mellitus; AD: Alzheimer's Disease; HFD-STZ: High Fat Diet and Streptozotocin; PZ: Pioglitazone; MWM: Morris Water Maze; PA: Passive Avoidance; HOMA-IR: Homeostasis Model of Insulin Resistance; OGTT: Oral Glucose Tolerance Test.

Introduction

Diabetes mellitus (DM) is a gradually rising major health problem worldwide. It is generally categorized into type 1 (T1DM) and type 2 (T2DM). T1DM is caused due to insufficient secretion of insulin by pancreatic β -cells whereas T2DM results from ineffective insulin uti-

lization by body cells. T2DM is an extremely common disease nowadays and is generally associated with obesity. Chronic hyperglycemia and dyslipidemia are the major hallmarks of the disease [1]. Frequent consumption of foods rich in saturated fats and sugar accompanied by physical inactivity causes insulin resistance and obesity leading to a variety of metabolic disorders like T2DM. Most of the diabetic cases are being diagnosed for T2DM which is due to both ineffective insulin action (Insulin resistance) and impaired insulin secretion by pancreatic β -cells, which results in hyperglycemia [2].

Insulin is an anabolic peptide hormone, the key regulator of metabolism in the human body. It is secreted by pancreatic β cells that play a key role in carbohydrate, protein and fat metabolism. Insulin receptors are differentially distributed in various body tissues and exhibit a multiorgan signal transduction pathway by tyrosine kinase activity. Insulin enters brain via receptor mediated active transport through blood brain barrier (BBB). It is an adenosine triphosphate (ATP) dependent process. This transport demonstrates saturable kinetics and therefore the transport decreases with increase in blood insulin level [3]. In brain, insulin plays a key role in learning and memory by modulating neuronal function by regulating energy metabolism, growth survival and differentiation. Alzheimer's disease (AD) patients exhibit impaired insulin signaling that is similar to that observed in T2DM [4]. According to recent studies, insulin along with other growth factors like brain derived neurotrophic factor (BDNF) transmits the signals to regulate cognitive functions mediated by hippocampus. Insulin resistance caused during diabetes down-regulates the signaling pathways of insulin in hippocampus and therefore leads to impairment in memory and synaptic plasticity [5].

T2DM and AD both are prevalent with aging. Individuals with type 2 diabetes mellitus, obesity and other metabolic syndromes are at high risk to develop AD that may be due to mitochondrial dysfunction [6]. T2DM leads to structural as well as physiological change that causes cognitive deficits and the condition is known as diabetic encephalopathy [7]. Persistent high blood glucose levels in diabetics damages small blood vessels which leads to microvascular complications like diabetic neuropathy, nephropathy and retinopathy whereas damage to large blood vessels leads to macrovascular complications like cerebrovascular disease, cardiovascular disease and stroke [8]. Over a course of time, high blood sugar due to insufficient action of insulin (Insulin resistance) starts affecting brain cognitive functions which lead to mild cognitive impairment (MCI) in early stage and AD in later stage hence, AD has been considered as brain type diabetes [9].

Withania Somnifera is a plant of Solanaceae family which is commonly found in western India. In traditional system of medicine, the roots of *Withania somnifera* plant are categorised as rasayanas which literally means the path of juice (Rasa: Juice, Ayana: Path). They are phytochemicals that are reputed to promote health and longevity by augmenting defence against disease, arresting the aging process, revitalising the body in debilitated conditions, increasing the capability of the individual to resist adverse environmental factors and creating a sense of mental well-being [10]. The consumption of specific rasayanas in a recommended pattern is supposed to increase the rasa in the cells which rejuvenates the system [11]. Both roots and leaves of the plant are reported to contain several alkaloids that contribute to its various pharmacological activities. Withaferin A is the major alkaloid present in the plant and has been shown to produce anti-inflammatory, immunomodulatory, anti-tumor, anti-stress, anticonvulsant, anti-hyperglycemic, anti-oxidant and hepatoprotective effects [12].

Currently, there is no drug therapy that provides definite solution for prevention or management of cognitive deficit induced by T2DM. However, Insulin resistance (IR) is the major therapeutic target for the development of new drugs for T2DM. Hence, the present study was designed to investigate the effects of *Withania somnifera* root extract on experimental model of type 2 diabetes mellitus induced Alzheimer's disease and the possible mechanisms mediating these effects.

Material and Methods

Subjects

Sprague Dawley (SD) rats (200-250 g) were used for the study and each experimental group was comprised of 6 animals. The rats were housed under standard laboratory conditions (22 ± 2°C, 12 hour light/dark cycle – lights on at 0800 hours). Care of animals has been taken as per guidelines of CPCSEA for use of animals in Scientific Research with approval of Institutional Animals Ethics Committee (IAEC).

High fat diet- streptozotocin (HFD-STZ) induced T2DM in rats

After one week of acclimatization period, Sprague Dawley (SD) rats (200-250 g) were divided into two groups. Twelve animals were fed with normal pellet diet for 8 weeks and 24 animals were fed with in-house prepared high fat diet (HFD) (60% calories as fat) the diet composition includes powdered (Normal Pellet Diet) NPD (425g/kg), lard (310 g/kg), casein (250g/kg), cholesterol (10 g/kg), DL-methionine (3g/kg), yeast powder (1g/kg) and sodium chloride (1g/kg) for a period of 8 weeks. After 8 weeks of dietary manipulation all animals were kept on overnight fasting, HFD animals were injected intraperitoneally (i.p) with a low dose of streptozotocin (STZ) 35 mg/kg dissolved in 0.1 M citrate buffer pH 4.5, while the normal pellet diet fed rats were treated with vehicle citrate buffer, i.p. [13,14]. The rats were allowed to continue to feed on their respective diets until the end of the study. The STZ induced hypoglycaemic mortality was avoided by feeding the HFD-STZ animals with 20% glucose solution for 24 hrs [15].

After 4 weeks of STZ administration, the fasting blood glucose level was determined from the tail vein by glucometer. Rats with fasting glucose level ≥200 mg/dL were considered as diabetic and selected for further pharmacological screening. Blood (1.0 ml) was collected using capillary from retro-orbital plexus of the rats under mild anaesthesia. The serum was separated and the levels of triglyceride and cholesterol were assessed by semi-automatic analyser. After 4 weeks of STZ administration, body weight and fasting blood glucose levels of all the animals were measured and animals fed normal pellet diet were divided into two groups (Group I and II) whereas diabetic animals were segregated into four groups (Group III-VI) and treated with *Withania somnifera* or pioglitazone. The standardized *Withania somnifera* (WS) root extract was procured from the Natural Remedy, Bangalore. *Withania somnifera* (WS) was administered in the doses of 100 mg/kg and 300mg/kg in separate groups. The doses were selected on the basis of previous studies. The earlier studies by Singh, *et al.* [16] have reported LD50 of *Withania somnifera* root extract to be 1750 mg/kg when administered orally in mice. Further, Kojah and Hafez [17] recently reported that the administration of ashwagandha root extract in doses up to 2000/mg/kg/day to rats for 28 days has no toxicity.

The various groups were treated for 8 weeks as mentioned below:

- Group I, Normal Control: normal pellet fed animals were treated with 1% Na-CMC suspension orally
- Group II, (NC+WS-300): normal pellet fed rats in this group were treated with *Withania somnifera* (WS) root extract in the dose of 300 mg/kg/day orally.
- Group III, (Diabetic control): Diabetic rats were orally administered with Na-CMC suspension
- Group IV, (WS-100): Diabetic rats treated with the root extract of WS 100 mg/kg/day, orally.
- Group V, (WS-300): Diabetic rats orally administered with the root extract of WS 300 mg/kg/day.
- Group VI, (PZ-20): Diabetic animals were treated with Pioglitazone 20 mg/kg/day, orally.

Parameters assessed

After 8 weeks of treatment with test and standard drugs the animals were screened with neurobehavioral and biochemical parameters. The specific parameters assessed were body weight, fasting blood glucose levels, triglycerides and total cholesterol. Oral Glucose

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Tolerance Test (OGTT) was performed and area under the curve was calculated according to the method described by Matthews., *et al.* [18]. The neurobehavioral parameters were assessed by Passive avoidance test and Morris water maze test. After assessment of neurobehavioral parameters, animals were sacrificed and blood samples were collected for serum insulin level assay by ELISA.

Biochemical parameters

Body weight, fasting blood sugar, serum triglyceride and serum cholesterol levels of each animal were recorded at the end of 12th week i.e., after four weeks of STZ administration (before starting drug treatment). At the end of 20 weeks, all the animals were kept on 12 hours fasting and their body weights and fasting blood sugar levels were assessed by tail vein using glucometer.

Oral glucose tolerance test (OGTT)

After determination of body weight and fasting blood sugar levels, rats of all the groups were administered orally with glucose solution of 2 mg/kg. Blood glucose of each rat was determined from the tail vein by glucometer at 0, 30, 60, 90, 120 minutes [1] and area under the curve was calculated as described by Matthews., *et al.* 1990 [18].

Neurobehavioral studies

After 12 hrs of OGTT, rats were subjected to neurobehavioral studies which include Passive avoidance and Morris water maze test.

Passive avoidance test

Animals of all groups were screened for memory retention using a passive avoidance apparatus. The apparatus consisted of a two compartment dark/light shuttle box with a guillotine door separating the compartments. The dark compartment had a stainless steel shock grid floor. During the acquisition trial, each rat was placed in the light chamber. After a 60 s habituation period, the guillotine door was opened, and the initial latency of animals to enter the dark chamber was recorded. Rats with an initial latency time of more than 60 s were excluded from further experiments. Immediately after the rat entered the dark chamber, the guillotine door was closed and an electric foot shock (75 V, 0.3 mA, 50 Hz) was delivered to the floor grids with a stimulator for 3s. Five seconds later, the rat was removed from the dark chamber and returned to its home cage. After 24 h, the retention latency time was measured in the same way as in the acquisition trial, but foot shock was not delivered and the latency time will recorded to a maximum of 600s. Short latencies indicated the poorer retention [19].

Morris water maze test (MWM)

Animals were subjected to the MWM to analyze cognitive function at the end of treatment with drugs. In brief, a circular tank (160 cm diameter and 50 cm height) was filled with warm water (22-24 °C) to a height of 30 cm. The pool area was divided into four quadrants (NE, SE, SW and NW) by four points (E, S, W and N), equally spaced along the circumference of the pool. A circular escape platform (12 cm diameter and 28 cm height) was fixed in the middle of quadrant NE, 2 cm below the surface of water for the non-visible trial. All the rats received the four non-visible platform trials (one from each starting point) everyday for the first 4 days and a probe trial on the 5th day by removing the platform. In the non-visible platform trials, if the animal was unable to reach the platform in the given time interval (120 s), it was guided gently to the platform and allow to remain there for 30 seconds. The time taken by the animal to reach the platform (escape latency time) was recorded on day 1-4. On day 5, the time spent in target quadrant (TSTQ) in search of the missing platform was recorded using stopwatch and was used for further statistical analysis. TSTQ is an indicator of memory retention [19,20].

Serum insulin, triglyceride, cholesterol levels

After neurobehavioral studies, the animals were sacrificed by ketamine overdose and blood samples were taken by cardiac puncture and serum was separated. Serum insulin level was determined by ELISA and triglyceride and cholesterol levels were determined by using commercial kits according to the manufacturer's protocol.

Determination of Homeostasis Model of Insulin Resistance (HOMA-IR):

HOMA-IR was calculated as follows [21,22]

HOMA-IR = Fasting blood glucose (mg/dL) × Fasting blood insulin (μU/ml)/405.

Statistical analysis

The data is expressed as Mean + SEM. Comparisons among different groups is analyzed by one way ANOVA followed by Tuckey's post hoc analysis. Non-parametric statistical tests (Mann-Whitney U test) is used for analyzing data wherever appropriate. A 'P' value of less than 0.05 is considered as the level of significance for all statistical tests.

Results

Effect of HFD-STZ and *Withania somnifera* on body weight of animals

The initial body weight of rats of Normal control group was 227.80 ± 2.9g and HFD-STZ group was 224.35 ± 3.03g. After 8 weeks of HFD, the mean weight of the animals increased to 402.0 ± 9.64g whereas the Normal control group body weight was 244.0 ± 3.87g. Thus, there was significantly more weight gain in HFD group as compared to Normal controls. After 4 weeks of STZ administration (to induce diabetes in HFD rats) the body weight of diabetic controls decreased drastically as compared to their initial body weight. The results are shown in table 1. After development of diabetes as verified by metabolic parameters (Table 1) the animals were treated with WS or positive control for 8 weeks. After 20 weeks of study i.e., after 8 weeks of treatment with respective drugs, it was found that there were no significant changes in the body weight of animals treated with WS-100, WS-300 and PZ-20 when compared with diabetic control animals. The results are summarized in table 2.

Effect of HFD-STZ and *Withania somnifera* on fasting blood glucose levels

At 12 weeks of study i.e., after four weeks of STZ administration, fasting blood sugar level was found to be increased significantly in Diabetic control rats when compared with Normal control animals as shown in table 1. At 20 weeks of study, the fasting blood sugar levels remained high in diabetic rats as compared with control group. Administration of WS-100 and WS-300 in diabetic rats for 8 weeks significantly reduced the fasting blood glucose levels when compared with the diabetic animals, the results were comparable with pioglitazone as summarized in table 2.

Effect of HFD-STZ and *Withania somnifera* on serum triglyceride and cholesterol level

At the end of 12th week of the study, diabetic control animals showed a significant increase in serum triglyceride level as compared to normal control group as described in table 1. After 20 weeks of study period, animals of diabetic group continued with increased serum triglyceride level when compared with control group. Diabetic animals treated with WS (100 and 300 mg/kg) and pioglitazone (20 mg/kg) for 8 weeks showed a reduction in the triglyceride level when compared with the diabetic control group. The results are described in table 2. Similarly, diabetic control animals showed a significant increase in serum total cholesterol level as compared to normal control

group as described in table 1. At the end of 20 weeks, animals of diabetic group continued with elevated total cholesterol level as compared to Normal control group. Treatment with (WS 100 and 300 mg/kg) and standard (Pioglitazone 20 mg/kg) groups have shown a decrease in the cholesterol levels after the treatment when compared with the diabetic control group. The results are depicted in table 2.

Group	Parameters			
	Body weight (g)	Fasting blood glucose (mg/dl)	Serum TG (mg/dl)	Serum Cholesterol (mg/dl)
After 8 weeks				
Normal Control	232.3 ± 3.87	96.82 ± 4.02	89.04 ± 3.43	71.55 ± 2.24
Diabetic control	402.0 ± 9.64	169.76 ± 5.27**	113.34 ± 2.80*	108.44 ± 5.65*
After 12 weeks				
Normal Control	246.30 ± 2.95	92.21 ± 2.25	86.14 ± 2.76	73.23 ± 3.24
Diabetic control	239.0 ± 1.34##	228.20 ± 3.26***	126.50 ± 2.21**	103.70 ± 1.90*

Table 1: Comparison of metabolic parameters between Normal controls and Diabetic rats at the end of 8 and 12 weeks of study
 TG: serum triglycerides; * $p < 0.01$, ** $p < 0.001$ when compared with the respective Normal controls;
 # $p < 0.05$, ## $p < 0.01$ when compared with diabetic control group at 8 weeks.

Group	Parameters (At the end of 20 weeks)			
	Body weight (g)	Fasting blood glucose (mg/dl)	Serum Triglycerides (mg/dl)	Serum Cholesterol (mg/dl)
Normal Control	257.50 ± 5.20	98.30 ± 5.92	96.24 ± 2.77	73.54 ± 3.66
NC+WS 300	252.5 ± 3.22	103.60 ± 6.80	88.94 ± 3.91	70.17 ± 2.57
Diabetic (D) control	236.40 ± 3.03	256.0 ± 4.92***	168.6 ± 4.44**	115.8 ± 2.27***
D + WS-100	247.10 ± 3.59	206.40 ± 6.01#	156.20 ± 2.29	99.18 ± 2.59#
D+ WS-300	250.00 ± 2.44	164.30 ± 6.95##	140.0 ± 3.23#	88.49 ± 3.42##
D + PZ-20	254.30 ± 2.29	135.70 ± 3.53###	147.30 ± 2.24#	97.12 ± 2.23#

Table 2: Effect of 8 weeks of treatment with *Withania somnifera* (WS) on various metabolic parameters in diabetic rats.
 *** $p < 0.001$, ** $p < 0.01$ when compared with Normal controls; # $p < 0.05$, ## $p < 0.01$,
 ### $p < 0.001$ when compared with Diabetic controls.

Effect of treatment on glucose tolerance

The results for oral glucose tolerance test (OGTT) showed that at the end of treatment period, the blood glucose levels at 30, 60, 90 and 120 minutes were significantly higher in diabetic control group ($p < 0.01$) as compared to that in Normal control group, suggesting impaired glucose tolerance in diabetic animals. The OGTT curve declined gradually after the treatment with *Withania somnifera* in a dose related manner and the results were comparable with that achieved by Pioglitazone. The results are summarized in figure 1.

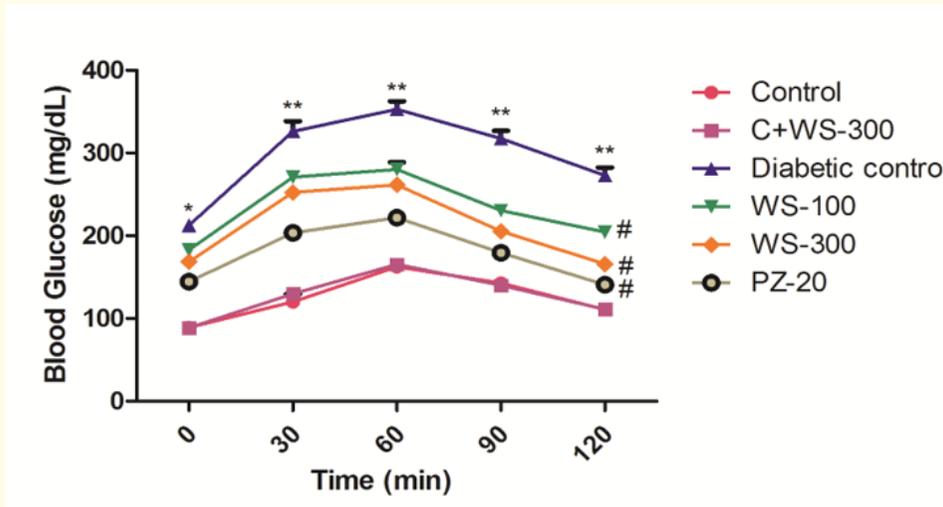


Figure 1: Effect of 8 weeks of treatment with *Withania somnifera* (WS) on blood glucose level during oral glucose tolerance test (OGTT).

** $p < 0.01$ when compared with control group. # $p < 0.05$, ## $p < 0.01$ when compared with diabetic control group.

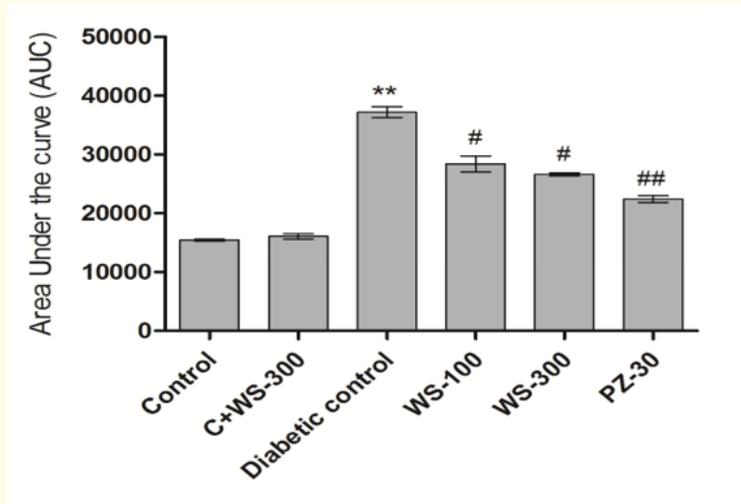


Figure 2: Effects of 8 weeks of treatment with *Withania somnifera* (WS) on Area under the curve of glucose concentration measured over 120 minutes during OGTT.

** $p < 0.01$ when compared with control group. # $p < 0.05$, ## $p < 0.01$ when compared with diabetic control group.

Effects of *Withania somnifera* on spatial learning of rats

The mean time of the animal to find the hidden platform i.e., Escape latency time (ELT) from day 1 to day 4 and Time spent in target quadrant (TSTQ) on day 5 of Morris water maze (MWM) test were measured. Latency to reach the submerged platform i.e., Escape latencies, decreased gradually in all the groups during four days of training in water maze (Table 3). In the probe trial i.e., on day 5, the time

spent in target quadrant (TSTQ) was decreased significantly ($p < 0.01$) in diabetic group as compared to the control group. *Withania somnifera* treated groups have (WS-100 and WS-300) shown significant increase in TSTQ ($p < 0.5$, $p < 0.01$ respectively) as compared to the diabetic group. Pioglitazone treated group has shown a significant increase in TSTQ ($p < 0.5$) as compared to the diabetic control group. Results are shown in figure 3.

Groups	Escape latency time (ELT) (in sec)			
	Day 1	Day 2	Day 3	Day 4
Normal Control (NC)	80.68 ± 7.02	52.12 ± 4.86	36.87 ± 3.89	31.37 ± 4.07
NC+WS-300	57.12 ± 7.59	56.00 ± 6.76	41.26 ± 4.56	27.30 ± 5.38
Diabetic (D) Control	90.50 ± 5.26	76.50 ± 3.74	61.75 ± 3.02	72.56 ± 2.27**
D + WS-100	79.75 ± 5.22	64.74 ± 3.71	62.31 ± 1.85	50.81 ± 3.27##
D + WS-300	55.62 ± 8.44	56.25 ± 6.71	51.06 ± 5.32	43.06 ± 4.57##
D + PZ-20	59.62 ± 5.63	55.25 ± 6.28	60.00 ± 6.75	57.75 ± 7.40#

Table 3: Effect 8 weeks of treatment with *Withania somnifera* (WS) on Escape latency time during Morris water maze (MWM) test. ** $p < 0.01$ when compared with Normal controls at Day 4; # $p < 0.05$, ## $p < 0.01$ when compared with diabetic control group at Day 4.

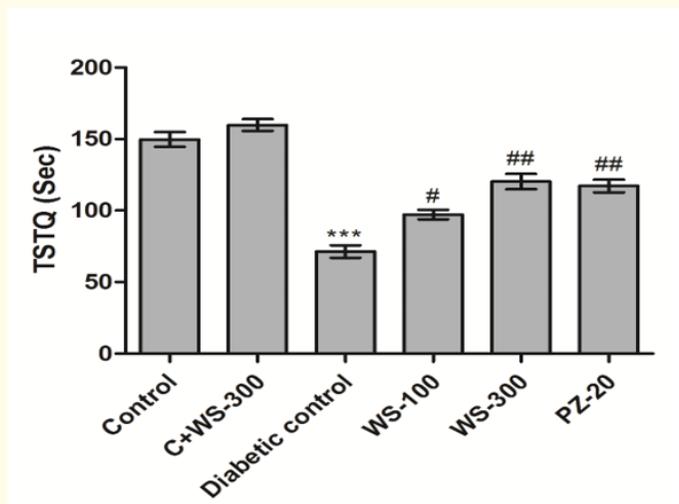


Figure 3: Effect of 8 weeks of treatment with *Withania somnifera* on time spent in target quadrant (TSTQ) on day 5 of MWM test in type 2 diabetic rats. *** $p < 0.001$ when compared with control group. # $p < 0.05$, ## $p < 0.01$ when compared with diabetic control group.

Effects of *Withania somnifera* on passive avoidance response

The retention latency of the diabetic group animals was found to be decreased significantly ($p < 0.001$) as compared to the control group. Treatment with *Withania somnifera* and Pioglitazone significantly increased the retention latency ($p < 0.05$, $p < 0.01$ respectively) as compared to the diabetic group animals. There was no significant changes have been found in the acquisition latency in all the groups as shown in figure 4.

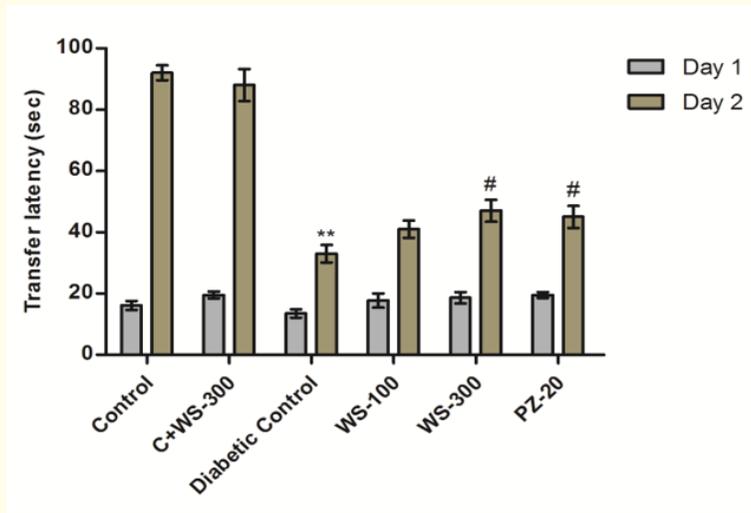


Figure 4: Effect of 8 weeks of treatment with *Withania somnifera* on transfer latency on day 1 and day 2 during Passive avoidance test in type 2 diabetic rats. ** $p < 0.01$ when compared with control group. # $p < 0.05$ when compared with diabetic control group.

Effect of *Withania somnifera* on serum insulin level and insulin resistance

Administration of Streptozotocin with HFD resulted in significant ($p < 0.01$) increase in the serum insulin level as well as insulin resistance (measured by HOMA-IR) when compared to normal control rats. Treatment with *Withania somnifera* (300 mg/kg) for 8 weeks reverted the serum insulin level towards normalcy and the values were significantly less than that of diabetic control group. Similarly, the insulin resistance was significantly decreased ($p < 0.01$) as compared to diabetic controls. The results were similar to that of pioglitazone which showed a significant ($p < 0.01$) decrease in serum insulin and insulin resistance as compared to diabetic controls. The results are summarized in figure 5 and figure 6.

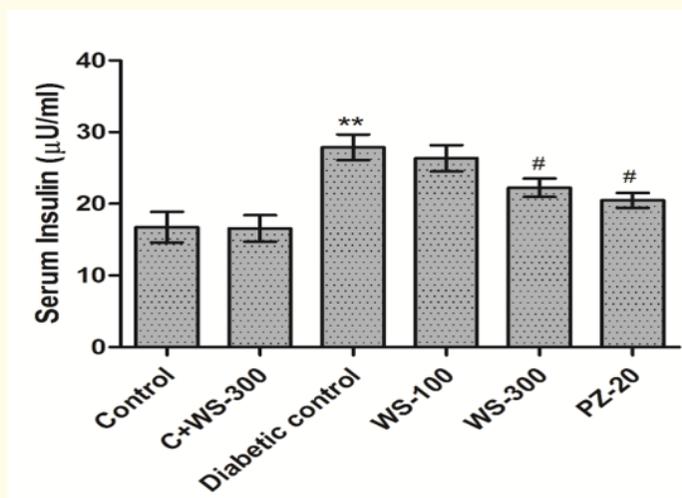


Figure 5: Effect of *Withania somnifera* on serum insulin level in type 2 diabetic rats. ** $p < 0.01$ when compared with control group. # $p < 0.05$, ## $p < 0.01$ when compared with diabetic control group.

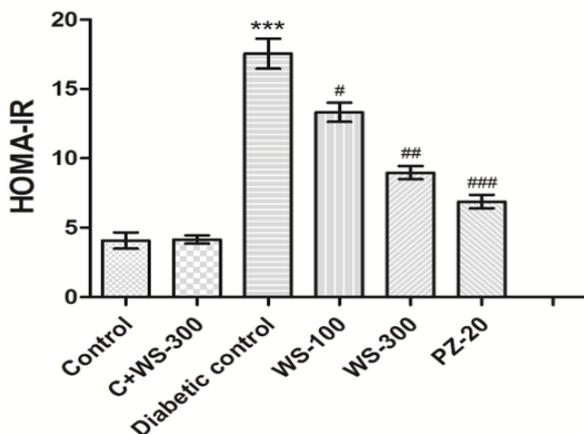


Figure 6: Effect of *Withania somnifera* on peripheral insulin resistance (HOMA-IR) in HFD-STZ induced type 2 diabetic rats. *** $p < 0.001$ when compared with control group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ when compared with diabetic control group.

Discussion

Metabolic syndrome has various risk factors viz obesity, hypertension, lipid abnormalities and impaired insulin and glucose metabolism. Sedentary life style and excess intake of fat/sugar can lead to obesity and type 2 diabetes mellitus (T2DM) accompanied by insulin resistance (IR) and β -cell dysfunction. In animals, the T2DM model is induced by giving a combination of high fat diet and streptozotocin (STZ) to mimic the pattern of the disease. Diet modification i.e., feeding rats with high fat diet causes insulin resistance (IR) and administration of low dose STZ causes mild degeneration of β -cells. IR also plays an important role in pathophysiology of T2DM induced neurodegenerative changes like Amnesia and Alzheimer's disease [23]. Insulin resistance is characterized by elevated levels of blood insulin along with reduced brain insulin levels [24]. Therefore, AD is also known as insulin resistant brain state or type 3 diabetes.

The primary action of insulin is to maintain glucose homeostasis by transporting the glucose molecules in muscles and adipose tissues. Various experimental evidences suggest that brain is now recognized as insulin sensitive organ and responsible for cognitive changes due to insulin resistance. Insulin receptors are widely expressed in certain brain regions like Hypothalamus, Hippocampus and Cerebral cortex in discrete concentrations and brain insulin resistance causes the cognitive dysfunction associated with the metabolic syndrome or T2DM [25]. Aberrant insulin signaling due to metabolic syndrome is reported to cause AD like pattern of reduced cerebral glucose metabolic rate in the brain [26].

In the present study, high fat diet followed by low dose streptozotocin (HFD-STZ) was used to induce T2DM like condition which was characterized by elevated blood glucose (FBG), triglycerides (TG) and cholesterol (TC) levels and an increase in insulin resistance. It was also observed that development of type 2 diabetes deteriorated the cognitive functions of the animals as assessed by Morris water maze test and passive avoidance test. This finding is supported by a study conducted by De Felice and Ferreira [26] that proved negative effects of diabetes on memory. The results are also corroborated by an earlier study which showed that administration of low dose of STZ induced peripheral and brain insulin resistant state and produced AD like behavioral changes [27].

Withania somnifera (WS) has been used in traditional system of medicine for more than 2500 years for its beneficial effects in the treatment of various ailments. It is also known as Ashwagandha and has been used widely for treating various neurological and metabolic

disorders. Withaferin A is the active constituent (withanolide) and is attributed for the beneficial properties of WS [28]. In the present study, after confirmation of diabetes in HFD-STZ rats, effect of two different doses of WS extract was evaluated on type 2 diabetes mellitus and associated cognition deficit. After 8 weeks of treatment with WS (i.e., after 20 weeks of the study) FBG, TG and TC levels of the animals were found to be reduced and body weight of the animals improved significantly as compared to diabetic animals. The results obtained were comparable with the standard anti-diabetic drug Pioglitazone. Treatment with WS also prevented memory impairment caused by T2DM. Similar findings have also been reported by Dar, *et al.* [29] who proved and concluded that WS improved the cognitive and psychomotor performances in humans.

Glucose intolerance is the major risk factor for the development of diabetes. The glucose intolerance was estimated by oral glucose tolerance test OGTT and it was observed that diabetic animals maintained a higher glucose concentration throughout the observation period as compared to control animals and therefore showed a significantly lowered rate of glucose disposal. Interestingly, animals treated with WS showed significantly lower glucose concentration with time and hence showed better glucose tolerance as compared to diabetic animals. These results support the earlier mentioned observation of reduced levels of FBG, TG, TC.

The HFD-STZ animals were subjected to neurobehavioural evaluations by Morris water maze (MWM) and passive avoidance (PA) tests. MWM is used to test the spatial memory of the rodents to locate the hidden or submerged platform in a pool of water by using visual cues. In MWM, the escape latency time (ELT) i.e, the time taken by the animal to reach the submerged platform is taken as a parameter of learning or acquisition. Shorter latencies indicate better learning by the animals and vice versa. The time spent in the target quadrant (TSTQ) is taken as parameter for the memory retention during the probe trial [30]. The obtained results showed that diabetic animals had greater ELT during the period of acquisition trial (Day 1 to Day 4) i.e., they took longer time to reach the submerged platform as compared to the control animals. The animals treated with WS showed significantly shorter latencies as compared to diabetic animals. Further, diabetic animals spent a significantly less time in target quadrant (low TSTQ) as compared to control animals. Treatment with WS for 8 weeks resulted in significant increase in time spent in the target quadrant (in search of the submerged platform) as compared to untreated diabetic rats. These results suggested that T2DM has a negative impact on learning as well as on memory retention that may be due to IR in the brain. WS treatment improved the acquisition and retention in the diabetic rats which may be due to reduction in the metabolic symptoms of T2DM and their impact on brain.

Passive avoidance test is an aversive (emotional) conditioning paradigm which is dependent on hippocampal functioning. The test evaluates the suppression of innate preference of the animals for the dark compartment of the test apparatus [31]. In step through avoidance test, animal makes a decision to avoid or enter in the dark compartment where it previously received an aversive stimulus like electric shock. The entry of the animal into such dark compartment is known as transfer latency and it is a parameter of memory retention. The animal has the option to avoid the dark compartment which is based on its memory to discriminate between the safe and unsafe area. Shorter transfer latencies indicate poor retention and vice versa [32]. In the present study it was observed that diabetic animals had poor retention and therefore they showed significantly shorter latencies as compared to control animals. Diabetic animals treated with WS showed significantly higher transfer latencies as compared to untreated diabetic rats which indicated better memory retention in these animals.

Type 2 diabetes is also known as insulin resistant diabetes because insulin resistance (IR) is one of the main components of its pathophysiology. It is characterized by the reduced sensitivity of the body tissues to the action of insulin that may be due to downregulation of insulin receptors or inability of the receptors to bind to the circulating insulin hormones or may be due to defect in the insulin signaling cascade. Insulin resistance i.e., impairment of glucose uptake in peripheral tissue is a main factor for the progression of T2DM. In our study, the IR was calculated by homeostasis model assessment of insulin resistance (HOMA-IR) as described by Anwer, *et al.* [21]. It was found that diabetic animals have significantly more IR as compared to control animals. It is supported by a previous study by Yang, *et al.* [33]. This indicated that HFD-STZ treatment increased the IR in rats and thereby induced T2DM. Interestingly, treatment with WS for 8 weeks significantly reduced the IR in diabetic animals.

In brain, insulin receptors are mainly concentrated in hippocampus and frontal cortex (the memory centers of the brain), and are responsible for regulating glucose uptake, learning and memory, food intake, weight control etc [34]. In insulin resistance there is a disruption of insulin signaling which adversely affects the glucose transport and thus inhibition of energy production due to glucose oxidation. Insulin may protect neurons against A β induced toxicity and it modulates the synaptic transmission by increasing the expression of NMDA (N-methyl -D-aspartic acid) receptors in the brain and alters long term potentiation (LTP) [35]. Decreased insulin signaling, including altered kinase activity and IRS expression in AD gets worse with disease progression. Abnormal serine phosphorylation of IRS-1 is the key signature of insulin resistance that is evident in the AD brain [36]. These reports suggest that insulin resistance is the key link between T2DM and AD which was modulated effectively by WS.

Conclusion

Taken together, the results showed that root extract of *Withania somnifera* improved the metabolic dysfunction by reducing the levels of cholesterol, triglycerides, fasting blood sugar and ameliorated insulin resistance with improvement in glucose tolerance. Further, behavioural analysis showed that WS improved the memory and spatial learning in cognitive impaired diabetic rats. Thus, WS can be a potential lead for the drug development for the management of T2DM associated cognition deficit.

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Conflict of Interest

Authors have declared that they have no conflicts of interest to disclose.

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