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# Effect of a Novel Polyherbal Formulation on Diabetes Induced Memory Deficits in Rats

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## Abstract

Epidemiological, clinical and experimental studies have shown a strong correlation between Diabetes mellitus (DM) and neurodegenerative disorders. Type II diabetes mellitus (T2DM) leads to elevated levels of plasma glucose and causes neurological complications including impairment of learning and memory processes. The pathogenesis mainly includes metabolic dysfunction due to brain insulin resistance, oxidative stress, inflammation and vascular complications. In this study we explored a new pharmacological intervention by investigating the neuroprotective effects of a standardized polyherbal formulation (PF) [(*Bacopa monnieri* (20 mg/kg), *Hippophae rhamnoides* (25 mg/kg), and *Dioscorea bulbifera* (15 mg/kg), p.o] on passive avoidance learning (PAL) and memory in normal and streptozotocin (STZ) induced diabetic rats. Treatment was started after the onset of hyperglycemia following STZ (60 mg/kg, i.p) injection. PAL and memory were assessed 30 days after treatment, followed by retention test 24 h after training. At the beginning and end, body weights and blood glucose measurements of the animals were done. The results showed STZ induced diabetic rats had severe impairments in acquisition and retrieval processes of PAL and memory. Treatment with the PF significantly improved cognitive deficits, increased body weight and lowered plasma glucose levels in the treated as compared to untreated diabetic rats. We hypothesize that acetyl choline enhancing, antioxidant, hypoglycemic and hypolipidemic properties of phytomolecule such as bacosides, quercetin, and diosgenin in the PF might be responsible for the nootropic effects of polyherbal formulation. Our study highlights the therapeutic potential of this polyherbal formulation for multi-targeted treatment of diabetes induced cognitive impairment.

**Keywords:** Polyherbal formulation; Diabetes mellitus; Streptozotocin; Passive avoidance; Memory; Blood glucose; Cognitive impairment

## Introduction

Growing evidence (experimental, clinical and epidemiological studies) have established that diabetes mellitus a chronic metabolic disorder is associated with neurological complications, cognitive impairments and lead to sporadic Alzheimer's disease (AD) or Type III diabetes [1-6]. It is posited that insulin / Insulin like Growth Factor (IGF) resistance in the brain results in starvation and disrupts downstream signalling pathways that results in neuronal death [7-9]. It initiates a cascade of neurodegenerative changes that is propagated by increased oxidative and endoplasmic reticulum (ER) stress, mitochondrial dysfunction, neuroinflammation, impaired energy metabolism, disrupted metabolic functions, and dysregulated lipid metabolism [10]. This cellular dysfunction result in AD specific molecular changes which include plaque formation, kinase activation that aberrantly phosphorylate tau protein and lead to accumulation of neurofibrillary tangles, accumulation of neurotoxic amyloid beta precursor protein peptides (AbPP-Ab) and cholinergic dyshomeostasis [11,12]. The prevalence rate of sporadic AD is alarming and is considered the most common cause of dementia in elderly [13]. Over the last decade the Food and Drug Administration (FDA) has not approved any new drugs for the treatment of AD; those which are in the market include acetylcholineesterase (AChE) inhibitors - Donepezil, Rivastigmine, and Galantamine or glutamate receptor antagonist Memantine [14,15]. However these drugs have many side effects, provide only symptomatic relief and do not halt or delay the progression of the disease [16,17]. Hence, there is an urgent need to explore novel therapies.

Herbal drugs are gaining worldwide popularity because of lesser side effects, cost effectiveness and easy availability to poor people particularly in developing countries [18,19]. Ayurveda, the Indian system of medicine have utilised the medicinal properties of several herbs to improve memory and cognitive function and to treat neurodegenerative diseases [20-24]. Many studies have therefore, assessed the therapeutic potential of novel phytomolecules to improve cognition, special learning and memory in streptozotocin diabetic rat model that induces AD-type neurodegeneration [25-30]. Since AD is associated with multiple pathologies such as oxidative stress, neuroinflammation and deficiency of acetylcholine, hence it is advantageous to use a multi-targeted therapeutic approach for treatment [31-34]. Hence, taking lead from ancient wisdom our group has standardized a polyherbal formulation (PF) using hydro-alcoholic extracts of *Bacopa monnieri, Hippophae rhamnoides* and *Dioscorea bulbifera* and have recently reported its positive therapeutic effects in elderly AD patients with cognitive deficits in a clinical study [35].

In the present study we aimed to assess the nootropic effects of PF [36] on passive avoidance learning paradigm and study its effect on blood glucose levels and body weight in streptozotocin diabetic rat model. Streptozotocin (STZ) is a nitrosamine-related compound

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that induces brain insulin resistance and insulin deficiency which results in cognitive impairment and AD-type neurodegeneration [37]. Therefore this animal model has been used to study potential therapeutic molecules for treatment of cognitive deficits induced by diabetes [6,38]. This is a novel study since earlier reports were mainly focused on monotherapy, while in this study we assessed the medicinal properties (e.g., anti-oxidant, anti-inflammatory, antihyperlipidemic, antihyperglycemic and neuromodulatory) of three plants in a combined herbal formulation [39-41]. We envisage that this study will be useful in exploring new multi- targeted treatment strategies to improve the neurological complications induced by diabetes.

## Methods

### Drugs

Streptozotocine (STZ) was purchased from (Sigma-Aldrich chemical Co. India) and dissolved in 1 ml saline, immediately before use. Other chemicals, reagents and solvents were procured from Biotech India Private Ltd. and were of analytical grade.

### Animals

Male Wistar rats with body weight of 200-250 g were procured from the central animal house of Banaras Hindu University. The animals were kept at 23°C ± 2°C with a relative humidity at 50 ± 10%, in separate cages under 12-h light: 12-h dark light cycle. They were given standard food pellets and water ad libitum, during the entire duration of the experiment. Each experimental group consisted of six animals. Animal ethical committee approval was taken and all procedures for treatments of laboratory animals were done in accordance with the criteria outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health (NIH) publication 86–23; revised 1985; http://www.oacu.od.nih.gov/regs/guide/guidex.htm). Prior to the experiment all animals were acclimatized to laboratory conditions.

### Experimental design

The animals in this study were randomly divided into four groups (n=6); two control [control (C) and control group receiving polyherbal formulation (C+P)] and two diabetic [diabetes (D), diabetic group receiving polyherbal formulation (D+P)]. The control rats received intra peritoneal injections of physiological saline. Diabetes was induced by a single i.p injection of STZ (60 mg/kg) prepared in citrate buffer, pH 4.5, this experimental model has been validated by several previous studies [42-44]. Three days after STZ injection, fasting blood glucose was determined using blood glucose monitor (Accuchek; Roche, Mannheim, Germany). Animals were considered diabetic if plasma glucose levels exceeded 250 mg/dl. After confirmation of diabetes the animals were orally administered polyherbal formulation [(*Bacopa monnieri* (20 mg/kg), *Hippophae rhamnoides* (25 mg/kg), and *Dioscorea bulbifera* (15 mg/kg)] [36] using gavage needle to control and diabetic groups for 30 days. After the treatment period, learning and memory were evaluated using Passive avoidance learning (PAL) test. All animals were weighed and plasma glucose levels were determined at the beginning and end of the experiment.

### Behavioural test

**Passive avoidance learning (PAL) test:** The step through passive avoidance test is based on contextual fear conditional learning [45] and is used to evaluate emotional memory. The animals learn to avoid a specific place associated with an aversive event. A reduction in step-through latency (STL) was used as an indication of impaired memory. The apparatus consisted of one bright chamber (20 × 20 × 30 cm) made up of transparent plastic and one dark chamber (20 × 20 × 30 cm) with walls made of dark opaque plastic. These chambers were separated by a guillotine door (6 × 8 cm). The floors of both chambers contained stainless steel rods spaced 0.5 cm apart and an electric shock generator was used to electrify the floor of the dark chamber and the test was performed as previously described [27,30].

**Training:** Animals in all the experimental groups were given two test trials to acclimatize them with the apparatus. The rat was first placed in the bright compartment facing away from the door, 5 s later the guillotine door was raised to allow the rat to explore and move into the dark compartment as a natural preference. Upon entering the dark compartment the door was closed and the rat was allowed to stay there for 30 s and then put back into its home cage. The habituation trial was repeated 30 min later and after the same interval the first acquisition trial was performed. The time taken by the rat to spontaneously enter the dark compartment was denoted as entrance latency (STL-a) and time was noted only when the animal had placed all four paws in the dark compartment. After entry, the guillotine door was closed and a mild electrical shock (0.5 mA) was applied for 3 s. After 30s the rat was returned to the home cage. This procedure was again repeated after 2 min. The rat received a foot shock each time it entered the dark compartment. Training was terminated when the rat remained in the bright compartment for 120 sec and the number of trials, i.e., entry into the dark compartment was recorded.

**Retention test:** Twenty four hours later, the rat was placed in the bright compartment as in the PAL acquisition trial. The guillotine door was raised after 5 s, the retention latency (STL-r) and time spent in the dark compartment (TDC) was recorded for up to 300 s. The time taken for a mouse to enter the dark compartment after door opening was defined as latency. If the rat did not enter the dark chamber within 300s, the retention test was terminated and a max score of 300 s was recorded.

### Statistical analysis

One-Way ANOVA followed by Tukey as post hoc test was done to determine statistically significant differences between experimental groups. All the results were expressed as Mean ± SEM (standard error of the mean) and statistical significance was considered at $p<0.05$. All analyses of the data were performed by using Graph Prism Pad software version 5.0.

## Results

### Effects of treatments on the PAL acquisition

During first acquisition trial (before receiving electrical shock), the entrance latency time (STL-a) between the control and the diabetic groups were statistically insignificant [($p=0.8188$), Figure 1A]. This indicated that rats in different experimental groups had similar exploratory behaviour in dark. However, we observed significant differences in the number of trials to acquisition criteria between control and diabetic experimental groups [($p<0.0001$), Figure 1B]. Tukey's Multiple Comparison test showed that number of trial to acquisition in control groups were significantly less than the diabetic groups. Number of trial in group C was significantly lower compared to group D ($P<0.001$) and D+P ($P<0.05$). Administration of polyherbal formulation resulted in no significant changes between groups C vs. C+P ($p>0.05$); however, there was significant decrease in the trial number between groups D vs. D+P ($P<0.05$).

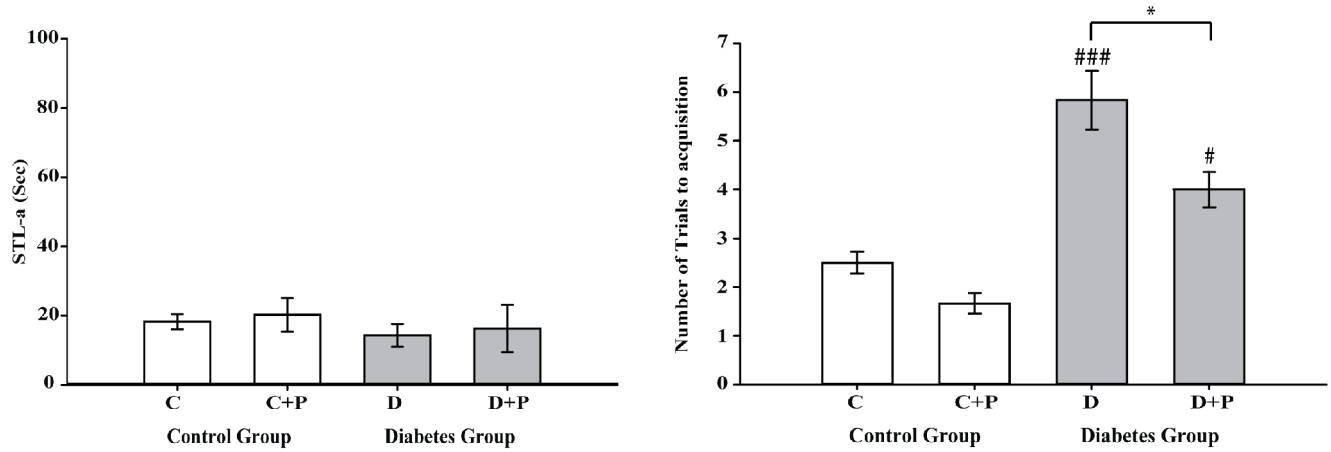


**Figure 1:** Effect of oral administration of polyherbal formulation on PAL test in acquisition trial.
Passive avoidance learning (PAL) test was performed following 30 day treatment period with saline (C) and saline + polyherbal formulation (C+P) to control groups and with streptozotocin and streptozotocin + polyherbal formulation (D+P) for diabetic groups.
A. Graph represents step-through latency in the acquisition trial (STL-a) of passive avoidance learning (PAL) task in the control and diabetic groups. Columns represent Mean ± SEM of step-through latency time (s).
B. Graph represents number of trials to acquisition of passive avoidance learning (PAL) task in the control and diabetic groups. Columns represent mean ± SEM. Tukey's multiple comparison tests was done to compare values between groups. *** indicates P<0.001, ** indicates P<0.01, * indicates P<0.05 and ns indicates P>0.05. # indicates comparison of all groups with control group C.

### Effects of treatments on the PAL retention

The PAL retention test was conducted 24 h after training process. We observed significant difference in the retention latency time (STL-r) between the experimental groups (P<0.0001). The STL-r time for group C was significantly greater than group D (P<0.001) and D+P (P<0.01); however administration of PF significantly increased the retention latency in group C+P vs. group C (P<0.05). In addition, we observed a major difference in STL-r between saline treated diabetic group D compared to group D+P that received PF (P<0.01). We also observed statistically significant differences in time spend in the dark compartment (TDC) between the control and diabetic groups [(P<0.0001) (Figure 2B)]. Tukey's multiple comparison test showed that TDC in saline treated control group C was significantly less compared to the saline treated diabetic group D (P<0.001) and PF treated diabetic group D+P (P<0.05). This result was consistent with finding that diabetes induces cognitive impairment. However, upon PF treatment TDC in diabetic group D+P was found to be significantly lower than saline treated diabetic group D (P>0.05) due to improvement in memory and retention of learned behaviour.

### Effects of treatments on the body weight and plasma glucose

We analysed the blood glucose levels of rats in different experimental groups at the beginning and end of the treatment period (Figure 3A). Statistical analysis by one way ANOVA showed that there was no significant differences between the control and diabetic group at the beginning (P=0.828). We performed similar analysis for the experimental groups at the end of 30 days treatment and observed statistically significant differences in plasma glucose levels between the control and diabetic groups (P<0.0001). Tukey post hoc test revealed that plasma glucose levels of diabetic group D was significantly higher than control group C and PF treated group C+P (P<0.001). Additionally, analysis showed that administration of PF to diabetic rats significantly reduced plasma glucose levels (group D+P) in comparison with untreated diabetic rats in group D (P<0.001). We also compared the body weight of the rats between groups at the beginning and end of 30 days treatment period (Figure 3B). We observed no significant changes in the body weight between the control and diabetic groups at the beginning of the study (P=0.201) by one way ANOVA analysis. Similar analysis was done for comparing body weight among experimental groups at the end of the experiment and we observed a significant reduction in the body weight of rats in the saline treated diabetic group D compared to the saline treated control group C (P<0.001). However, PF treated diabetic rats showed significant increase in body weight as compared to untreated diabetic rats (P<0.01).

## Discussion

Several experimental and clinical studies have established a strong association of diabetes with neurodegenerative disorders such AD [46]. It is proven that the molecular and biochemical features of sporadic AD correspond with features of Type 1 (insulin deficiency) and Type 2 (insulin resistance) diabetes; therefore AD is essentially a metabolic disease which is also referred to as Type 3 diabetes [3]. It is established that insulin and Insulin like Growth Factor (IGF) resistance in the brain affects glucose uptake and utilization that causes imbalance in energy production, leads to mitochondrial dysfunction, oxidative and ER stress that propagates cell death cascades, neuroinflammation, dysregulation of lipid metabolism leading to accumulation of toxic lipids (ceramides) and cholinergic dyshomeostasis [47-49]. At the molecular level, down regulation of insulin receptors (IR) at the blood brain barrier causes lower insulin transport to the brain, thus causing insulin resistance. Inappropriate activation of kinases result in inhibitory phosphorylation that affects PI3K and the MAPK insulin mediated signalling cascades, both of which are involved in the maintenance of synaptic plasticity and cell stress response [2,7,50]. These processes intensely effects neuronal survival by disrupting neurotransmitter homeostasis and neuronal cytoskeleton, causes accumulation of toxic oligomeric fibrils (neurofibrillary tangles) and insoluble aggregates (amyloid beta plaques) and compromise learning and memory functions [12]. These studies indicate the multifactorial pathogenesis of neurodegeneration that is caused due to insulin resistance and signals the need to assess innovative approaches for drug discovery and development.

In the present study streptozotocin was used to induce diabetes

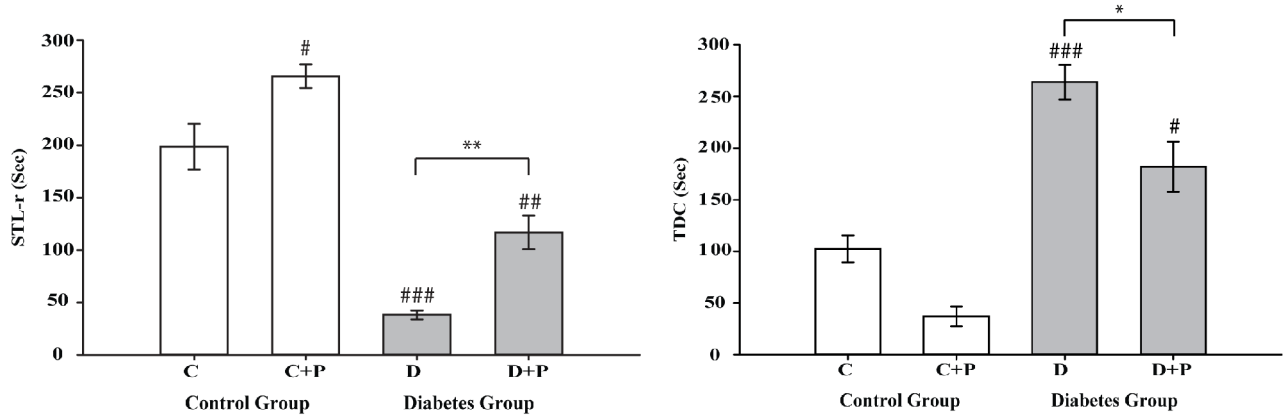


**Figure 2**: Effect of oral administration of polyherbal formulation on PAL test in retention trial.
Passive avoidance learning (PAL) test was performed following 30 days treatment period with saline (C) and saline + polyherbal formulation (C+P) to control groups and with streptozotocin and streptozotocin + polyherbal formulation (D+P) for diabetic groups.
A. Graph represents step-through latency in the retention trial (STL-r) of passive avoidance learning (PAL) task in the control and diabetic groups. Columns represent Mean ± SEM of step-through latency time (s).
B. Graph represents time spend in the dark compartment (TDC) of passive avoidance learning (PAL) task in the control and diabetic groups. Columns represent mean ± SEM.
Tukey's multiple comparison tests was done to compare values between groups. *** indicates P<0.001, ** indicates P<0.01, * indicates P<0.05 and ns indicates P>0.05. # indicates comparison of all groups with control group C.

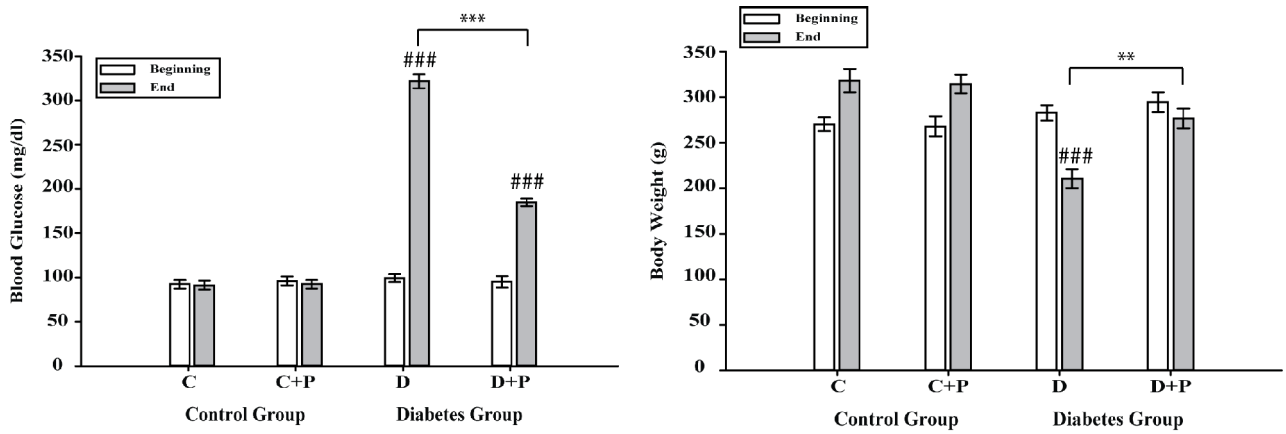


**Figure 3:** Effect of oral administration of polyherbal formulation on plasma glucose levels and body weight.
Blood glucose and body weight of rats were recorded at the beginning and end of the 30 day treatment period; experimental groups involved saline treated (group C) or polyherbal formulation treated (group C+P) control groups; streptozotocin at a dose 60 mg/kg of was used to induce diabetes which were then treated with either saline (group D) or polyherbal formulation (group D+P) for diabetic groups.
A. Graph represents blood glucose levels at the beginning and end of the study. Columns represent Mean ± SEM of blood glucose (mg/dl).
B. Graph represents body weight at the beginning and end of the study. Columns represent Mean ± SEM of body weight (g).
Tukey's multiple comparison tests was done to compare values between groups. *** indicates P<0.001, ** indicates P<0.01, * indicates P<0.05 and ns indicates P>0.05. # indicates comparison of all groups with control group C.

associated learning and memory impairments in rats to test the therapeutic potential of a novel PF. Streptozotocin at a low dose induces insulin resistant like brain state and is used widely as a method of inducing sporadic AD [37]. It produces both behavioural and neurochemical features that resembled human AD. Therefore, we evaluated the effect of PF on learning and memory deficits upon PAL paradigm in STZ diabetic rats. By monitoring the blood glucose levels, we found that STZ treatment induced diabetes in rats. In the PAL paradigm these rats showed disrupted learning and memory functions. There was a significant increase in the number of trials to acquisition, reduction in STL-r time, and increase in time spend in the dark compartment (TDC) as compared to the non-diabetic rats. However, treatment with PF containing hydro-alcoholic extracts of *Bacopa monnieri, Hippophae rhamnoides* and *Dioscorea bulbifera* for 30 days significantly improved PAL and memory in diabetic rats. We also observed a significantly lowered blood glucose level and increased body weight of diabetic rats at the end of the treatment. In PAL acquisition task a significant decrease in the number of trials in the PF treatment groups in control and diabetic rats was evidence of improvement in learning and memory functions. Similarly in the PAL retention task, a significant increase in latency time (STLr) and decrease in TDC were indicative of memory enhancing and nootropic effects of the PF treatment.

The patented PF studied here was inspired from Indian system of traditional medicine which indicated a long history of the traditional

use of the three plants *Bacopa monnieri, Hippophae rhamnoides* and *Dioscorea bulbifera* in the PF [20,22,36]. *Bacopa monnieri,* commonly known as *bhramhi* is a widely used and experimentally studied plant for improving brain functions and treatment of epilepsy and depression [51]. Studies have shown that Bacoside A is the main bioactive component and consists of a mixture of saponins (Bacoside A3, bacopaside II, jujubogenin isomer of bacopasaponin C and bacopasaponin C) [52]. Several experimental studies have demonstrated the neuro-protective properties of *Bacopa monnieri* such as regulation of cholinergic system, enhancing acetylcholine levels, reducing neuroinflammation, amyloid levels and oxidative stress [52-57]. *Hippophae rhamnoides*, commonly known as sea buckthorn or amlavetas, also has been traditionally used for its medicinal properties [58]. The fruits and seeds are rich in anti-oxidants, carotenoids, flavonoids, organic acids, amino acids, vitamins, micro and macronutrients, phytosterols, and fatty acids omega-3 and 6 [59-61]. Several studies have reported a wide range of medicinal properties of *Hippophae rhamnoides* fruit and seed preparations that include immuno-modulatory, anti-hypertensive, cardioprotective, antiglycemic, antilipidemic, anticholesterolemic, antioxidant and anti-apoptotic properties [41,62-65]. Quercetin is the bioactive constituent in *Hippophae rhamnoides* that modulate neurotransmitter levels by inhibiting AChE and monoamine oxidase B activity [66,67]. It acts as a neuroprotective agent by reducing oxidative stress and promotes learning, memory and neuron survival [68-70]. *Dioscorea bulbifera*, commonly known as air potato or *varahikand* is widely used for its antidiabetic properties [71-73]. Extracts from this plant are rich in phenolic and flavonoid content and the major phytoconstituents are diosgenin and diosgenin acetate [74]. Studies have shown that extracts of *Dioscorea bulbifera* inhibit amylase and glucosidase and mediate its anti-hyperglycemic effects [73]. Other medicinal properties reported include cardio-protective, anti-oxidant, and anti-apoptotic properties [75-77]. *In silico* analysis have shown that diosgenin has an affinity to bind to amyloid peptides in monomeric and fibril form and can induce calcium influx in human cortical neuronal cell line [78,79]. These studies indicate a possible mechanism by which this molecule could affect neuronal structure and function.

The notable medicinal properties of the PF attest the effects we observed on learning and memory in diabetic and control rats after the treatment. This result was in accordance with our previous clinical study, in which we showed significant improvement in cognitive deficits associated with AD in elderly subjects [35]. The novelty of this study lies in the fact that the PF is a combination treatment, with multi-targeted drug action [34]. This is in contrast with other experimental studies which focus on single targeted therapeutic action of natural compounds [25-29]. Secondly, this study supports the reverse pharmacology approach which claims to reduce costs, time and toxicity issues in drug development [80]. However there is scope to understand the mechanism of action and optimize safety, efficacy and acceptability of the PF as a drug. Further experiments are needed to study the effectiveness of the PF on different animal behavioural models such as Morris water maze test, elevated maze test, novel object recognition test etc. along with biochemical investigations to assess the antioxidant and anti-inflammatory marker levels considering the medicinal properties of the plants in PF. Additionally, the effect of PF on cholinergic deficits, taupathy, amyloid plaques should be studied to decipher the molecular basis of the therapeutic action. Since the PF is poly-molecular in nature, hence deciphering the molecular mechanisms underlying its cognitive benefits is a challenge. Future studies should utilize systems biology, *in silico* and high throughput genomics approaches to decipher molecular pathways affected by the PF.

Estimates indicate millions of people suffering from dementia and diabetes will increase several fold in the next millennium. These numbers suggests that as the population ages, T2DM associated cognitive dysfunction and dementia will become a huge burden on the caretakers and society. Research in the last 2 decades have provided valuable insights into understanding the pathophysiology of these diseases, however the ultimate goal is to offer patients preventive treatment that can protect them against accelerated cognitive decline. Current treatments option available for dementia are very limited (cholinesterase inhibitors, NMDA receptor antagonist) [14]; they only provide short term symptomatic relief and do not prevent disease progression. Also, over the last decade a large number of drug candidates have failed in clinical trials [15]. The paucity of treatment is due to the multi-factorial nature of diabetes associated cognitive dysfunction. There is a need to develop combination therapies, however adverse effects associated with modern synthetic drugs could prove to be prohibitive when trying to develop them as combination treatments [16,17]. The key advantage of the PF used in this study is its muti-targeted therapeutic action and lower side effects. However, clinical trials are warranted to assess its efficacy and safety in T2DM patients with cognitive dysfunction.

In conclusion, our study demonstrates that administration of a novel PF containing hydro-alcoholic extracts of *Bacopa monnieri, Hippophae rhamnoides* and *Dioscorea bulbifera,* to diabetic rats for 30 days protects against the diabetes induced cognitive deficits and reduces blood glucose levels. The PF contains multiple active constituents and may impart its therapeutic action by enhancing acetylcholine concentration, attenuating hyperglycemia, reducing oxidative stress and neuroinflammation. This treatment may be developed into a therapeutic that will provide a new potential for treatment and prevention of diabetes associated neurodegeneration and cognitive dysfunction.

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