Learning and Memory Enhancing Activity of Polyherbal Formulation on Streptozotocin Induced Memory Impairment in Rats via Reducing Mitochondria–Targeted Cytochrome

Deepa Shukla1,2, Sajal Srivastava2,*, Talha Jawaid3

ABSTRACT

Introduction: Neurodegenerative diseases and/or brain injury may occur due to mild to severe memory disorders in which Alzheimer’s disease (AD) is defined as degradation of neurons and there is no effective therapy or cure for the disease. The intention behind this study was to determine memory enhancing effect of an Ayurvedic polyherbal formulation (PHF) in Streptozotocin-induced memory deficit experimental animal models. Methods: Polyherbal formulation (PHF) was a suspension of three plant extracts i.e. Alpinia galanga, Glycerichiza glabra and Convolvulus pluricaulis along with other excipients. Different behavioral animal models like Social recognition test (SRT), Morris water maze (MWM) test, Pole climbing test (PCT) and Elevated plus maze (EPM) test were used to evaluate efficacy of Polyherbal formulation (PHF) in learning and memory process of animals. Gene expression was performed by RT-PCR and western blot. Results: Results indicated that time of social recognition during trial 2 (SIT2) was appreciably reduced in PHF (200mg/kg). NR1, NR2B and GAP-43 proteins leads to reduction showed p53 regulates the Bcl-2 family proteins its expression in the given experiment was

INTRODUCTION

Alzheimer’s disease (AD) is defined as degradation of neurons and there is no effective therapy or cure for the disease.1 It has affected nearly 40 million people around the globe and that is expected to increase in the coming years.2 People with disturbed cognition is in a transition stage between declining due to aging and other clinical syndromes associated with Alzheimer’s disease.3 Neurodegenerative diseases and/or brain injury may occur due to mild to severe memory disorders, all are linked to acquisition, consolidation and retrieval.4

A possible step towards standard therapy for the treatment is to slowdown the progression of disease. Thus, research on disease origin and pathophysiology is required along with the testing of new drugs. The medications for treatment of cognitive impairment may cause various side effects and other consequences. To avoid these undesired effects to drugs, the extensive research has been going on medicinal plants and natural supplements due to their wide history of neuroprotective and brain boosting drugs with no side effects. Medicinal plants have been used in various cognitive improving abilities like memory, motivation, attention, intelligence, and concentration.5 They are also referred as nootropic drugs that enhance cognition and maintain healthy emotional state, process of learning and motor control.6 Ayurveda as an ancient system of medicine of India has gaining importance worldwide due to its disease preventive and health promoting approach.7 In the present study, an Ayurvedic Polyherbal formulation was prepared by incorporating three traditional herbs i.e. Alpinia galanga (AG), Glycyrrhiza glabra (GG) and Convolvulus pluricaulis (CP). The above-mentioned herbs were selected due to their uses as folk medicine in cognition enhancing and memory boosting effects. Alpinia galanga, it is a well-known medicinal plant containing mild spicy fragrance belongs to family Zingiberaceae.8 It has been used in the Asian countries for the treatment of oxidative stress, stomachache, ulcer, inflammation, diabetes mellitus, spasm and microbial and insecticidal infection.9 It is a class of edible group and popular nerve tonic herb in India. Glycyrrhiza glabra L. is a Leguminosae plant, possess several bioactive compounds like glycyrrhizin, glycyrrhetic acid, octanoic acid etc. that are responsible for neuroprotective, hepatoprotective, immunomodulatory and anticancer activities.10,11 Convolvulus pluricaulis Linn. is a native plant of India used as a neuroprotective and memory enhancer herb since ancient times.12 It belongs to family
Convolvolaceae and used in the treatment of diabetes, ulcer, anxiety, liver diseases and oxidative stress. Therefore, in this study, the memory boosting efficacy of Polyherbal formulation (PHF) was evaluated in different behavioral models with a thought to neurodevelopmental origins in memory deficit disorder such as Schizophrenia, Dementia, Alzheimer’s and Autism. In addition, to evaluate the biochemical level of Polyherbal formulation (PHF) in brain by estimating enzymatic levels and level of metabolic enzyme acetylcholinesterase (AChE).

**MATERIAL AND METHODS**

Chemicals and reagents

Donepezil, Streptozotocin, Propyl paraben, Methyl paraben, Tween 80 and Sodium carboxy methyl cellulose were purchased from Sigma Aldrich, USA. All other solvents were of analytical grade and distilled water was used throughout the study. Albino Wistar rats were used for evaluating the effect of poly herbal formulation (PHF) in different experiment models.

Preparation of formulation made up of herbs

The formulation of herbs was prepared by mixing 0.5014 g, 0.3343 g and 0.3343 g of *Alpinia galanga* (AG), *Glycerhiza glabra* (GG) and *Convolulus pluricaulis* (CP) extracts, respectively followed by addition of parabens (Propyl paraben, 0.02%, w/v and Methyl paraben, 0.2%, w/v), tween 80 (0.1%, w/v) and a little amount of sodium carboxy methyl cellulose (0.5%, w/v) then uniformly triturated to form a smooth paste. The paste was rinsed with distilled water (100 ml) and vortexed using mechanical stirrer (500 rpm) to get suspension. The formulation was found stable upto 90 days of storage.

Experimental design and drug administrations

The effect of *Alpinia galanga* (AG), *Glycerhiza glabra* (GG) and *Convolulus pluricaulis* (CP) extracts and their formulation was evaluated in experimental models of memory impairment due to streptozotocin (STZ) administration. The test drug along with standard and vehicle were giving for 14 days. Intracerebroventricular (ICV) streptozotocin (STZ) administration. The test drug along with standard and vehicle were given for evaluating the effect of poly herbal formulation (PHF) in different experiment models.

Streptozotocin (STZ) induced memory impairment and grouping of animals

The test compounds and standard drug were administered for 14 days and behavioral assessments were carried out from 14th day onwards. Animals were anesthetized using ether and STZ (3 mg/kg) was given to animals on 1st and 3rd day through intracerebroventricular (ICV) route. Injections for intracerebroventricular (ICV) route were made with hypodermic needle that was attached to Hamilton microliter syringe. The needle was perpendicularly inserted to the skull of animals to reach into the brain. Streptozotocin (STZ) was prepared freshly by dissolving into cerebrospinal fluid (CSF) (25 mg/ml) solution. The concentration was adjusted in such a way to deliver only required amount of drug (10 μl) through injection.

Animals were divided into seven groups each containing six animals. Group – 1 Control, Vehicle treated, intra-peritoneal (p.o.); Group – 2 Toxic group, STZ (icv); Group – 3 Donepezil (5 mg/kg, i/p); Group – 4, 5 and 6 received extract of AG, GG and CP, respectively in the dose of 200 mg/kg, p.o.; and Group – 7 and 8 received PHF (200 and 400 mg/kg, p.o., respectively).

Assessment of learning and memory

Social recognition test (SRT)

Social Recognition Test (SRT) was performed on male Wistar rats weighing 220–240 g. Animals memory were impaired by administration of STZ. STZ was administered on 14th day to the test animals. The effect of test drugs were determined by introducing unfamiliar juvenile in the cages of animals as social stimuli. The time recorded as T1 that test animal spent with the juvenile for 5 min interval and then juvenile rat was removed. T2 was recorded after 2 h when same juvenile was reintroduced into their cages. SRT of test animal was determined by significant reduction of interaction time in trial 2 (T2) as compared to T1. The recognition index (RI) was calculated using following formula:

\[ RI = \frac{T1}{T2} \]

**Morris water maze (MWM) test**

Morris Water Maze (MWM) test was performed on male Wistar rats weighing 220–240 g. Animals memory were impaired by administration of STZ on 14th day of treatment. The arrangement was made with this test by placing the rats in a circular pool with dimensions of 45 × 26 × 20 cm which was filled with water. The pool was marked with four different starting points i.e. N-E-ES-WN. The animals were allowed to stay for 30 s if they were unable to escape themselves from the pool in 120 s. The prior trials were given to each animal; daily four trials were given for five days before placing them into the pool. The effect of test drugs was determined by noting the escape latency time of the animals.

**Elevated plus maze (EPM) test**

Elevated Plus Maze (EPM) test apparatus was used to study the effect of test compounds on STZ induced memory impaired animals. The apparatus consists of a 25 cm × 25 cm × 40 cm chamber along with dim light and sound box. The electric shock was given to animals be grid floor of the chamber. The animals avoided electric stimulus by jumping onto the pole. This jumping was noted as an escape time of the animals to avoid foot shock. However, jumping on to the pole before shock due to buzzer sound was considered as avoidance. The experiment was terminated after 10 trials with intervals of 30 s. The animals were given the respective treatment and again subjected to the test procedure for 5 consecutive days after the completion of training. A significant reduction in escape latency time was considered as successful retention of avoidance memory.

**Biochemical estimation**

Malondialdehyde (MDA) estimation

Malondialdehyde was estimated according to the method of Colado et al., 1997. Briefly, TCA and HCl (5N) were mixed with brain homogenate in which 2% TBA was added. The mixture was then heated at 900℃ for 15 min followed by centrifugation for 10 min at 12000 × g. The supernatant was isolated and measured at 532 nm using ELISA plate reader (Biotek, USA). The determined MDA in supernatant was expressed as nmol/mg of protein. It was calculated using standard curve of Tetra ethoxy propane (TEP).

Glutathione (GSH) estimation

Gultathione level in animals were determined according to the method of Sharm and Gupta, 2001. Briefly, trichloroacetic acid was mixed with
The effect of PHF was determined on 14th day after memory impairment assay was done. The results were significantly different at *p<0.05, as determined by One-way ANOVA and after that dunnett’s multiple comparison test. Results are expressed as mean ± S.E.M. Statistical analysis was carried out according to the method of Livak and Schmittgen.

Western Blot Analysis of protein expression

Western Blot Analysis was performed on brain tissues obtained from the hippocampal region of the animals after 24 h of treatment. The tissues were homogenized with the buffer containing NaCl (50 mM/L), EDTA (1 mM/L), SDS (0.5%), Triton X-100 (1%), Tris HCl (20 mM/L) and sodium deoxycholate (0.5%). It was centrifuged for 20 min at 15,000 × g. The samples (50 µg) were run on polyacrylamide gel and then transferred to PVDF membrane. It was blocked by milk solution (5%) for 2 h. Incubation of membrane was performed at 4°C along with the specific antibodies i.e. mouse monoclonal anti-β-actin (1:10000; dilution), NR2B (1:10000, dilution), NR1 (1:10000, dilution), GAP-43 (1:1000, dilution) and β-actin. Mastercycler ep realplex was used for quantitative RT-PCR reactions for data analysis. The expression of gene after data analysis was calculated according to the method of Livak and Schmittgen.

Biochemical estimations

The MDA level was increased in the cortex and hippocampus regions of the memory impaired rats significantly as compared to the control and vehicle groups. Treatment with donepezil reduced MDA levels in both brain regions significantly as compared to STZ group. Administration of 200 mg/kg of plant extracts significantly decreased MDA levels in cortex and hippocampus of STZ-injected rats. PHF treatment also caused significant decrease in MDA level in cortex and hippocampus of STZ injected rat brain.
Shukla, et al.: Learning and Memory Enhancing Activity of Polyherbal Formulation on Streptozotocin Induced Memory Impairment in Rats via Reducing Mitochondria–Targeted Cytochrome

Figure 1: Effect of plant extracts and PHF on cognition impaired rats using the social recognition test. Results were presented as mean ± S.E.M., significantly different at **P<0.01 and ***P<0.001 as compared to trial 1 of the respective group.

Figure 2: Effect of plant extracts and PHF on recognition index of STZ treated animals. Results were presented as mean ± S.E.M., significantly different at #P<0.05 as compared to control and vehicle groups and *P<0.05 and **P<0.01 as compared to the STZ group.
Figure 3: Effect of plant extracts and PHF in memory impaired rats using Morris water maze test. Results were presented as mean ± S.E.M., significantly different at (*P < 0.05, **P < 0.01 and ***P < 0.001 as compared to session 1 of the respective groups.

Figure 4: Effect of plant extracts and PHF in memory impaired rats using pole climbing test. Results were presented as mean ± S.E.M., significantly different at (*P < 0.05, **P < 0.01 and ***P < 0.001 as compared to day 1 of the respective groups.
Effect of PHF on GSH level in memory impaired rats

Effect of PHF was calculated by estimating the level of GSH in brain regions of rat using the calibration curve obtained with different concentration of glutathione. STZ administration caused reduction in GSH levels in brain regions as compared to control and vehicle groups. As shown in Figure 7, donepezil prevented reduction in GSH level in cortex and hippocampus regions significantly (P<0.01). Administration of plant extract prevented STZ induced reduction in GSH levels. AG, CC and GG extracts increased GSH levels significantly in STZ treated rat brain regions.

Effect of PHF on AChE level in STZ-induced amnesic rat brain

Results exhibited that administration of STZ caused increase in the AChE activity in both cortex and hippocampus regions of treated animals. Preventive treatment with donepezil significantly inhibited AChE activity in cortex (P<0.05) and hippocampus (P<0.01) of STZ injected rats (Figure 8). Administration of plant extract prevented elevation of AChE activity. AG, CC and GG extracts significantly decreased AChE activity in impaired memory rats. There was significant decrease in the AChE activity in brain regions of the rats treated with 200 mg/kg PHF (Figure 8).

Effect of PHF on mRNA expression and protein expression

Expressions of Bcl-2, Bak, and Bax mRNA are demonstrated in for excluding the variations due amount and nature of RNA, the results recorded were adjusted according to the expression of GAPDH. Hippocampal tissue in STZ group was indicated by considerably amplified the levels Bak & Bax and significantly declined the levels of Bcl-2. Their levels were appreciably inverted in PHF (200mg/kg). PHF 200 group showed best results which were almost comparable to STZ group. In comparison to STZ group, PHF (200mg/kg) demonstrated more significant effect in up-regulation of Bcl-2 protein and down-regulation of p53 protein. In mitochondrial pathway cell apoptosis chiefly involves the Bcl-2 g. Bcl-2 and Bax (both are belong toBcl family) controls the secretion of prosapoptotic factors from mitochondria. In the present study, Bax and Bak miRNA (proapoptotic) were down-regulated, while Bcl-2 mRNA and protein which are antiapoptotic were up-regulated in PHF (200mg/kg) group opposite to STZ group. Also, p53 regulates the Bcl-2 family proteins its expression in the given experiment was appreciably reduced in PHF (200mg/kg) group opposite effect was recorded in STZ group. These findings indicated that PHF could exert its memory enhancement by interacting with these proteins (Figure 9).

DISCUSSION

Pathogenesis of cognition impairment starts from temporal lobe or dysfunction of prefrontal part of brain. It can be ranging from mild cognitive impairment to dementia. Learning and memory processes are based on electro-chemical signaling, acts as a network within the brain. The signaling may occur through biogenic monoamines, acetylcholine, amino acid, neuropeptides and other gene families. Memory formation in the brain can be divided into different consecutive
Figure 6: Effect of plant extracts and PHF (200 mg/kg) on the malondialdehyde (MDA) levels in animals. Results were presented as mean ± S.E.M., #Significant difference (#P < 0.05 and ##P < 0.01) in comparison to the respective brain region of control and vehicle groups and *Significant difference (*P < 0.05, **P < 0.01 and ***P < 0.001) in comparison to the respective brain region of STZ group.

Figure 7: Effect of plant extracts and PHF on the glutathione (GSH) levels in animals. Results were presented as mean ± S.E.M., significantly different at #P < 0.05 and ##P < 0.01 as compared to control and vehicle groups and significantly different at *P < 0.05, **P < 0.01 and ***P < 0.001 as compared to STZ administered group.
phases such as acquisition, consolidation, retrieval and in some condition, reconsolidation or extinction phases. The acquisition phase in the learning of new behavior, consolidation phase is the stabilization or storage of earned memory, retrieval phase is accessing the stored memory, reconsolidation is re-evaluation of prior gained memory and extinction phase is inhibition of stored memory. Serial acquisition of memory is required for learning new information, however, memories are also acquired from past knowledge. Generalization and interference are the memory slots that are produced due to interaction of two different memory traces from behavioral point of view.

The Ayurvedic Polyherbal formulation (PHF) was used for memory enhancement in experimental behavioral animal models due to wide utilization of selected folk medicines and phytotherapy as well. The protocol of the study was designed to evaluate efficacy of these herbs (A. galanga, G. glabra and C. pluricaulis) in the form of PHF as brain tonic against mental illnesses with a thought to be an effective herbal supplement for neurological disorders. Herbal approach has offered several opportunities to produce new drugs for memory disorders. In this cognitive behavioral study, the PHF was found effective in boosting learning and memory process of animals in different behavioral models i.e. SRT, MWM, PCT and EPM. Results exhibited that STZ losses animal memory while testing in different behavioral models. It has reported that STZ mimics biochemical parameters in animals. PHF was found effective in reducing STZ induced toxicity (learning and memory deficit) in animal models along with restoring biochemical enzymatic levels of MDA and GSH. PHF was also found effective in reducing metabolic enzyme activity of acetylcholinesterase. The test drug enhances the level of acetylcholine in the animal brain. In several
behavioral studies the neuroprotective drugs aimed at preventing the production of free radicals in the brain. Production of free oxidative radicals indicate progression of cognitive decline. PHF reduced AChE activity at both cortex and hippocampus regions of the brain in a dose-dependent manner. Our findings were matched with the work of Parfitt et al., 2012, that hippocampus consolidate information from short-term memory to long-term memory and plays role in forming, organizing and storing memories. Bcl2, Bax and Bcl-2 mRNA expression in PHF treated group was significant as compared control and STZ groups. Pro-apoptotic function in cells is showed by Bax and Bak1, however, Bcl-2 is anti-apoptotic in function. Bcl-2 expression in inversely proportional to apoptotic rate that causes cell apoptosis. Present study showed expression of Bak, Bax and Bcl-2 in brain, however the mechanism of expression is yet to be investigated. The western blot technique was used to evaluate NR1 and NR2B expression. Results showed that PHF increased NR1 and NR2B expression. It has reported that NMDA receptor plays immense role in plasticity of synapse as seen in learning and memory. GAP-43 plays role in regulation of growth, neurite outgrowth and synaptic plasticity. The reduction of GAP-43 level in brain is seen due to STZ administration. It was raised by PHF and it may be attributed to recovery of synaptic plasticity in the brain.

CONCLUSION

It can be concluded from the study that PHF alleviated amnesia in STZ treated rats by improving cholinergic function, reduction of oxidative stress and enhancement of behavior. Thus, PHF may be an alternative compound for the treatment of memory loss memory deficit cases.

DECLARATION

Author declare no conflict of interest.

ACKNOWLEDGEMENT

Authors are thankful to the institute for providing all facilities required during the work.

REFERENCES

ABOUT AUTHORS

Deepa Shukla is Ph D scholar in Amity Institute of Pharmacy, Amity University Uttar Pradesh, Lucknow Campus, India. She completed her B Pharm and M Pharm from Dr. A.P.J. Abdul Kalam Technical University, Uttar Pradesh. She has been teaching and researching for more than 6 years.

Dr. Sajal Srivastava is currently working as a Dy Director & Associate professor at the Department of Pharmaceutical Chemistry, Amity Institute of Pharmacy, Amity University Uttar Pradesh, Lucknow Campus, India. He is Head of Department of Pharmaceutical Chemistry. He has over 17 years of experience in teaching Phytochemistry, Medicinal Chemistry and Instrumental Techniques. Dr. Srivastava’s main research area is medicinal plants and chemistry of heterocyclic compound. He has also acquired expertise in the development of nasal dosage form. He has authored many book chapters and research articles in peer reviewed journals. In his research area many post-graduate scholars are pursuing their master and doctoral degree.

Dr TALHA JAWAID is an Assistant Professor of Pharmacology college of Medicine at AL-IMAM BIN SAUD ISLAMIC UNIVERSITY, Riyadh, Kingdom of Saudi Arabia. He graduated from Rajiv Gandhi University of health sciences, Bangalore and finished his PhD from Integral University, Lucknow. He Participated in teaching Pharmacology to all medical professional school students. He has 15 years of teaching and research experience. Dr. Talha Jawaid main research area is neuropharmacology and cardiovascular pharmacology. He has published various research paper in peer review journals. He has published one book in his credit.