

Neuroprotective Activity of Ethanolic Extract of Polyherbal Formulation on Streptozotocin Induced Alzheimer's Disease in Mice.

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ABSTRACT:

Objective: To investigate the effect of Polyherbal Formulation on cholinergic dysfunction in STZ induced Alzheimer's disease (AD).

Methodology: The current study was designed to investigate the protective effect of Polyherbal Formulation (PHF) 200 and 400mg/kg for 21 days. Swiss albino mice were injected with STZ (0.5mg/kg) on 14th day of pretreatment with PHF. Behavior pattern was assessed using Morris water maze, Y maze and Open field test. Biochemical estimations, namely brain Acetylcholinesterase (AChE), reduced glutathione (GSH), Catalase, Super oxide dismutase (SOD) and TBARS levels were performed.

Results: The study showed that STZ significantly impaired learning and memory with a significant enhancement in brain AChE, TBARS and reduction in brain GSH, Catalase and SOD levels. Treatment of PHF significantly attenuated STZ induced behavioral and biochemical changes. The inhibitory effect of PHF on STZ induced dementia of AD type might be associated with its AChE inhibition and Free radical scavenging activity.

Conclusion: The present study revealed that Polyherbal formulation significantly reduced AChE levels, oxidative stress, lipid peroxidation and cognitive impairment. The neuroprotective effect of Polyherbal Formulation might be associated with its antioxidant and anti-cholinesterase activities.

INTRODUCTION

Dementia is a brain disorder characterized by impairment of memory and loss of intellectual ability. The dementing condition, that has gained much attention in the recent years is Alzheimer's disease (AD), which is a progressive neurodegenerative disorder associated with loss of neurons in distinct brain areas (Sharma et al., 2008). Besides the neuropathological hallmarks of this disease, namely neurofibrillary tangles and neuritic plaques, it is characterized neurochemically by a consistent deficit in cholinergic

neurotransmission, particularly affecting cholinergic neurons in the basal forebrain (Pulok et al., 2007). All forms of dementia of Alzheimer's type are characterized by abnormalities in glucose metabolism, reduced glucose utilization and levels of energy rich phosphates like ATP, ADP, etc (Arnaiz et al., 2001; Nitsch et al; 1991). Disturbed energy metabolism is intricately associated with increased oxidative stress that results in oxidation of biomolecules and initiates excitotoxic neuronal cell damage (Martinez et al., 1994; Olanow et al., 1993). The nature provides a new opportunity to regain one's full mental capacity.

A number of herbs traditionally employed in the Indian System of Medicine "Ayurveda," have yielded positive results with limited side effects. The poly herbal formulation contain Plants of *Elaeocarpus ganitrus*, *Evolvulus alsinoides* L, *Ocimum sanctum* Linn (Tulsi), Gomutra (Distilled cow urine) and Honey to investigate the Neuroprotective Activity of Polyherbal Formulation against Intracerebroventricular (ICV) streptozotocin induced AD which causes oxidative stress, cholinergic dysfunction and lipid peroxidation.

METHODOLOGY

Drugs and chemicals:

Donepezil, Film coated tablets from Alkem laboratories Pvt Ltd, India. Streptozotocin, DTNB (5, 5'-dithiobis (2-nitrobenzoic acid)), Eserine, Acetylthiocholine iodide, Reduced Glutathione were purchased from Sigma Aldrich, USA. 2-Thiobarbituric acid was purchased from Himedia laboratories Pvt Ltd, Mumbai. All other reagents of Analytical grade are from S. D Fine chemicals Ltd, India. Polyherbal formulation is prepared by adding equal quantities of ethanolic extracts of *Elaeocarpus ganitrus*, *Evolvulus alsinoides*, *Ocimum sanctum* and to this Honey and Gomutra (Distilled cow urine) is added (Raman et al., 2012).

Experimental animals:

Swiss albino mice, weighing 20-35g, at the age 3-4 weeks were used in the study. They were maintained on standard laboratory pellet chow diet; Provimi limited (India), provided water *ad libitum* and were kept under standard conditions at 23-25 °C, 35 to 60% humidity, and 12hr light /dark cycle. The mice were acclimatized to the laboratory conditions a week prior to experiment. The experimental protocol was duly approved by institutional animal ethics committee (IAEC) and care of the animals was carried out as per the guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA) (Protocol. No: I/IAEC/LCP/005/2014/SM-30♂).

Acute toxicological studies:

Acute oral toxicity was performed as per the OECD 423 guideline. The administered dose was assigned as toxic if mortality was observed in two or three animals. The same dose was repeated again if mortality was observed in one animal to confirm the toxic dose. The animals were observed for toxic symptoms of behavioural changes, locomotion, convulsions and mortality. The Polyherbal Formulation was found to be non-toxic at 2000mg/kg and the LD50 of 2000 mg/kg and above is said to be unclassified according to OECD 423. 1/10th (200 mg/kg) and 1/5th (400 mg/kg) of this dose were selected for In vivo pharmacological study.

Induction of neurotoxicity and grouping:

Neurotoxicity is induced by ICV injection of STZ (0.5 mg/kg) by identifying the bregma point in skull. In brief, bregma point was identified (Laursen et al., 1986) by rubbing the point of needle over the skull (approximately 1–3 mm rostral to the line drawn through anterior base of ears), then at 45° angles the needle was inserted 2 mm lateral to midline and STZ was injected. Animals were randomly divided into five groups of each six mice. Group I: vehicle control received Distilled Gomutra and honey (p.o), Group II: negative control received only with i.c.v injection of STZ, Group III and IV animals were injected with i.c.v STZ and treated with 200 and 400mg/kg (p.o) Polyherbal Formulation, Group V: standard group received i.c.v injection of STZ and treated with 5mg/kg (p.o) Donepezil. Except vehicle control group all the animals received i.c.v injection of STZ on 14th day of Polyherbal formulation pre-treatment and the treatment was continued upto 21 days. The negative control animals received only i.c.v injection of STZ.

In-vivo Pharmacological evaluation

Behavioural studies

Morris water maze (MWM):

Morris water-maze test was employed to assess learning and memory of the animals (Parle et al., 2004; Morris et al., 1984). It consists of large circular pool (150 cm in diameter, 45 cm in height, filled to a depth of 30 cm with water maintained at 28±1°C). The water was made opaque with white colored non-toxic dye. A submerged platform (10×10 cm), painted in white was placed inside the target quadrants of this pool, 1 cm below surface of water. Each animal was subjected to four consecutive training trials on each day with inter-trial gap of 5 min. Day 4 escape latency time (ELT) to locate the hidden platform in water maze was noted as an index of acquisition or learning.

Video tracking in Y Maze:

Y-maze task used to measure the spatial working memory in rats. The maze is made of gray plastic. Each arm is 40 cm long, 13 cm high, 3 cm wide at the bottom, 10 cm wide at the top, and converged at an equal angle. Each mouse was placed at the end of one arm and allowed to move freely through the maze for 8 mins. Mice tend to explore the maze systematically, entering each arm in turn. The ability to alternate requires that the mice know which arm they have already visited. The series of arm entries, including possible returns into the same arm is recorded. Alteration is defined as the successive entries into the three arms, on overlapping triplet sets. The percentage of alteration is calculated as the ratio of actual alterations to possible alterations, defined as the total number of arm entries minus two, and multiplied by hundred. Typically,

mice exhibit an alteration percentage of 60-70%, and perform 25-35 arm entries within the 8 min session (Reddy et al., 1997).

Open field habituation:

The exploratory behaviour of the mice was evaluated by open field habituation task method. Mice was placed in a 40 cm×50 cm×60 cm open field whose brown linoleum floor was divided into 12 equal squares by white lines and left to explore it freely for 5 minutes. The number of line crossings and head dippings were counted (Maria et al., 2005).

STATISTICAL ANALYSIS

All data are presented as Mean ± S.E.M. The significance of difference among the groups were assessed using one way analysis of variance (ANOVA) followed by Tukey's test using Graph pad PRISM software and $p \leq 0.05$ was considered significant.

RESULTS

Behavioural parameters

Morris water Maze :

The spatial working memory was assessed by Escape Latency Time (ELT) in Morris water Maze and the effect of Poly Herbal Formulation on ELT were shown in Table: 1. Administration of STZ showed significant ($P < 0.001$) increase in ELT in Group II when compared with Group I, Group III, IV and Group V showed significant ($P < 0.001$) decrease in ELT when compared with Group II.

Y-Maze :

Table: 2 shows the results for the performance of mice in Y-Maze task in which spatial recognition memory performance as alternation behaviour can be examined. The percentage alterations of Group II significantly ($P < 0.001$) lowered when compared with Group I, there was a significant ($P < 0.01$, $P < 0.001$), and ($P < 0.001$) increase in percentage alterations when Group III, Group IV and Group V are compared with Group II. The statistical significance ($P < 0.01$) observed when Group I is compared with Group

Catalase enzyme activity:

The catalase levels were shown in Table 5 and Fig: 8. The enzyme levels were significantly ($P < 0.001$) decreased in Group II when compared with Group I, there was a significant ($P < 0.01$, $P < 0.001$) and ($P < 0.001$) increase in catalase activity when Group III, Group IV and Group V were compared with Group II. Group III and Group IV showed statistical significance ($P < 0.001$) and ($P < 0.01$) in enzyme levels when compared with Group I.

III and statistical significance ($P < 0.01$) was observed when Group III is compared with Group V.

Open field habituation test:

Effect of Poly Herbal Formulation on open field exploration were shown in Table: 3. The exploratory behaviour (i.e.) the number of line crossings and head dippings decreased significantly ($P < 0.001$) in Group II on comparison with Group I, Group III, Group IV and Group V showed significant ($P < 0.01$, $P < 0.001$) and ($P < 0.001$) increase in line crossings and head dippings when compared with Group II. Group III showed statistical significance ($P < 0.01$) in head dippings when compared with Group I.

Acetyl cholinesterase enzyme activity:

Effect of Poly Herbal Formulation on AchE was depicted in Fig: 1. Administration of STZ significantly ($P < 0.001$) increased the AchE activity when compared with Group I, Group III, Group IV and Group V and significantly ($P < 0.05$, $P < 0.001$) and ($P < 0.001$) attenuated the raise in enzyme level when compared with Group II.

Total protein:

Effect of Poly Herbal Formulation on total protein was shown in Fig: 1. The protein levels were significantly ($P < 0.001$) decreased upon administration of STZ when compared with Group I, Group III, Group IV and Group V and significantly ($P < 0.05$, $P < 0.001$) and ($P < 0.001$) attenuated the fall in protein levels when compared with Group II. There was observed statistical significance ($P < 0.001$) among Group III and Group V.

Anti-Oxidant parameters

Reduced Glutathione (GSH):

The results were shown in Table 5 and Fig: 7, the GSH levels were significantly ($P < 0.001$) decreased in Group II when compared with Group I. There was observed significant ($P < 0.01$, $P < 0.001$) and ($P < 0.001$) increase in enzyme levels in Group III, Group IV and Group V on comparison with Group II. Statistical significance ($P < 0.01$) was observed when Group III was compared with Group V in the increased enzyme level.

There was a dose dependant significant ($P < 0.01$) increase in enzyme levels observed among Group III and Group V.

Superoxide dismutase (SOD):

The effect of Poly Herbal Formulation on anti-oxidant enzyme SOD was shown in Table 5 and Fig: 9 The enzyme levels were significantly ($P < 0.001$) decreased in Group II when compared with Group I, there was a significant ($P <$

0.01, $P < 0.001$) and ($P < 0.001$) increase in enzyme levels in Group III, Group IV and Group V when compared with Group II.

Thiobarbituric acid reactive species (TBARS):

Effect of Poly Herbal Formulation on lipid peroxidation markers were shown in Table 6 and Fig: 10. The brain TBARS significantly ($P < 0.001$) increased in Group II when compared with Group I. Group III, Group IV and Group V significantly ($P < 0.01$, $P < 0.001$) and ($P < 0.001$) decreased TBARS when compared with Group II. Group III showed statistical significance ($P < 0.01$) with Group I and significance ($P < 0.001$) observed when Group III compared with V.

Table 1: Effect of PHF on Escape Latency Time in Morris water Maze

Group	Escape latency (Time in sec)
Group I (Vehicle)	24.50±3.29
Group II (STZ)	113.1±11.98 ^{***}
Group III (Donepezil 5mg/kg+STZ)	25.14±1.68 ^{&&&}
Group IV (LD 200mg/kg+ STZ)	36.7±4.13 ^{###}
Group V (HD 400mg/kg+ STZ)	29.52±3.12 ^{\$\$\$}

Values are expressed as mean ± SEM of n=6 animals. ^{***} $P < 0.001$ indicates comparison of negative control group with control group. ^{###} $P < 0.001$ indicates comparison of low dose group with negative control. ^{\$\$\$} $P < 0.001$, indicates comparison of high dose group with negative

Table 2: Effect of Poly Herbal Formulation on percentage alterations in Y-Maze

Group	Y-Maze (Percentage alterations)
Group I (Vehicle)	43.62±0.83
Group II (STZ)	18.06±2.55 ^{***}
Group III (Donepezil 5mg/kg+STZ)	38.55±1.406 ^{&&&}
Group IV (LD 200mg/kg+ STZ)	31.75±2.28 ^{###}
Group V (HD 400mg/kg+ STZ)	41.60±1.89 ^{\$\$\$}

Values are expressed as mean ± SEM of n=6 animals. ^{***} $P < 0.001$ indicates comparison of negative control group with control group. ^{###} $P < 0.01$ indicates comparison of low dose group with negative control ^{\$\$\$} $P < 0.001$ indicates comparison of high dose group with negative control. ^{&&&} $P < 0.001$ indicates comparison of standard with negative control.

Table 3: Effect of Poly Herbal Formulation on open field exploration

Group	Line crossings	Head dippings
Group I (Vehicle)	126.7±4.41	25.17±2.71
Group II (STZ)	77.83±3.458 ^{***}	8.500±1.40 ^{***}
Group III (Donepezil 5mg/kg+STZ)	122.4±4.137 ^{&&&}	21.83±1.92 ^{&&&}
Group IV (LD 200mg/kg+ STZ)	102.2±3.02 ^{##}	20.63 ±1.60 ^{##}
Group V (HD 400mg/kg+ STZ)	117.7±6.00 ^{\$\$\$}	23.83 ±1.79 ^{\$\$\$}

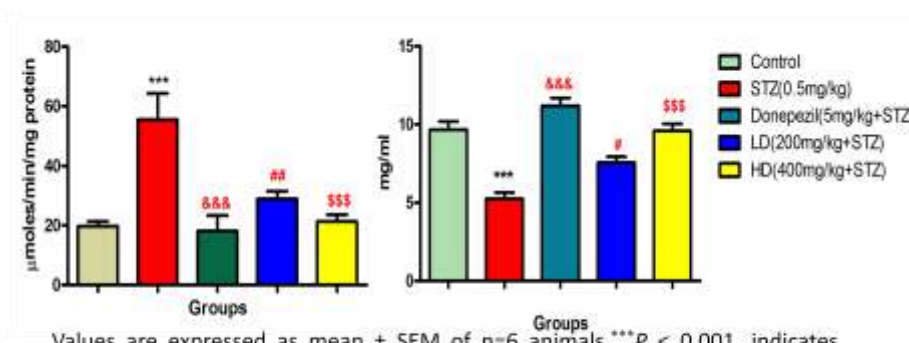
Values are expressed as mean ± SEM of n=6 animals. ^{***} $P < 0.001$ indicates comparison of negative control group with control group. ^{##} $P < 0.01$ indicates comparison of low dose group with negative control ^{\$\$\$} $P < 0.001$ indicates comparison of high dose group with negative control. ^{&&&} $P < 0.001$ indicates comparison of standard with negative control.

Table:4 Effect of Poly Herbal Formulation on TBARS

Group	TBARS (nmol/mg protein)
Group I(Vehicle)	2.41±0.26
Group II (STZ)	5.25±0.24 ^{***}
Group III (Donepezil5mg/kg+STZ)	2.17±0.20 ^{&&&}
Group IV(LD 200mg/kg+ STZ)	3.53±0.32 ^{###}
Group V (HD 400mg/kg+ STZ)	2.55±0.21 ^{\$\$\$}

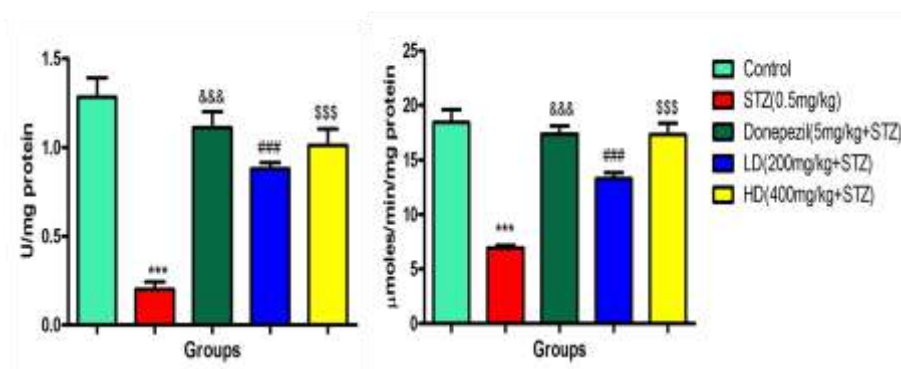
Values are expressed as mean ± SEM of n=6 animals. ^{***}*P* < 0.001 indicates comparison of negative control group with control group. ^{###}*P* < 0.001, ^{###}*P* < 0.001 indicates comparison of low dose group with negative control. ^{\$\$\$}*P* < 0.001, indicates comparison of high dose group with negative control. ^{&&&}*P* < 0.001 indicates comparison of standard with negative control.

Figure:1 Effect of PHF on AchE and Total Protein



Values are expressed as mean ± SEM of n=6 animals. ^{***}*P* < 0.001, indicates comparison of negative control group with control group. ^{##}*P* < 0.01, [#]*P* < 0.05 indicates comparison of low dose group with negative control. ^{\$\$\$}*P* < 0.001, indicates comparison of high dose group with negative control. ^{&&&}*P* < 0.001, ^{&&}*P* < 0.01 indicates comparison of standard with negative control.

Figure:2 Effect of PHF on SOD and Catalase



Values are expressed as mean ± SEM of n=6 animals. ^{***}*P* < 0.001 indicates comparison of negative control group with control group. ^{##}*P* < 0.01 indicates comparison of low dose group with negative control. ^{\$\$\$}*P* < 0.001, indicates comparison of high dose group with negative control. ^{&&&}*P* < 0.001 indicates comparison of standard with negative control.

DISCUSSION:

Neurochemically Alzheimer's disease (AD) is characterized by abnormalities in glucose metabolism, reduced glucose utilization and levels of energy rich phosphates like ATP, ADP etc (Arnaiz et al., 2007; Nitsch et al., 1991). Disturbed energy metabolism is intricately associated with increased oxidative stress that results in oxidation of biomolecules and initiates excitotoxic neuronal cell damage (Olanow et al., 1993; Raman et al., 2012). Numerous studies have provided clear evidence that memory function is highly dependent on the cholinergic system's functionality (Drachman et al., 1980). The degeneration of forebrain cholinergic projections has been demonstrated to be one of the most salient neurobiological and neuropathological features of AD (Coyle et al., 1983; Davies et al., 1976; Gibson et al., 1981; Wurtman et al., 1992), although alterations in other neurotransmitter systems may also contribute to memory function (Perry et al., 2001; Perry et al., 1986; Quirion et al., 1990).

The intracerebroventricular (i.c.v) Streptozotocin (STZ) mice model is an appropriate animal model used for study of AD type dementia (Lannert et al., 1998; Nitsch et al., 1991; Sharma et al., 2001). It has been shown to induce memory deficits along with increase in brain oxidative stress levels and brain Acetylcholinesterase (AChE) activity (Kaur et al., 2009). Acetylcholine (ACh) could be considered to be a neurotransmitter which is highly involved in learning and memory processes (Dunnett et al., 1990). AChE is a key enzyme responsible for the hydrolysis of ACh at the cholinergic nerve terminals. Selective vulnerability of cholinergic neurons with increased AChE, decreased Choline Acetyltransferase (ChAT) and decreased ACh levels are the neurochemical alterations associated with dementia of AD type (Sidharth et al., 2011). The cholinergic system appears to be interconnected with Nor-epinephrine, dopamine, serotonin, GABA, opioid peptides, substance P and angiotensin II. The interaction between the cholinergic system and the other neuromodulators could be essential for the formation of memory (Gasbarri et al., 1993; Levin et al., 1994; Riekkinen et al., 1993; Sahgal et al., 1993; Tanila et al., 1994; Decker et al., 1995). In the present study treatment with two different doses of Polyherbal formulation improved spatial recognition memory and exploratory behaviour as evident by improved performance in Morris water Maze, Y-Maze and Open field test.

In the current study PHF increased AChE activity in the brain of STZ treated mice was observed and the treatment with Polyherbal formulation significantly restored AChE activity. This effect might be influenced by its anti-cholinesterase activity from chemical constituents present in it. Oxidative stress contributes to increased neuronal damage which plays an important role in the advancement of aging and age related neurodegenerative disorders such as AD (Tourandokht et al., 2011; Badruzzaman et al., 2012). Oxidative stress occurs when the anti-oxidant defences are broken down by the overproduction of reactive oxygen species and the release of proinflammatory mediators. Expanding the antioxidant capacity of neurons will provide a potential strategy to protect

neurons from oxidative damage (Ahlenmeyer et al., 2001). Endogenous antioxidants such as reduced glutathione (GSH), catalase and superoxide dismutase (SOD) offer antioxidant resistance to prevent oxidative damage (Badruzzaman et al., 2012). ICV STZ treatment causes marked reduction in brain glucose/energy metabolism and shows a progressive trend towards oxidative stress. The evidences indicate that STZ treatment generates reactive oxygen species that result in increased oxidative stress (Sidharth et al., 2011). In the present study we demonstrated that PHF treatment significantly attenuated STZ induced oxidative stress by decreasing Thiobarbituric acid reactive species and by increasing endogenous antioxidant enzyme levels like GSH, Catalase and SOD in brain. The beneficial role might be attributed for its antioxidant property by polyphenolic rich fractions (Ellman et al., 1961) and free radical scavenging activity from its flavonoid constituents (Sanja et al., 2012). PHF in various studies have been reported to exert antioxidative and potential anti-neuroinflammatory actions and the role of inflammation in the Pathophysiology of AD is well documented. Therefore with support from the literature and data it may be proposed that extracts of polyherbal formulation mediated beneficial effect in STZ dementia may be attributed to its multiple effects including antioxidant, anticholinesterase and anti-neuroinflammatory actions.

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