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Original Article

Evaluation of In Vivo Anti Alzheimer's Activity of *Vigna radiata* and *Vigna pilosa* using Beta Amyloid Induced Neurotoxicity in Rats

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ABSTRACT

AD is the most common form of dementia. Extracellular senile (amyloid) plaques and intracellular neurofibrillary tangles (NFTs) are hallmarks of the disease. *Vigna radiata* and *Vigna pilosa* are plants used in many Ayurvedic formulations used in the management of dementia and related conditions. The present study was aimed to evaluate the neuroprotective effect of these plants on an amyloid-β (Aβ) 1-42 model of Alzheimer's disease in rats. Duration of the study was 21 days. After the recovery period post Aβ_{1-42} ICV administration, from the 8thday, the ethyl acetate extract of *Vigna radiata* and ethanolic extract of *Vigna pilosa* (200mg/kg, 400mg/kg), Donepezil (3mg/kg) treatments were made once daily p.o till the 21st day. Cognitive behavioural studies were conducted using radial maze test, Step-through Passive Avoidance Test. The animals were further subjected to euthanasia and the brain were collected and evaluated for antioxidant parameters and brain cytokine levels. The brain tissues were subjected to histopathological examination. The treatment with the extracts significantly improved the cognitive capability of the rats in the Radial arm maze task and step through passive avoidance test. It also reduced oxidative stress, which was evident by the lower levels of lipid peroxide and nitric oxide as well as elevated levels of antioxidant enzymes like catalase, superoxide dismutase and reduced glutathione. The treatment alleviated neuroinflammation in rats by decreasing the concentration of neuroinflammatory markers in a dose-dependent manner. From the results it can be concluded that the plants *Vigna radiata* and *Vigna pilosa* has beneficial effects in the improvement of cognitive impairment AD, by reducing oxidative stress and neuroinflammation.

Key Words: Alzheimer's disease, *Vigna radiata, Vigna pilosa,* amyloid-β .

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INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia. It is characterized by memory impairment and cognitive dysfunction. AD is mainly characterized by the deposition of amyloid β (Aβ) plaques and intracellular neurofibrillary tangles in the brain, along with neuronal degeneration and high levels of oxidative stress[1]. Current AD treatments do not stop or reverse the disease progression, highlighting the need for new and more effective therapeutics[2]. With the rapid increase of aging population in the recent years, senile dementia has become one of the most important public health problems in the world. Alzheimer's disease (AD), as the most common type of dementia, accounts for up to 70% of all cases of dementia[3]. Herbal medicines have their origins in ancient cultures, including those of the Egyptian, Indian and Chinese. It involves the use of medicinal plants to treat AD and enhances general health and wellbeing. In fact, many pharmaceutical drugs are based on the synthesized adaptations of naturally occurring compounds found in plants[4]. Ayurvedic medicine is a system of traditional medicine native to India, and Ayurvedic practitioners have developed a number of medicinal preparations and surgical procedures for the treatment of various ailments. The Physicians use medicinal plants and their constituents to strengthen the functional activity of the nervous system and for restoration of memory[5].

Vigna radiata and *Vigna pilosa* belonging to the family Fabaceae are routinely included in many ayurvedic formulations used to treat memory impairment and related conditions. This study aims to find out the possible *in vivo* anti Alzheimer's activity of these plants.

MATERIALS AND METHODS

Plant materials

The whole plants of *V. radiata* and *V.pilosa* belonging to the family Fabaceae were collected in December 2021 from Varam village, Kannur district, Kerala. The plants were authenticated by the Taxonomist of M/s. Arogya Medicare, a GMP-certified Ayurvedic drug manufacturing located in Kannur, Kerala. The voucher specimens of the plants are maintained at the herbarium (AHAMS 172 dated 21/12/2021 and AHAMS196 dated 21/12/2021). Morphological and molecular methods were employed to authenticate the plant.

Preparation of extract

The shade dried whole plants were ground and sieved through sieve number 22 and subjected to successive solvent extraction, using hexane, chloroform, ethyl acetate, ethanol and water.

Phytochemical Screening

The different extracts of the plants *V. radiata* and *V. pilosa* were subjected to preliminary phytochemical screening[6-7].

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Evaluation of anti-alzheimer's activity of the leaf extracts of in intracerebroventricular (ICV) injection of Amyloid-β model in rats (ICV-Amyloid-β Alzheimer's model)

Based on the acute toxicity studies reports and according to OECD 423 guidelines 200 mg/kg ($1/10^{th}$ of 2000 mg/kg) dose of leaf extracts of were selected for the pharmacological screening evaluation for Alzheimer's disease.

Animals

Wistar albino rats (175-200g, wt.) were obtained from Kerala Veterinary and Animal Science University, Mannuthy, Thrissur. They were maintained in controlled conditions at the animal house of AlShifa College of Pharmacy, Kizhattoor, Perinthalmanna, Kerala, India, with a temperature of 23° ± 2 C, a 12-hour light-dark cycle, and a relative humidity of 60 ± 5%. Animal customizations were done before the surgery. Experimental procedures were done in between 9:00 am and 5:00 pm. Food was withdrawn 12 to 18 hours prior to the surgical procedure. The Institutional Animal Ethics Committee (IAEC) of Al Shifa College of Pharmacy approved all experimental procedures (Approval no. IAEC 073/21), ensuring adherence to internationally accepted principles for laboratory animal research.

Preparation of $A\beta_{1-42}$ solution

Artificial cerebrospinal fluid (aCSF) which is widely employed as a vehicle for the administration of substances to the CNS of test animals. aCSF was prepared prior to the surgery. The composition of aCSF: 2.9 mM KCl:147mM NaCl; 1.6 mM $MgCl_2$; 2.2 mM dextrose; and1.7 mM $CaCl_2$. Aβ1–42 peptide was dissolved in the prepared aCSF to get a concentration of 5 mg/ml and it was further incubated at 37°C for 72 h to increase aggregation. 2.0µl (5µg/µl) was given to each animal on each site of bregma through ICV injection with the help of stereotaxic apparatus.

Preparation of plant extracts

Ethyl acetate extract of *Vigna radiata* and ethanolic extract of *Vigna pilosa* were weighed equivalent to make the dose 100 mg/kg and 200 mg/kg and were then reconstituted in the 0.3% Carboxy Methyl Cellulose (CMC) for oral administration.

Experimental Design

Groups and treatments

The rats were divided into 7 groups (Table 1) and each group consisted of 6 animals. All the animals were subjected to behavioural studies.

Study design

The study was conducted in experimental animals for a total of 21 days. The day of $A\beta_{1-42}$ infusion was considered as 0^{th} day. A week after preliminary $A\beta_{1-42}$ ICV infusion, the animals were subjected to a week of recovery. After this, from the 8^{th} day, the extract treatments were carried out once daily to the respective groups and the same was

Table 1: Groups and treatments.

Groups	Treatments	No. of animals
I	Sham operated (SO) (ICV-2µl aCSF/site)	6
II	Amyloid-β ($A\beta_{1-42}$) (ICV- 10µg/2µl aCSF/site	6
III	$A\beta_{1-42+}$ DPZ (3mg/kg)	6
IV	$A\beta_{1-42}$ + ETAC-VR(100mg/kg)	6
V	$A\beta_{1-42+}$ ETAC-VR(200mg/kg)	6
VI	$A\beta_{1-42}$ + EtOH-VP(100mg/kg)	6
VII	$A\beta_{1-42+}$ EtOH-VP(200mg/kg)	6

Table 2: Effect of ethyl acetate extracts of *Vigna radiata* (ETAC-VR) & *Vigna pilosa* (EtOH-VP) on Reference Memory Error (RME) of Radial Arm Maze (RAM) test in ICV-Aβ rat model of Alzheimer's disease.

S. No	Groups	RME Score
1	SO	1.43±0.42
2	Aß(1-42)	5.77±0.29***
3	DPZ (3mg/kg)	2.41±0.52###
4	ETAC-VR(100mg/kg)	3.75±0.41#
5	ETAC-VR(200mg/kg)	2.97 ±0.38##
6	EtOH-VP(100mg/kg)	3.10 ±0.44##
7	EtOH-VP(200mg/kg)	2.31±0.63###

Values are represented as Mean ±SEM. Superscript***denotes p<0.001; **denotes p<0.01; denotes p<0.05 vs SO group and ### denotes p<0.001; ## denotes p<0.01; # denotes p<0.05 vs Aβ1-42 treated groups respectively. (SO-Sham Operated, Aβ-Amyloidbeta, DPZ-Donepezil)

Table 3: Effect of ethyl acetate extracts of *Vigna radiata* (ETAC-VR) & *Vigna pilosa* (EtOH-VP) on Correct Working Memory Error (CWME) of Radial Arm Maze (RAM) test in ICV-Aβ rat model of Alzheimer's disease Correct Working Memory Error (CWME).

S. No	Groups	CWMEScore
1	SO	1.67±0.53
2	Aß(1-42)	6.23 ±0.31***
3	DPZ (3mg/kg)	2.89±0.47###
4	ETAC-VR (100mg/kg)	3.97±0.53#
5	ETAC-VR (200mg/kg)	3.15 ±0.27##
6	EtOH-VP (100mg/kg)	3.37 ±0.21##
7	EtOH-VP (200mg/kg)	2.55±0.87###

Values are represented as Mean ± SEM. Superscript***denotes p<0.001; **denotes p<0.01; denotes p<0.05 vs SO group and ### denotes p<0.001; ## denotes p<0.01; # denotes p<0.05 vs Aβ1-42 treated groups respectively. (SO-Sham Operated, Aβ-Amyloid beta, DPZ-Donepezil)

Table 4: Effect of ethyl acetate extracts of *Vigna radiata* (ETAC-VR) & *Vigna pilosa* (EtOH-VP) on Incorrect Working Memory Error (IWME) of Radial Arm Maze (RAM) test in ICV-Aβ rat model of Alzheimer's disease.

S. No	Groups	IWME Score
1	SO	2.27±0.73
2	Aß(1-42)	7.21 ±0.42***
3	DPZ (3mg/kg)	3.15±0.52###
4	ETAC-VR (100mg/kg)	6.28±0.77
5	ETAC-VR (200mg/kg)	4.27 ±0.31##
6	EtOH-VP (100mg/kg)	6.72±0.53
7	EtOH-VP (200mg/kg)	4.25 ±0.17##

Values are represented as Mean ± SEM. Superscript *** denotes p<0.001; ** denotes p<0.01; * denotes p<0.05 vs SO group and ### denotes p<0.001; ## denotes p<0.01; # denotes p<0.05 vs Aβ1-42 treated group respectively (SO-Sham Operated, Aβ-Amyloid beta, DPZ-Donepezil)

Table 5: Effect of ethyl acetate extracts of *Vigna radiate* (ETAC-VR) & *Vigna pilosa* (EtOH-VP) on initial latency (IL) period of Passive Avoidance Test (PAT) test in ICV-Aβ rat model of Alzheimer's disease.

S. No	Groups	Initial Latency(Sec)
1	SO	29.56±2.45
2	Aß(1-42)	52.31±1.77***
3	DPZ (3mg/kg)	34.15±1.97###
4	ETAC-VR(100mg/kg)	42.83±1.59#
5	ETAC-VR(200mg/kg)	35.35±2.14###
6	EtOH-VP(100mg/kg)	40.56±1.58##
7	EtOH-VP(200mg/kg)	36.18±2.17###

Table 6: Effect of ethyl acetate extracts of *Vigna radiata* (ETAC-VR) & *Vigna pilosa* (EtOH-VP) on step through latency (STL) period of Passive Avoidance Test (PAT) test in ICV-Aβ rat model of Alzheimer's disease.

S. No	Groups	Step-through Latency(Sec)
1	SO	211.78±11.28
2	Aß(1-42)	133.54±9.87***
3	DPZ (3mg/kg)	198.56±12.83##
4	ETAC-VR(100mg/kg)	153.47±11.11
5	ETAC-VR(200mg/kg)	185.23±8.69#
6	EtOH-VP(100mg/kg)	147.89±13.57
7	EtOH-VP(200mg/kg)	189.56 ±12.31#

Values are represented as Mean ± SEM. Superscript *** denotes p<0.001; ** denotes p<0.01; *denotes p<0.05 vs SO group and ### denotes p<0.001; ## denotes p<0.01; # denotes p<0.05 vs Aβ1-42 treated group respectively. (SO-Sham Operated, Aβ-Amyloid beta, DPZ-Donepezil)

Table 6: Effect of ethyl acetate extracts of *Vigna radiata* (ETAC-VR) & *Vigna pilosa* (EtOH-VP) on step through latency (STL) period of Passive Avoidance Test (PAT) test in ICV-Aβ rat model of Alzheimer's disease.

S. No	Groups	Malondialdehyde(uMofMDA/gtissue)
1	SO	110.87±9.65
2	Aß(1-42)	412.33±17.86***
3	DPZ (3mg/kg)	268.98±8.67###
4	ETAC-VR(100mg/kg)	253.21±14.81###
5	ETAC-VR(200mg/kg)	189.26±13.58###
6	EtOH-VP(100mg/kg)	222.14±10.89###
7	EtOH-VP(200mg/kg)	154.78±11.58###

Values are represented as Mean ± SEM. Superscript *** denotes p<0.001; ** denotes p<0.01; *denotes p<0.05 vs SO group and ### denotes p<0.001; ## denotes p<0.01; # denotes p<0.05 vs Aβ1-42 treated group respectively. (SO-Sham Operated, Aβ-Amyloid beta, DPZ-Donepezil)

Table 7: Effect of ethyl acetate extracts of *Vigna radiata* (ETAC-VR) & *Vigna pilosa* (EtOH-VP) on brain Lipid Peroxide (LPO) level in ICV-Aβ rat model of Alzheimer's disease.

S. No	Groups	Malondialdehyde(uMofMDA/gtissue)
1	SO	110.87±9.65
2	Aß(1-42)	412.33±17.86***
3	DPZ (3mg/kg)	268.98±8.67###
4	ETAC-VR(100mg/kg)	253.21±14.81###
5	ETAC-VR(200mg/kg)	189.26±13.58###
6	EtOH-VP(100mg/kg)	222.14±10.89###
7	EtOH-VP(200mg/kg)	154.78±11.58###

Values are represented as Mean ±SEM. Superscript *** denotes p<0.001; ** denotes p<0.01; * denotes p<0.05 vs SO group and### denotes p<0.001; ## denotes p<0.01; # denotes p<0.05 vs Aβ1-42 treated group respectively. (SO-Sham Operated, Aβ-Amyloid beta, DPZ-Donepezil)

Table 8: Effect of ethyl acetate extracts of *Vigna radiata* (ETAC-VR) & *Vigna pilosa* (EtOH-VP) on brain Nitric Oxide (NO) level in ICV-Aβ rat model of Alzheimer's disease.

S. No	Groups	Nitrate/ Nitrite(uM/gtissue)
1	SO	25.63 ±1.87
2	Aß(1-42)	85.56±2.23***
3	DPZ (3mg/kg)	46.87±1.93###
4	ETAC-VR(100mg/kg)	67.26±1.87##
5	ETAC-VR(200mg/kg)	37.28±2.71###
6	EtOH-VP(100mg/kg)	62.57±1.56##
7	EtOH-VP(200mg/kg)	29.17±2.11###

Values are represented as Mean ± SEM. Superscript *** denotes p<0.001; ** denotes p<0.01; * denotes p<0.05 vs SO group and ###denotes p<0.001; ## denotes p<0.01; # denotes p<0.05 vs Aβ1-42 treated group respectively. (SO-Sham Operated, Aβ-Amyloid beta, DPZ-Donepezil)

Table 9: Effect of ethylacetate extracts of *Vigna radiata* (ETAC-VR) & *Vigna pilosa* (EtOH-VP) on brain Superoxide dismutase (SOD) level in ICV-Aβ rat model of Alzheimer's disease.

S. No	Groups	SOD(U/gtissue)
1	SO	12.81 ±1.12
2	Aß(1-42)	2.57 ±0.87***
3	DPZ (3mg/kg)	7.89 ±0.73##
4	ETAC-VR(100mg/kg)	6.63±0.77#
5	ETAC-VR(200mg/kg)	8.75±0.53###
6	EtOH-VP(100mg/kg)	7.12±0.93#
7	EtOH-VP(200mg/kg)	9.67±1.13###

Values are represented as Mean ± SEM. Superscript *** denotes p<0.001; ** denotes p<0.01; * denotes p<0.05 vs SOgroup and ### denotes p<0.001; ## denotes p<0.01; # denotes p<0.05 vs Aβ1-42 treated group respectively.(SO-Sham Operated, Aβ-Amyloid beta, DPZ-Donepezil)

Table 10: Effect of ethyl acetate extracts of *Vigna radiata* (ETAC-VR) & *Vigna pilosa* (EtOH-VP) on brain Catalase (CAT) level in ICV-Aβ rat model of Alzheimer's disease.

S. No	Groups	Catalase(U/gtissue)
1	SO	8.98±0.79
2	Aß(1-42)	1.98 ±0.59***
3	DPZ (3mg/kg)	5.57 ±0.21##
4	ETAC-VR (100mg/kg)	5.75 ±0.58##
5	ETAC-VR (200mg/kg)	6.83±0.67###
6	EtOH-VP (100mg/kg)	5.12±0.77#
7	EtOH-VP (200mg/kg)	7.15±0.69###

Values are represented as Mean ± SEM. Superscript *** denotes p<0.001; ** denotes p<0.01; * denotes p<0.05 vs SO group and ###denotes p<0.001; ## denotes p<0.01; # denotes p<0.05 vs Aβ1-42 treated group respectively. (SO-Sham Operated, Aβ-Amyloid beta, DPZ-Donepezil)

Table 11: Effect of ethyl acetate extracts of *Vigna radiata* (ETAC-VR) & *Vigna pilosa* (EtOH-VP) on brain Glutathione (GSH) level in ICV-Aβ rat model of Alzheimer's disease.

S. No	Groups	GSH(U/gtissue)
1	SO	25.33 ±1.17
2	Aß(1-42)	4.47±0.97***
3	DPZ (3mg/kg)	15.45±1.13###
4	ETAC-VR (100mg/kg)	14.22±1.41###
5	ETAC-VR (200mg/kg)	18.73±1.77###
6	EtOH-VP (100mg/kg)	16.14±1.69###
7	EtOH-VP (200mg/kg)	19.87±1.83###

Values are represented as Mean ± SEM. Superscript *** denotes p<0.001; ** denotes p<0.01; * denotes p<0.05 vs SO group and ###denotes p<0.001; ## denotes p<0.01; # denotes p<0.05 vs Aβ1-42 treated group respectively. (SO-Sham Operated,Aβ-Amyloid beta, DPZ-Donepezil)

Table 12: Effect of ethyl acetate extracts of *Vigna radiata* (ETAC-VR) & *Vigna pilosa* (EtOH-VP) on brain interleukin-1β (IL-1β) level in ICV-Aβ rat model of Alzheimer's disease.

S. No	Groups	IL-1β(pg/ml)
1	SO	123.47±13.18
2	Aß(1-42)	377.25±12.58***
3	DPZ (3mg/kg)	305.17±14.15##
4	ETAC-VR (100mg/kg)	299.17±13.89##
5	ETAC-VR (200mg/kg)	197.85±13.33###
6	EtOH-VP (100mg/kg)	347.17±12.89
7	EtOH-VP (200mg/kg)	166.87±12.67###

Values are represented as Mean ±SEM. Superscript *** denotes p<0.001; ** denotes p<0.01; * denotes p<0.05 vs SO group and ### denotes p<0.001; ## denotes p<0.01; # denotes p<0.05 vs Aβ1-42 treated group respectively (SO-Sham Operated,Aβ-Amyloid beta, DPZ-Donepezil)

Table 13: Effect of ethyl acetate extracts of *Vigna radiata* (ETAC-VR) & *Vigna pilosa* (EtOH-VP) on brain tumor necrosis factor alpha (TNF-α) level in ICV-Aβ rat model of Alzheimer's disease.

S. No	Groups	TNF-α(pg/ml)
1	SO	175.23±11.58
2	Aß(1-42)	456.71±17.87***
3	DPZ (3mg/kg)	371.83±13.37##
4	ETAC-VR (100mg/kg)	405.87±14.89
5	ETAC-VR (200mg/kg)	296.53±11.72###
6	EtOH-VP (100mg/kg)	399.17 ±18.13
7	EtOH-VP (200mg/kg)	275.81±10.16###

Values are represented as Mean ± SEM. Superscript *** denotes p<0.001; ** denotes p<0.01; * denotes p<0.05 vs SO group and ### denotes p<0.001; ## denotes p<0.01; # denotes p<0.05 vs Aβ1-42 treated group respectively.(SO-Sham Operated, Aβ-Amyloid beta, DPZ-Donepezil)

continued (every 24hrs) till the 21st day. Then, three days (15-17th day), the experimental animals were subjected to a habituation trail for radial arm maze (RAM) test and 18-19th day, the animals were given with habituation trail of step through avoidance test (PAT). After trail, the animals were subjected to PAT test to measure initial latency (IL) on 20th day and step through latency (STL) on 21st day respectively. After PAT test, on 21st day, the animals were evaluated for RAM test. After behavioural studies, the animals were scarified and the brain samples were harvested and the hippocampus region was micro-dissected for neurobiochemicals, cytokines, neurotransmitters estimation and histopathological evaluation.

Experimental Induction of Alzheimer's disease (ICV-$A\beta_{1-42}$) model

Anesthesia was induced in rats with ketamine (80mg/kg) and xylazine (10mg/kg) administration. Following the induction of anesthesia Intra Cerebro Ventricular (ICV) administration of $A\beta_{1-42}$ was done with the help of chemotaxic apparatus. The scalp of the rats were incised and the position of the head was adjusted in order to keep the bregma and lambda in the same horizontal plane. After that the skull was opened and burr holes were drilled for the Aβ1–42 intra-hippocampal injections (anteroposterior: -3.8 mm from Bregma, medial/lateral: ±2.2 mm and dorsal/ventral: -2.7 mm). Aβ1–42 (2.0 µl per side) was injected bilaterally into the lateral ventricles through a stainless-steel cannula using a Hamilton micro syringe. The same volume of normal saline was injected into the SHAM rats. The duration of injection was 5 minutes. To ensure full infusion, the syringe was left in place for 2 minutes. To recover, the rats were left for 7 days post-surgery with daily application of antibiotics (mupirocin). After that the rats were subjected to behavioral evaluation and estimation of neuro biochemicals[8-9].

Cognitive Behavioural studies

Radial Arm Maze Task

To analyse the working memory and reference memory of rats, Radial arm maze was used. The radial arm maze has eight arms of 4 feet long, connected to a circular central platform. This maze was elevated to 90cm from floor. In case of trail period each arm was allotted for food reward and the pattern of baited and un-baited arms stayed same throughout test sessions. At the edge of each arm a food container was kept with food pellets as a reward for the rat and at the end of the arm a protection the goal box. Rails (2.5 cm high) were made for preventing the animal from falling.

Animals were trained to become accustomed to the RAM before the measurement of the behaviour was conducted. The animals were kept in fasting for one night to boost their appetite. For three days, the animals were maintained in the maze for fifteen minutes daily. The animal had eight days of habituation training before entering all eight arms or a ten-minute period. The animal was permitted to stay in the goal box for a minute. Errors of reference memory were denoted as entry numbers of into an un-baited arm. When the rat just pushed its head through the wrong opening and it remained there for longer than a minute, it was promptly replaced at the maze's centre. Each arm was washed with 70% ethanol to remove any prior animal traces (faces and urine). The latency to find the goal box was used to gauge overall behavioural performance during the retention and acquisition phases. The recorded metrics are detailed below[10].

Errors correct working memory is denoted number of baited arm re-entries and Errors of incorrect working memory are denoted as number un-baited arm re-entries.

Step-through Passive Avoidance Test

Passive avoidance test was used to gauge retention levels after the radial arm maze task was completed for two to three days. The apparatus's design resembled a chamber with a guillotine door and lighting. A stimulator delivered an electric shock to the grid's floor. The animal was placed inside the room by opening the door (guillotine) after the two-minute familiarisation period. Animals that displayed IL for more than 60 seconds were not included in the investigation. These rats were re-inserted into the lit room and the retention trail was run after one day. Step-through latency was determined for the positioning of the irradiation chamber and the entrance to the dark chamber. The step through latency cut off was set at a maximum of 60s[11].

Neurobiochemicals Evaluation

Anti-oxidant parameters

Brain Isolation

The rats were euthanized after the behavioural evaluations were completed and this was followed by a fast separation of the brain samples, the hippocampus region was carefully dissected, washed with cold saline, and stored at -8°C pending further analysis. The brain samples were homogenised using 10% ice-cold KCl (100 l of KCl for every 10 mg of tissue) for anti-oxidant parameter analysis[12].

Lipid peroxide (LPO)

With a few minor adjustments from the thiobarbituric acid (TBA) test established by Ohkawa et al. (1979), lipid peroxidation was assessed by measuring the TBAR concentration. The incubation mixture includes 0.5 ml of aliquot, 0.2 ml of sodium dodecyl sulphate solution at 8%, 1.5 ml of thiobarbituric acid aqueous solution at 0.9%, and double distilled water bath for 30 minutes. The red chromogen was extracted into 5 ml of n-butanol and pyridine (15.1 v/v) after cooling, and the mixture was centrifuged at 4000 rpm for 10 min. The organic layer's absorbance was measured at 532 nm (UV, Shimadzu, Japan). As an external standard, 1,1,3,3-tetra ethoxy propane was utilised at concentrations between 80 and 240 nmol[13].

Nitric oxide (NO)

Nitric oxide (NO) was measured indirectly as nitrate and nitrite by taking 0.2 ml of 10% homogenate and then adding 1.8 ml of saline and 0.4 ml of 35% sulfosalicylic acid to the protein precipitation. Centrifuge at 4000 rpm for 10 min to remove precipitate. Take a 1 ml aliquot of the supernatant and add 2 ml Griess reagent (dissolve 1 g sulfonamide in a small amount of water, add 2 ml orthophosphoric acid and 100 mg naphthylethylenediamine and make the volume to 100 ml). The mixture was left in the dark for 20 minutes. Color intensity was read at 540 nm (UV, Shimadzu, Japan). Standard calibration drawn using sodium nitrite in the concentration range of 200-1000 ng[14].

Superoxide dismutase (SOD)

Add 0.3ml sodium pyrophosphate buffer (0.025M, pH 8.3) to 0.05ml homogenate. To this mixture, 0.025 ml and 0.075 ml of PMS (186 µM) and NBT (300 µM buffer, pH 8.3) were added. Start the reaction by adding 0.075ml NADH. The mixture is then incubated at 30°C for 90 seconds. 0.25 ml of glacial acetic acid was added to stop the reaction continuously. n-Butanol (2 ml) was shaken frequently with the reaction mixture; The mixture is then centrifuged at 4000 rpm for 1 minute. Colorimetric analysis using a spectrophotometer at 560 nm with n-butanol (1.5 ml) as blank[15].

Catalase (CAT)

A small amount of brain homogenate (100 µl) or sucrose (0.32 M) was prepared with 65 mM potassium phosphate buffer, pH 7.8 (2.25 ml) for 30 min at 25 °C. The reaction was initiated by the addition of hydrogen peroxide (7.5 mM; 650 µl). Absorbance change measured at 240 nm over 2 to 3 min (UV, Shimadzu, Japan) [16].

Reduced glutathione (GSH)

GSH content was estimated according to the method of Jollow et al. (1974). Add 0.25 ml of brain homogenate to an equal volume of ice-cold 5% TCA. Centrifuge at 4000 rpm for 10 min to remove precipitate. Add 0.25 ml of 0.2 M phosphate buffer, pH 8.0, and 0.5 ml of DTNB (0.6 mM in 0.2 M phosphate buffer, pH 8.0) to a 1 ml portion of the supernatant and mix well. Read absorbance at 412 nm using a spectrophotometer[17].

Inflammatory cytokines estimation

The hippocampal region of the brain sampled was resuspended in buffer containing 0.1% BSA, 81 mM NaHPO, 19 mM NaHPO, 50 mM NaCl and 0.1% Triton to measure inflammatory cytokines. α level was measured using an ELISA kit (Quantikine and Invitrogen, USA) according to the manufacturer's instructions[12].

Neurotransmitter – Glutamate estimation

Hippocampus was homogenized in 0.1 N HCl in 80% ethanol (200 µl ethanol per 10 mg brain), transferred to a polypropylene tube, and centrifuged 20 times at 4500 rpm. For 20 minutes with the temperature maintained at 25°C. The resulting supernatant is transferred to a microcentrifuge tube for glutamate estimation. Glutamic acid content was determined by HPTLC (CAMAG - version 1.3.4, USA). Chromatographic conditions: silica gel GF254 as stationary phase, n-butanol: glacial acetic acid: water as mobile phase (65:15:25 v/v); Applicator: Linomat V; Scanner: CAMAG TLC Scanner III; Growth chamber: Double-well glass chamber (20×10); Development mode: Escalation mode (multi-development); Detection reagent: 0.2% ninhydrin acetone solution; Scanning Wavelength 486 nm; Test operation: 25 ± 2 °C room temperature, 55-65% relative humidity. Preparation of L-glutamic acid standard solution (20-200 ng/point) for use in drawing calibration curves[12].

Histopathology - Hematoxylin and Eosin staining

Brain samples were fixed by placing them in formalin and soaked in alcohol to remove lipid residues. These samples were then fixed in paraffin and 5µm coronal sections were obtained from the CA1 hippocampal region of the brain. The sections were processed and stained with hematoxylin and eosin (H&E). After drying the residue using ethanol, the slices were then examined under a microscope under a 40X objective lens, and images of the CA1 hippocampus region of the brain were taken to understand the effects of the drug[18].

Statistical analysis

The values were expressed as the Mean ± SEM. All the data were statistically analyzed by using Graphpad Prism 6.0 software. Statistical significance was determined by One Way ANOVA followed by Tukey's multiple comparison tests to assess statistical differences between the groups. Values were considered statistically significant if $P<0.05$.

RESULTS

Based on the acute toxicity study reports, 200 mg/kg (1/10th of 2000 mg/kg) and 100 mg/kg (1/20th of 2000 mg/kg) does of ethyl acetate extracts of *Vigna radiata* (ETAC-VR) & *Vigna pilosa* (EtOH-VP) respectively were selected for the pharmacological evaluation for Alzheimer's disease in ICV-Aβ rat model.

Cognitive Behavioural Studies

Radial Arm MazeTest (RAM)

a. Reference Memory Error(RME)

Intracerebroventricular administration of Aβ1-42 in rats has significantly increased ($p<0.001$) the RME score in comparison to SO rats. Treatment with DPZ (3 mg/kg) ($p<0.001$), ETAC-VR (100mg/kg) ($p<0.05$), ETAC-VR (200 mg/kg) ($p<0.01$), EtOH-VP (100 mg/kg) ($p<0.01$) and EtOH-VP (200 mg/kg) ($p<0.001$) have notably decreased the RME score in cognitive declined rats indicates the cognitive enhancement potential of above extract treatments.

b. Correct Working Memory Error (CWME)

In comparison to SO group, the Aβ1-42 administered group ($p<0.001$) has shown significant increase in CWME score indicating the loss of cognitive function upon ICV administration of Aβ1-42 in rats. Treatment with DPZ (3 mg/kg) ($p<0.001$), ETAC-VR (100 mg/kg) ($p<0.05$), ETAC-VR (200 mg/kg) ($p<0.01$), EtOH-VP (100 mg/kg) ($p<0.01$) and EtOH-VP (200 mg/kg) ($p<0.001$) have remarkably decreased the CWME score in Aβ1-42 administered rats providing evidence of the cognitive enhancement activity of above extracts in Alzheimer's like conditions.

c. Incorrect Working Memory Error (IWME)

ICV injection of Amyloid-β has significantly ($p<0.001$) increased the IWME score in comparison to SO vehicle treated rats. Administration of DPZ (3 mg/kg) ($p<0.001$), ETAC-VR (200mg/kg) ($p<0.01$) and EtOH-VP (200mg/kg) ($p<0.01$) in Amyloid-β infused rats have shown considerable decline in IWME score in RAM tests indicating the cognitive enhancement potential of above extracts.

Step through Passive Avoidance Test

d. Initial latency

When compared to SO group, the Aβ1-42 administered group ($p<0.001$) has shown significant increase in IL period which denotes the declining of memory function after ICV administration of Aβ1-42 in rats. Treatment with DPZ (3 mg/kg) ($p<0.001$), ETAC-VR (100 mg/kg) ($p<0.05$), ETAC-VR (200 mg/kg) ($p<0.001$), EtOH-VP (100 mg/kg) ($p<0.01$) and EtOH-VP (200 mg/kg) ($p<0.001$) have notably reduced the IL period in Aβ1-42 infused rats evidences the cognitive enhancement activity of above extracts in Alzheimer's like pathology.

e. Step through latency

ICV administration of Aβ1-42 in rats has significantly reduced ($p<0.001$) the STL period in comparison to SO vehicle treated rats. Treatment with DPZ (3 mg/kg) ($p<0.01$), ETAC-VR (200mg/kg) ($p<0.05$) and EtOH-VP (200 mg/kg) ($p<0.05$) have significantly reduced the STL period in comparison to Aβ1-42 infused rats providing evidence of the

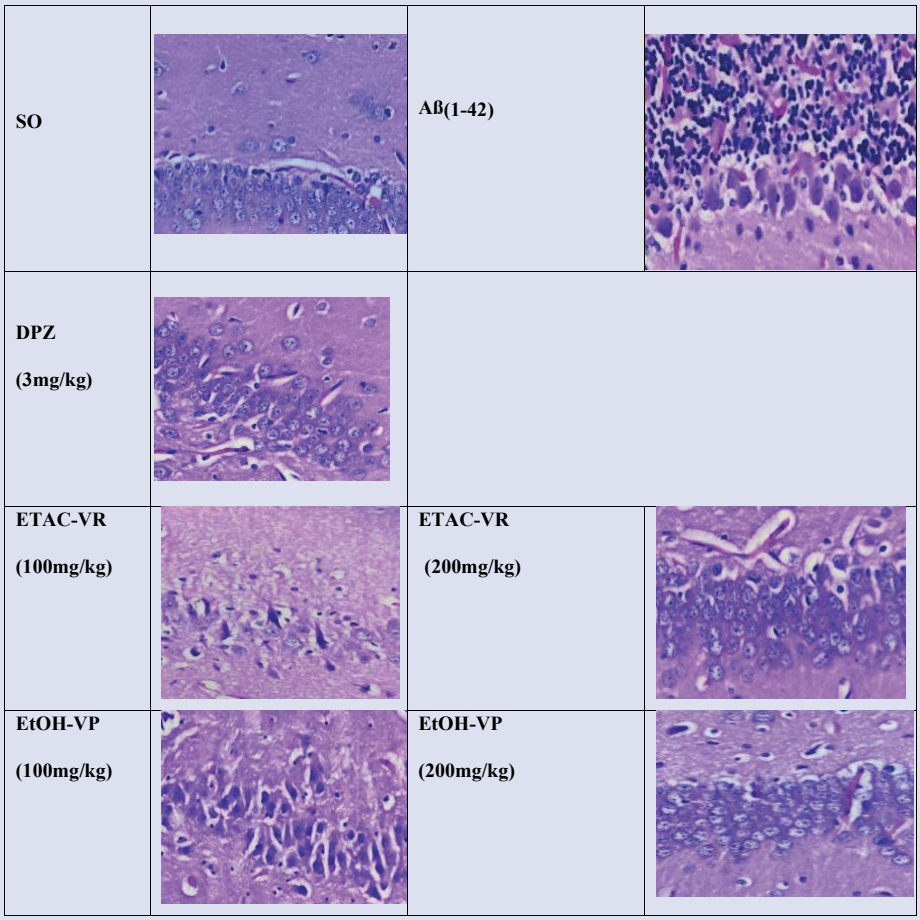

Figure 1: Sections of hippocampus region of brain stained with Hematoxylin and Eosin.

memory enhancement activity of above extract treatment in cognitive declined rats.

Neuro-biochemicals Estimation

a. Lipid Peroxide Level(LPO)

Central administration of Aβ1-42 in rats has remarkably increased (p<0.001) the LPO level in thebrain when compared to SO vehicle treated rats. Administration of DPZ (3 mg/kg) (p<0.001), ETAC-VR (100mg/kg) (p<0.001), ETAC-VR (200mg/kg) (p<0.001), EtOH-VP (100mg/kg) (p<0.001) and EtOH-VP (200mg/kg) (p<0.001) have significantly reduced the brain LPO level in comparison to Aβ1-42 infused rats indicating the anti-oxidant potential of above extracts in neurodegenerative conditions like Alzheimer's disease.

b. Nitric Oxide(NO)

ICV injection of Aβ1-42in rats has significantly elevated (p<0.001) the NO level in the brain incomparison to SO group. Treatment with DPZ (3 mg/kg) (p<0.001), ETAC-VR (100 mg/kg) (p<0.01), ETAC-VR (200mg/kg) (p<0.001), EtOH-VP (100mg/kg) (p<0.01) and EtOH-VP

(200 mg/kg) (p<0.001) in Aβ1-42 infused rats have shown significant reduction in brain NO level representing the anti-oxidant activity of above extracts in Alzheimer's like conditions.

c. Superoxide dismutase (SOD)

In comparison to SO rats, the Aβ1-42 administration in rats has remarkably decreased (p<0.001) the brain SOD level. Treatment with DPZ (3 mg/kg) (p<0.01), ETAC-VR (100 mg/kg) (p<0.05), ETAC-VR (200 mg/kg) (p<0.001), EtOH-VP (100 mg/kg) (p<0.05) and EtOH-VP (200 mg/kg) (p<0.001) have significantly increased the brain SOD level in comparison to Aβ1-42 infused rats indicating the restoration of anti-oxidant enzymes level after extracts treatment.

c. Catalase (CAT)

Aβ1-42 administration through ICV route has drastically attenuated (p<0.001) the brain CAT levelin comparison to SO group. Treatment with DPZ (3 mg/kg) (p<0.01), ETAC-VR (100 mg/kg) (p<0.01), ETAC-VR (200mg/kg) (p<0.001), EtOH-VP (100mg/kg) (p<0.05) and EtOH-VP (200 mg/kg) (p<0.001) have remarkably elevated the CAT level in the brain of Aβ1-42 infused rats indicating the radical scavenging activity and anti-oxidant enzymes restoration capacity of the above extracts in Alzheimer's like neurodegenerative conditions.

e. Reduced Glutathione (GSH)

In comparison to SO rats, the Aβ1-42administration in rats has remarkably decreased (p<0.001) the brain GSH level. Treatment with DPZ(3mg/kg) (p<0.001), ETAC- R (100mg/kg) (p<0.001), ETAC-VR(200mg/kg) (p<0.001), EtOH-VP (100mg/kg) (p<0.001) and EtOH-VP

(200 mg/kg) (p<0.001) have significantly increased the brain GSH level in comparison to Aβ1-42 infused rats indicating the restoration of anti-oxidant enzymes level after extracts treatment.

Brain Cytokines

a. IL-1β

Central Aβ1-42 administration through ICV route has significantly elevated (p<0.001) the brain IL-1β level in comparison to SO group indicates the intensity of neuroinflammation occurred upon Aβ1-42

ICV infusion. Treatment with DPZ (3 mg/kg) ($p<0.01$), ETAC-VR (100 mg/kg) ($p<0.01$), ETAC-VR (200 mg/kg) ($p<0.001$) and EtOH-VP (200 mg/kg) ($p<0.001$) have notably attenuated the IL-1β level in the brain of Aβ1-42 infused rats indicating the anti-neuroinflammatory potential of the above extracts in Alzheimer's like neurodegenerative conditions.

b. TNF-α

When compared to SO group, the ICV-Aβ1-42 administered rats have shown remarkable elevation in ($p<0.001$) the brain TNF-α level denotes the neuroinflammation progression after Aβ1-42 ICVinfusion. Administration of DPZ (3 mg/kg) ($p<0.01$), ETAC-VR (200 mg/kg) ($p<0.001$) and EtOH-VP (200 mg/kg) ($p<0.001$) have drastically attenuated the brain TNF-α level in the Aβ1-42 treated rats representing the anti-neuroinflammatory activity of above extracts in neurodegenerative conditions.

Histopathology-Hematoxylin and Eosin staining

The histopathology staining with Hematoxylin and Eosin in hippocampus region evidences that the neuronal structures of SO group residue are very intact and the neurons and organelles were present well without damage. The nuclei of neuronal cells were stained clearly and kept centered without injury. ICV injection of Aβ1-42 has caused significant neuronal morphology changes and the neurons were noticeably degenerated and became necrotic, swelled and their arrangement was messy. Treatment with DPZ (3 mg/kg), ETAC-VR (200 mg/kg) and EtOH-VP (200 mg/kg) has shown some degree of restoration of neuronal morphology from Aβ1-42 induced neurodegeneration and reduced the neuronal damage with decreased necrotic, swelling and neuronal degeneration with restored intact cells which are comparable with SO group. Treatment with ETAC-VR (100mg/kg) and EtOH-VP (100mg/kg) have shown partial protection of neurons with considerable morphological restoration in Hematoxylin and Eosin staining in Aβ1-42 infused rats.

DISCUSSION

Dementia is increasing globally due to various reasons and focus has been given to the prevention of cognitive impairment that leads to dementia[19]. The primary constituent of amyloid plaques is Aβ[20]. Aβ deposition further leads to many other major pathological modifications including NFT formation as well as neuronal[21]; alteration in the breakdown of amyloid precursor protein (APP) leading to the pathogenesis. APP is an integral protein of the plasma membrane. Its altered cleavage is caused by β-secretases (BACE1) and γ-secretases to produce insoluble Aβ fibrils. The Aβ further oligomerizes, and diffuses into synaptic clefts and alters the synaptic signaling. Further, it undergoes polymerization and gets converted to insoluble amyloid fibrils that aggregate into plaques[22].

It is evident that Aβ has a role in the pathogenesis and progression of AD. So the Aβ-injected rat model is regarded as the most dependable model for understanding the pathogenesis of AD[23].

Phytoconstituents from medicinal plants have great potential for the development of drugs for the effective management of neurological disorders including AD[24]. Various plant extracts and bioactive molecules with antioxidant property show protective effects against Aβ (1-42)-induced neurotoxicity.

Vigna radiata and *Vigna pilosa* are plants belonging to the family Fabaceae, routinely included in many Ayurvedic formulations used in the treatment of memory impairment and related complications.

The present study was aimed to evaluate the anti alzheimer's activity of ethyl acetate extract of *Vigna radiata* and ethanolic extract of *Vigna pilosa* against Aβ (1-42)-induced neurotoxicity in rats. The in-vitro acetyl choline esterase inhibitory action and in vitro neuroprotective activity against Aβ (1-42) induced neurotoxicity in SHY-5 neuroblastoma cell line had already been reported[25].

From the results it was evident that the cognitive impairment induced by the Aβ (1-42) as the Reference Memory Error and Correct Working Memory Error in the radial arm test was significantly reduced in the extract-treated groups.

In the Passive avoidance test, the Initial Latency and Step through latency which are indicators of cognitive impairment and resulted due to the Aβ (1-42) infusion was significantly reduced in the standard treated groups and the extract treated groups. This shows the protective effects of these plants against the neuro-degeneration caused by the Aβ (1-42).

The neuroprotective activity of the plants are evident after the estimation of neurochemicals of the brain. The Lipid Peroxide and Nitric Oxide levels were significantly less in the extract treated groups when compared to the test group indicating decrease in oxidative stress. Significantly higher levels of antioxidant enzymes viz catalase, superoxide dismutase and reduced glutathione in the brain homogenates of standard and extract treated groups. The brain cytokine levels in the extract and standard treated groups were also found to be significantly lower than the test group. This further confirms the antioxidant and neuroprotective effects of the plants.

Glutathione, SOD, CAT and MDA are the important non-enzymatic markers of injury. Glutathione plays a key role in coordinating innate antioxidant defense mechanisms. It is mainly concerned with the removal of free radical species such as hydrogen peroxide, superoxide radicals, alkoxy radicals and maintenance of membrane protein thiols and functions as a substrate for glutathione peroxidase and GST[26]. SOD and CAT enzymes are also important scavengers of super ion and hydrogen peroxide. They prevent the generation of hydroxyl radical and protect the cellular constituents from oxidative damage[27]. A reduction in activities of these enzymes is associated with the accumulation of highly reactive free radicals, leading to deleterious effects such as loss of integrity and function of cell membranes[28]. Malondialdehyde (MDA), a major reactive aldehyde resulting from the peroxidation of biological membrane polyunsaturated fatty acids (PUFA), is used as an indicator of tissue damage involving a series of chain reactions[29].

CONCLUSION

From the results it can be concluded that the treatment with ethyl acetate extract of *Vigna radiata* and ethanolic extract *Vigna pilosa* could ameliorate the effect of Aβ (1-42) on cognitive functions, attenuated oxidative stress and neuroinflammation in rats. The study demands further extensive research to develop potential therapeutic agents from these plants for the management of AD.

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