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NEUROPROTECTIVE EFFECT OF *VANDA TESSELLATA* AS "*RASNA*" SPECIES, ON ALUMINIUM CHLORIDE INDUCED ALZHEIMER'S IN RATS

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ABSTRACT

The present study focused on evaluation of neuroprotective effect of hydromethanolic extracts of *Vanda tessellate* (VT), also considered as Rasna. Aluminum Chloride ($AlCl_3$) induces neuroinflammation in rats and finally the development of AD. PASS online and molecular docking *insilico* studies were conducted with PPAR-γ for β-sitosterol and AChE for gigantiol. Total 36 trained Wistar rats were divided into VI groups 6 in each. Group I - normal control, Group II – Disease control, Group III – Rivastigmine (0.3mg/kg, p.o), Group IV and V – Hydromethanolic extract of VT (HMEVT, 150mg/kg, 300mg/kg, p.o) respectively, Group VI - Ayurvedic Formulation of Rasna (AFR) (1ml/kg, p.o). All the animals received Aluminum Chloride ($AlCl_3$) (300mg/kg, p.o) except group I. The rats were treated for 20 days but mean time behavioural study, body weight changes were monitored on 0^{th}, 7^{th}, 14^{th}& 20^{th} day. On 21^{th} day, rats were sacrificed, brains were isolated, then antioxidant enzymes levels, protein content and neurotransmitters levels were determined. Histopathology of cortex and hippocampus parts of the brain were studied. Group II animals showed reduction in locomotor activity, increased in the number of entries as well as time spent in closed arm and time taken to climb the pole was increased but it was reversed in groups treated with 150mg/kg, 300mg/kg doses of HMEVT and AFR. Increased level of protein content, malondialdehyde, reduction in body weight and antioxidants enzymes like superoxide dismutase, catalase, glutathione were observed in disease control group and it was due to free radicals generation and were corrected and restored in groups treated with HMEVT and AFR. Moreover, the histopathological report also showed cellular level protection efficacy found with HMEVT and AFR. The neuroprotective action of HMEVT was due to the active constituents and was proved in *insilico* study. The order of neuroprotective efficacy was HMEVT > AFR.

Keywords: Aluminium Chloride, Alzheimer's disease, antioxidants, Rasna, *Vanda tessellata*; *insilico*

INTRODUCTION

Alzheimer's disease (AD), a neurodegenerative disorder where neurofibrillary tangles (NFTs) and β-amyloid (Aβ) plaques are formed and gets accumulated in brain results in declining of cognitive function as well as memory **(Borai *et al*., 2017)**.The etiology is multifactorial, factors such as genetic factors, oxidative stress, brain inflammation, head trauma and environmental factors including exposure to toxic aluminium **(Zaky *et al*., 2017)**. Normal functions of neurons were interrupted upon the entry of aluminium via a specific transferrin receptor into blood-brain barrier result in memory loss **(Singh *et al*., 2018)** and development of AD is due to deposition of aluminium at hippocampus and frontal cortex where it causes toxic conformational change of cytoskeleton proteins in brain result in formation of Aβ and tau NFTs **(Hesham and Mustafa, 2020)**.
Researchers reported that 'nervines' are a category of plants in Ayurveda are used to strengthen the central nervous system and their chemical constituents play vital role on restoration of memory **(Doungue *et al*., 2018)**. One such Ayurvedic plants are "Rasna" - A controversial medicinal plant. Total 13 plants are listed under Rasna in which *Pluchea lanceolata* is an official name of Rasna. *Vanda tessellata* (VT) is called as Rasna in West bengal and also in many parts of India and are traditionally used for diseases of the nervous system and neurological disorders **(Palash *et al*., 2013)**. VT belongs to the family Orchidaceae, β-sitosterol and gigantiol are the active constituents responsible for the neuroprotective effects **(Kumar and Khanum, 2012)** but not yet scientifically proven by animal experimental models. So, the current study was focused to evaluate the neuroprotective effect of *V. tessellata* where the hydromethanolic extract were taken to prove the potential effect on Aluminium Chloride ($AlCl_3$) induced AD in rats.

MATERIAL AND METHODS

Plant material

The whole plant of VT was collected from Thiruvattar, Kanyakumari, Tamil Nadu, in the month of October 2019. Dr J Jameson, Plant taxonomist from Department of Botany, St Albert's College (Autonomous), Ernakulam , identified and authenticated meantime herbarium specimen also prepared and deposited (voucher specimen number is 479) at Department of Botany, St Albert's College (Autonomous), Ernakulam, Kerala , India.

Extraction procedure

Roots and leaves of VT were isolated and washed with water to free from soil particles and dried at room temperature (shade dry). Plant materials were powdered coarsely with a mechanical grinder in order to increase the contact between the plant materials with the solvent. The powdered materials (300g) were extracted by cold maceration using water and methanol (70:30) at room temperature for a period of 7 days with intermittent shaking until the soluble matter has dissolved or completion of extraction. After 7 days, the mixture was strained through muslin cloth and squeezed to remove all the remaining liquid and were passed through the whatmann filter and then solvent was recovered by using rota evaporator under reduced pressure. The crude extract of VT was named as hydromethanolic extract of VT (HMEVT) and stored in refrigerator at 4^0C in a well tight container for the further experimental purposes.

INSILICO STUDY

PASS Online

PASS (Prediction of Biological Activity Spectra for Substance) an online tool used to predict biological activities like pharmacological effects, biochemical mechanism, toxic and adverse effects, enzymes interaction, metabolic and transporters link, influence on gene expression etc. β-sitosterol and gigantiol were found to be important chemical constituents from VT. 2D structures of these compounds were chosen for the activity prediction. The value defining the likelihood for a given activity to be either revealed (Pa) or not revealed (Pi) **(Raju *et al*., 2021; Mathew *et al*., 2021).**

Molecular docking

The proteins (targets) selected for neuroprotective studies were PPAR-γ for β-sitosterol and AChE for gigantiol. Ligands (β-sitosterol & gigantiol) are an important chemical constituents from VT. Autodock vina PyRx was used to find binding energy expressed in Kcal/Mol. Number of hydrogen bonds between the ligand and receptor and amino acid sequence of attachment of ligands on targets were identified by using Pymol visualisation **(Raju *et al*., 2021).**

***In vivo* study**

Experimental animals

Adult male and female Wistar rats with body weights of 150-250g were used for the study. The animals were maintained under standard environmental conditions (23-25^{0}C, 12 hour light/12 hour dark cycle) and had free access to standard rodent pellet and water *ad libitum*. The animals were acclimatized in laboratory condition for a week before commencement of the study and were trained for behavioural study. The method of study, treatment and handling of animals were presented before IAEC and the committee approved the proposal number: SJCP/IAEC/2019-13/19 for the proceeding experiments of rats.

Treatment protocol

Treatment protocol was designed based **on Singh *et al*., 2018, Somasekar *et al*., 2017**. Total 36 well trained rats were grouped into six groups of six in each. All the rats in the groups 2-6 were administered Aluminium Chloride ($AlCl_3$, 300mg/kg, p.o) daily for 20 days except group 1 which was taken as normal control. Group I was considered as normal control, received only distilled water (p.o). Group II was considered as disease control ($AlCl_3$, 300mg/kg, p.o). Group III was considered as positive control and were treated with Rivastigmine (0.3mg/kg, p.o). Group IV animals were treated with HMEVT (150mg/kg, p.o). Group V animals were treated with HMEVT (300mg/kg, p.o) and group VI animals were treated orally with 1ml/kg of Ayurvedic formulation Rasna (AFR) for 20 days.

During treatment period, animals body weight and behavioural study were assessed on 0, 7th, 14th and 20th day. Elevated plus maze was used to assess memory, locomotor activity was assessed by using digital actophotometer, Cook's pole climbing apparatus was used to study of cognitive function, mainly a response to conditioned stimuli during learning & its retention. On 21st day, bloods was withdrawn immediately after euthanasia via retro orbital plexus to assess protein content as well as serum antioxidants enzymes catalase (CAT), reduced glutathione (GSH), superoxide dismutase (SOD) and malondialdehyde (MDA) level. Brain was isolated to measure aluminium content, neurotransmitter (dopamine, acetylcholine, noradrenaline and serotonin) estimation and histopathology of cortex and hippocampus.

Behavioural Studies

Before the commencement of the experiments, all rats were trained in actophotometer, elevated plus maze test and pole climbing instruments according to the procedure of **Cook and Weidley, 1957; Soman *et al*., 2004**.

a) Actophotometer Test

Locomotor activity was assessed by using digital actophotometer, the apparatus equipped with infrared light sensitive photocells. Each trained animals were kept in digital actophotometer and motor activities were observed for a period of 5min. When the beam of light falling on the photo cell is cut off by the animal that considered as one count and were recorded, values were expressed as number of counts per 5min. Locomotor activity assessment was made in all the groups on 0th day and after drug treatment on 7th, 14th, 20th day.

b) Elevated Plus Maze Test

EPM (exteroceptive behavioral model) is widely employed for evaluating the learning, memory and anxiety in rodents. It has four arms; two open arms and two closed arms are arranged opposite to central sheath which is elevated 50cm above the ground floor. Under silent and dark condition each rat were placed at the centre of the apparatus. The total number of entries as well as time spent in open and closed arms was recorded. The elevated pluz maze test were conducted for all the group of trained rats on 0th day and after drug treatment on 7th, 14th, 20th day, on each group of animals after drug treatment.

C) Pole Climbing Test

Cognitive function of a rat was evaluated by using Cook's Pole Climbing Apparatus where learning & memory retention were evaluated under conditional stimuli (CS). It was a wooden chamber (25 × 25 × 25cm) with stainless steel rods grid floor, 6mA shock is delivered to the floor. At the top lid, a pole (2.5cm width) was at the centre of the chamber. Each rats were placed for 45seconds to explore inside the chamber. A buzzer signal followed by an unconditioned stimulus i.e electric shock was supplied through steel rods grid floor for 45sec. Trained rats were learned to associate the buzzer sound followed by foot shock and try to escape from the foot shock by pole climbing after the buzzer signal. Cut off time to climbing reaction is 10sec. The pole climbing test was conducted for all the group of trained rats on 0th day and after drug treatment on 7th, 14th, 20th day.

Estimation of antioxidants

Preparation of brain homogenate

Animals were sacrificed after 20 days of treatment by Ketamine (80mg/Kg, i.p) + Xylazine (10mg/Kg, i.p). Brains were removed carefully. Parts of brain such as hippocampus and cortex were separated, weighed and homogenized with ice-cold phosphate buffer of pH 7.4 to prepare brain homogenate. It was centrifuged at 800×g for 5min at 4°C to remove the nuclear debris. The supernatant was used for the estimation of MDA content. The remaining supernatant was further centrifuged at 10,000×g for 30min at 4°C to get the post-mitochondrial supernatant which was used for the estimation of GSH, CAT and for SOD; the homogenate was centrifuged at 12000rpm for 20min at 400°C **(Raju and Sinchu, 2017)** and were estimated by using auto analyser with reagent kit.

Determination of total protein

In each tube were added 50µl of above homogenate, 2950µl of 0.9% NaCl and 3000µl of Biuret reagent and the total protein was estimated based on the procedure of **Mæhre *et al*., 2018.**

Estimation of brain neurotransmitters

Weighed brain tissue was homogenised in homogenizer with 5ml HCl- butanol solution for about 1min. Then it was centrifuged for 10min at 2000 rpm. Under identical condition 1ml of supernatant was added in a centrifuge tube and shaken with 2.5ml heptane and 0.3ml of 0.1M HCl for 10 min. Discard the organic layer and take the aqueous phase (0.2ml) to estimate serotonin, dopamine and noradrenaline **(Raju and Sinchu, 2018; Schlumpf *et al*., 1974)**. This procedure was carried out at 0°C and the estimation of AChE was performed according to the procedure of **Ellman (1959).**

Estimation of aluminium content

Weighed (30mg) brain tissue was added with 0.05ml nitric acid, 0.2ml H_2O_2 and0.1ml of polytetrafluoroethene and incubated at 120^{0}C for 2h. Atomic absorption spectrophotometer was used for the estimation of aluminium **(Arokiasamy *et al*., 2015).**

Histopathology brain

Hippocampus and cortex were isolated, washed and placed in 10% formaldehyde. Then they were stained with haematoxylin and eosin (H & E). Under light microscope parts were examined by a pathologist.

Statistical analysis

All the *Invivo* study data were expressed as the Mean ± SEM of six values. The difference between treatment groups was compared to disease control by One Way Analysis Of Variance (ANOVA) followed by Newman-Keul's multiple comparison test; where, $p < 0.05$ implied significance calculation.

RESULTS

Calculation of percentage yield of extracts

The percentage yield was calculated with standard formula and found to be 8.5 (%w/w) from 300g of crude plant materials.

***Insilico* study**

Figure 1 showed the predicted biological activity of the compound β-sitosterol from VT. It has predicted 8 different types of CNS activity, out of which Acetylcholine neuromuscular blocking agent activity scored Pa > 0.6, it means that the ligand exhibits the CNS activity at acetylcholine pathway was confirmed.

PASS online

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All Pa>Pi Pa>0,3 Pa>0,7

0,622	0,016	Acetylcholine neuromuscular blocking agent
0,427	0,015	Antiparkinsonian, rigidity relieving
0,524	0,116	Nootropic
0,395	0,055	Dementia treatment
0,314	0,028	Vascular dementia treatment
0,218	0,051	Neurotrophic factor enhancer
0,112	0,083	Neurotrophic factor
0,266	0,259	Neurotransmitter uptake inhibitor

Figure 1 PASS report of β-sitosterol from VT**.**

Figure 2 showed the predicted biological activity of the compound Gigantiol from VT. It has been predicted total 6 different types of CNS related biological activity, out of which Neurotransmitter antagonist agent scored Pa > 0.6, it means that the ligand revealed the CNS activity in experiment.

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All Pa>Pi Pa>0,3 Pa>0,7

0,646	0,006	Neurotransmitter antagonist
0,634	0,013	Acetylcholine neuromuscular blocking agent
0,474	0,021	Dementia treatment
0,412	0,134	Acute neurologic disorders treatment
0,424	0,192	Nootropic
0,389	0,005	Neurotrophic factor enhancer

Figure 2 PASS report of Gigantol from VT

Table 1 showed the docking score, number of hydrogen bonds and binding site of Gigantiol and β-Sitosterol on their respective receptor. Gigantiol bound with AChE site with the binding energy of -7.7 Kcal/mol and a hydrogen bond was identified at the position of 61 aspartic acid β-Sitosterol bound with PPAR-γ site with the binding energy of -9.1 Kcal/mol and a hydrogen bond was identified at the position of 376 alanine.

Table 1 Docking scores of Gigantiol and β-Sitosterol on AChE, PPAR-γ receptor

Plant Name	Ligand	Receptor	Binding score (Kcal/mol)	Hydrogen bonds	Binding site	PDB
Vanda tessallata	Gigantiol	AChE	-7.7	1	61 ASP	4PQE
	β-Sitosterol	PPAR-γ	-9.1	1	376 ALA	2HWQ

Figure 3 showed docking of β-Sitosterol at the active site region of PPAR-γ (PDB: 2 HWQ), with high binding affinity, as indicated by total docking scores of -9.1 and also showed strong molecular interactions formed between ALA376 residues of PPAR-γ.

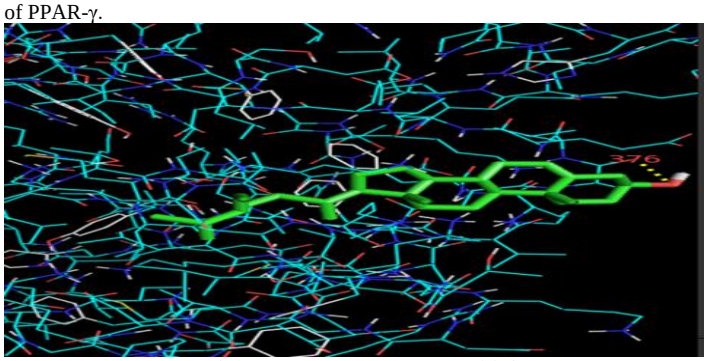


Figure 3 Visualisation of docking in pymol: PPAR-γ with β-Sitosterol

Figure 4 showed docking of Gigantiol at the active site region of AChE (PDB: 4PQE), shown high binding affinity, as indicated by total docking scores of -7.7 and also showed strong molecular interactions formed between ASP61 residues of AChE.

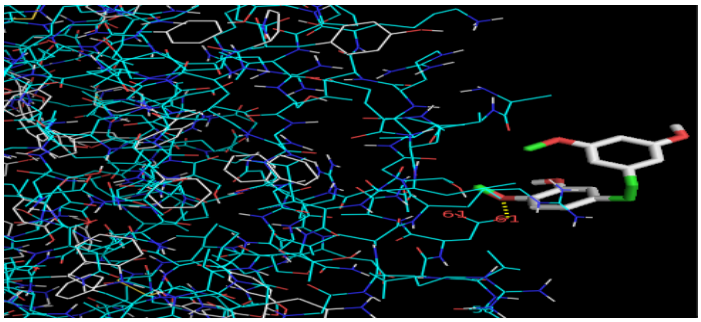


Figure 4 Visualisation of docking in pymol: AChE with Gigantiol

***In vivo* Study**

The locomotor activity of trained rats which were treated with HMEVT and AFR on $AlCl_3$ induced Alzheimer's disease was monitored on 0^{th}, 7^{th}, 14^{th} and 20^{th} day of study were illustrated in Tab 2. The locomotor activity on 0^{th} day was not significant ($p>0.05$) in between the trained rats in each group but on 7^{th} day, the locomotor activity of 300mg/kg HMEVT and 1ml/kg of AFR treated rats showed significant ($P<0.01$) effect when compared with disease control group whereas 150mg/kg HMEVT treated rats showed non-significant ($p>0.05$) effect. After 14 days of continuous treatment, 300mg/kg HMEVT showed more significant ($p<0.001$) effect than 150mg/kg HMEVT ($P<0.05$) and 1ml/kg of AFR ($p<0.01$) treated rats when compared with disease control group but on 20^{th} day of treatment the locomotor activity was more significantly ($p<0.001$) increased rats which were simultaneously treated with 300mg/kg HMEVT than 150mg/kg HMEVT ($P<0.05$) and 1ml/kg of AFR ($p<0.01$) treated rats when compared with disease control group.

Table 2 Effect of HMEVT and AFR on locomotor activity of $AlCl_3$ induced Alzheimer's disease in trained rats.

Groups	Number of counts / 5min			
	0^{th} Day	7^{th} Day	14^{th} Day	20^{th} Day
Normal Control	206.50 ± 2.29	220.00 ± 1.29	216.00 ± 0.89	223.83 ± 7.31
Disease Control ($AlCl_3$ 300mg/kg)	201.33 ± 1.35	130.66 ± 1.78	107.167± 1.90	89.83 ± 1.42
Rivastigmine (0.3mg/kg) + $AlCl_3$ (300mg/kg)	204.50 ± 1.72	167.83 ± 1.66	149.33 ± 1.35	128.66 ± 1.33
HMEVT (150mg/kg) + $AlCl_3$ (300mg/kg)	207.33 ± 2.04^{d}	180.33±3.19^{d}	181.66 ± 2.40^{c}	185.83 ± 2.31^{c}
HMEVT (300mg/kg) + $AlCl_3$ (300mg/kg)	205.00 ± 1.52^{d}	196.00 ± 1.46^{b}	187.00 ± 1.75^{a}	190.00 ± 1.50^{a}
AFR (1ml/kg) + $AlCl_3$ (300mg/kg)	208.16 ± 1.53^{d}	177.83 ± 2.31^{b}	157.50 ± 1.82^{b}	139.83 ±1.64^{b}

The data were expressed as mean ± SEM, n = 6. The data were analysed by One Way Analysis of Variance (ANOVA) followed by Newman-Keul's multiple comparison test where, a: $p<0.001$, b: $p<0.01$, c: $p<0.05$, d: $p>0.05$.

The table 3 illustrated the learning, memory ability and anxiety of trained rats which were received with HMEVT and AFR were recorded as number of entries in open and closed arms within 5min on $AlCl_3$ induced Alzheimer's disease. On 0^{th} day, it was not significant ($p>0.05$) in extract and AFR treated rats when

compared with disease control rats. After 7 days of continuous treatment, 300mg/kg HMEVT and 1ml/kg of AFR treated rats showed significant (p<0.01) effect on the number of entries in open and closed arm but it was progressively improved upon continuous 20 days treatment. At the end of the study, 300mg/kg HMEVT and 1ml/kg of AFR showed highly significant (P<0.001) whereas 150mg/Kg of HMEVT showed less significant (p<0.01) on learning, memory ability and anxiety than the disease control group animals.

Table 3 Effect of HMEVT and AFR on learning, memory and anxiety of $AlCl_3$ induced Alzheimer's disease in trained rats.

Parameters	Number of entries in open arm & closed arm / 5min							
Days	0		7		14		20	
Treatment groups	Open	Closed	Open	Closed	Open	Closed	Open	Closed
Normal Control	4.50±0.76	27.66±1.35	4.33±0.80	25.16±2.89	5.00±0.81	23.83±2.89	6.50±.76	18.33±2.02
Disease Control ($AlCl_3$ 300mg/kg)	3.83±0.60	21.50±1.72	3.00±0.68	27.66±1.80	2.33±0.49	33.16±0.94	1.66±0.33	37.50±0.99
Rivastigmine (0.3mg/kg) + $AlCl_3$ (300mg/kg)	6.33±1.14	23.16±1.74	17.50±0.92	18.00±1.84	20.33±1.33	15.33±1.46	23.16±1.22	12.83±1.51
HMEVT (150mg/kg) + $AlCl_3$ (300mg/kg)	2.83±0.60^{d}	17.00±1.77^{d}	7.16±0.87^{c}	11.67±1.68^{c}	10.16±1.13^{c}	7.50±1.47^{c}	9.00±1.57^{c}	5.3±0.88^{c}
HMEVT (300mg/kg) + $AlCl_3$ (300mg/kg)	6.16±1.19^{d}	15.50±1.40^{d}	10.83±1.24^{a}	8.66±1.11^{a}	14.66±1.25^{a}	4.33±0.76^{a}	17.00±1.06^{a}	2.16±0.47^{a}
AFR (1ml/kg) + $AlCl_3$ (300mg/kg)	2.3±0.49^{d}	22.50±1.82^{d}	10.83±1.42^{b}	14.66±1.70^{b}	16.83±1.30^{b}	12.83±1.74^{b}	19.80±1.30^{b}	10.16±1.64^{b}

The data were expressed as mean ± SEM, n = 6. The data were analysed by One Way Analysis of Variance (ANOVA) followed by Newman-Keul's multiple comparison test where, a: p<0.001, b: p<0.01, c: p<0.05, d: p>0.05.

The effect of HMEVT and AFR on learning, memory ability and anxiety of trained rats of $AlCl_3$ induced Alzheimer's disease were illustrated in Table 4. On 0th day, time spent in open and closed arms was not significant (p>0.05) in rats treated with two doses of HMEVT and AFR when compared with disease control rats. After 7 days of continuous treatment of 300mg/kg HMEVT and 1ml/kg of AFR showed significant (p<0.01) improvement effect whereas 150mg/kg of HMEVT showed less significant (p<0.05) on time spent in open and closed arms but it was progressively improved upon continuous 20 days treatment. At the end of the study, administration of 300mg/kg HMEVT (p<0.001), 1ml/kg of AFR (p<0.01) and 150mg/Kg of HMEVT (p<0.05) showed drastic change on learning, memory ability than the disease control group animals.

Table 4 Effect of HMEVT and AFR on learning, memory ability and anxiety (time spent in open and closed arm) of $AlCl_3$ induced Alzheimer's disease in trained rats.

Parameters	Time spent in open arm & closed arm / 5 min							
Days	0		7		14		21	
Treatment	Open	Closed	Open	Closed	Open	Closed	Open	Closed
Normal Control	0.90±0.15	2.41±0.250	1.01±0.230	2.60±0.481	0.97±0.122	2.98±0.504	1.08±0.348	2.53±0.401
Disease Control ($AlCl_3$ 300mg/kg)	0.45±0.047	3.06±0.588	0.27±0.043	3.37±0.520	0.127±0.039	3.76±0.223	0.03±0.021	4.15±0.257
Rivastigmine (0.3mg/kg) + $AlCl_3$ (300mg/kg)	0.75±0.06	2.51±0.162	0.82±0.047	2.31±0.103	0.93±0.065	2.10±0.107	1.01±0.049	1.91±0.093
HMEVT (150mg/kg) + $AlCl_3$ (300mg/kg)	0.48±0.041^{d}	2.83±0.066^{d}	0.72±0.046^{c}	2.41±0.051^{c}	0.77±0.059^{c}	2.21±0.051^{c}	0.87±0.037^{c}	2.03±0.064^{c}
HMEVT (300mg/kg) + $AlCl_3$ (300mg/kg)	0.55±0.058^{d}	2.81±0.059^{d}	0.63±0.036^{a}	2.77±0.046^{a}	0.66±0.04^{a}	2.56±0.059^{a}	0.72±0.054^{a}	2.33±0.084^{a}
AFR (1ml/kg) + $AlCl_3$ (300mg/kg)	0.65±0.03^{d}	2.61±0.056^{d}	0.80±0.05^{b}	2.48±0.05^{b}	0.90±0.05^{b}	2.18±0.070^{b}	0.96±0.052^{b}	2.31±0.073^{b}

The data were expressed as mean ± SEM, n = 6. The data were analysed by One Way Analysis of Variance (ANOVA) followed by Newman-Keul's multiple comparison test where, a: p<0.001, b: p<0.01, c: p<0.05, d: p>0.05.

The effect of HMEVT and AFR on change in conditioned avoidance response as time taken to claim the pole on $AlCl_3$ induced Alzheimer's disease was illustrated in Table 5.Time taken to climb the pole of trained rats was not significant (p>0.05) on 0th day in rats which were simultaneously treated with both doses of HMEVT and AFR when compared with disease control trained rats. After seven days treatment, time taken to climb the pole was reduced more significantly (p<0.001) with 300mg/kg HMEVT and 1ml/kg of AFR (p<0.01) and 150mg/Kg of HMEVT (p<0.05) when compared with disease control trained rats. Upon extension of continuous administration from day 14 to 20, the time taken to climb the pole outside 5min response was significantly (p<0.001) decreased in rats which were treated with HMEVT (300mg/kg), but 150mg/kg of HMEVT showed less significant (p<0.05) effect when compared with the disease control groups.

Table 5 Effect of HMEVT and AFR on conditioned avoidance response test of $AlCl_3$ induced Alzheimer's disease in trained rats.

Parameters & Treatment	Time taken to climb the pole (min)			
Days	0th	7th	14th	20th
Normal Control	2.56 ± 0.05	2.48 ± 0.03	2.45 ± 0.02	2.51 ± 0.02
Disease Control ($AlCl_3$ 300mg/kg)	2.51 ± 0.04	2.72 ± 0.07	2.92 ±0.06	3.10 ± 0.06
Rivastigmine (0.3mg/kg) + $AlCl_3$ (300mg/kg)	2.43 ± 0.02	2.23 ± 0.02	2.10 ± 0.03	1.90 ± 0.03
HMEVT (150mg/kg) + $AlCl_3$ (300mg/kg)	2.57 ± 0.07^{d}	2.46 ± 0.02^{c}	2.35 ± 0.02^{c}	2.26 ± 0.02^{c}
HMEVT (300mg/kg) + $AlCl_3$ (300mg/kg)	2.62 ± 0.03^{d}	2.38 ± 0.02^{b}	2.23 ± 0.02^{a}	2.02 ± 0.02^{a}
AFR (1ml/kg) + $AlCl_3$ (300mg/kg)	2.52 ± 0.02^{d}	2.31 ± 0.06^{b}	2.20 ± 0.06^{a}	2.12 ± 0.05^{a}

The data were expressed as mean ± SEM, n = 6. The data were analysed by One Way Analysis of Variance (ANOVA) followed by Newman-Keul's multiple comparison test where, a: p<0.001, b: p<0.01, c: p<0.05, d: p>0.05.

The effect of HMEVT and AFR on change in body weight $AlCl_3$ induced Alzheimer's disease in rats was illustrated in Table 6. The body weight was not significantly (p>0.05) changed in rats on 0th but continuous treatments, it was progressively improved on day 7th, 14th and 20th day. At the end of the study, body weight was significantly changed with 300mg/kg HMEVT (p<0.001) whereas 150mg/kg of HMEVT showed less significant (p<0.01) effect. Similarly AFR also made increase in body weight significantly (p<0.01) on AlCl3induced Alzheimer's disease when compared with disease control rats.

Table 6 Effect of HMEVT and AFR on change in body weight of $AlCl_3$ induced Alzheimer's disease in rats.

Parameters Treatment	Body Weight (g)			
Days	0th	7th	14th	20th
Normal Control	160.33 ± 9.00	162.66 ± 13.72	165.16 ± 13.62	167.83 ± 13.44
Disease Control ($AlCl_3$ 300mg/kg)	157.16 ± 6.45	140.33 ± 6.20	130.00 ± 6.10	116.66 ± 4.98
Rivastigmine (0.3mg/kg) + $AlCl_3$ (300mg/kg)	169.50 ± 9.73	193.66 ± 9.09	204.83 ± 7.91	221.83 ± 7.06
HMEVT (150mg/kg) + $AlCl_3$ (300mg/kg)	170.00 ± 4.85^{d}	176.50 ± 4.46^{d}	179.66 ± 4.41^{c}	182.00 ± 4.02^{C}
HMEVT (300mg/kg) + $AlCl_3$ (300mg/kg)	172.00 ± 8.27^{d}	184.16 ± 7.09^{a}	191.00 ± 7.03^{a}	195.66 ± 6.78^{a}
AFR (1ml/kg) + $AlCl_3$ (300mg/kg)	169.169 ± 5.40^{d}	186.00 ± 7.19^{b}	195.16 ± 7.14^{b}	206.50 ± 7.73^{b}

The data were expressed as mean ± SEM, n = 6. The data were analysed by One Way Analysis of Variance (ANOVA) followed by Newman-Keul's multiple comparison test where, a: p<0.001, b: p<0.01, c: p<0.05, d: p>0.05.

Table 7 showed the effect of HMEVT and AFR on protein content as well as aluminum concentration in brain of $AlCl_3$ induced Alzheimer's disease in rats. There was increased level of protein content and aluminum concentration in brain were found with disease control rats. Protein content in brain was significantly (p<0.001) reduced after 20 days of continuous administration of 150mg/kg and 300mg/kg HMEVT. Administration of 1ml/kg of AFR also shown significant

($p<0.01$) reduction in aluminum concentration and protein content of $AlCl_3$ induced Alzheimer's disease in rats.

Table 7 Effect of HMEVT and AFR on total protein and Aluminium content in brain of $AlCl_3$ induced Alzheimer's disease in rats.

Parameters Treatment	Total protein content (mg/g)	Concentration of Aluminium (µgm/gm)
Normal Control	36.76 ± 0.70	-
Disease Control ($AlCl_3$ 300mg/kg)	53.70 ± 0.74	9.1 ± 0.15
Rivastigmine (0.3mg/kg) + $AlCl_3$ (300mg/kg)	35.33 ± 0.68	2.3 ± 0.17
HMEVT (150mg/kg) + $AlCl_3$ (300mg/kg)	29.89 ± 0.70[a]	3.1 ± 0.29[a]
HMEVT (300mg/kg) + $AlCl_3$ (300mg/kg)	31.74 ± 0.72[a]	2.9 ± 0.19[a]
AFR (1ml/kg) + $AlCl_3$ (300mg/kg)	34.27 ± 0.91[a]	3.6 ± 0.20[b]

The data were expressed as mean ± SEM, n = 6. The data were analysed by One Way Analysis of Variance (ANOVA) followed by Newman-Keul's multiple comparison test where, a: $p<0.001$, b: $p<0.01$

Table 8 showed the effect of HMEVT and AFR on antioxidant status in brain of $AlCl_3$ induced Alzheimer's disease in rats. The CAT, SOD and GSH level were decreased in disease control group but they were significantly increased and restored in animals treated with 300mg/kg ($p<0.001$) and 150mg/kg of HMEVT ($p<0.01$). Like 150mg/kg of HMEVT, 1ml/kg of AFR also restored the antioxidant enzyme level in the brain of $AlCl_3$ induced Alzheimer's disease in rats. There was an increased level of MDA found in brain of disease control animals, 20 days continuous treatment with 300mg/kg ($p<0.001$) and 150mg/kg of HMEVT as well as , 1ml/kg of AFR ($p<0.01$) reduced the content significantly as that of normal in the brain of $AlCl_3$ induced Alzheimer's disease in rats.

Table 8 Effect of HMEVT and AFR on antioxidant status in brain of $AlCl_3$ induced Alzheimer's disease in rats.

Treatment & Parameters	CAT (U/Mol)	GSH (Millimole/gm)	MDA (nmoles of MDA/mg wet tissue)	SOD (U/gm tissue)
Normal Control	33.05 ± 0.71	53.67 ± 0.75	43.11 ± 0.68	71.34 ± 0.61
Disease Control ($AlCl_3$ 300mg/kg)	10.48 ± 0.84	16.87 ± 0.72	75.09 ± 0.72	31.58 ± 0.74
Rivastigmine (0.3mg/kg) + $AlCl_3$ (300mg/kg)	27.38 ± 0.60	50.00 ± 0.70	46.51 ± 0.76	66.91 ± 0.57
HMEVT (150mg/kg) + $AlCl_3$ (300mg/kg)	18.44 ± 0.77[b]	35.35 ± 0.77[b]	44.85 ± 0.65[b]	41.54 ±0.81[b]
HMEVT (300mg/kg) + $AlCl_3$ (300mg/kg)	29.05 ± 0.69[a]	38.97 ± 0.71[a]	38.82 ± 0.82[a]	54.71 ± 0.66[a]
AFR (1ml/kg) + $AlCl_3$ (300mg/kg)	22.37 ± 0.78[b]	32.64 ± 0.77[b]	45.61 ± 0.67[b]	55.36 ± 0.70[b]

The data were expressed as mean ± SEM, n = 6. The data were analysed by One Way Analysis of Variance (ANOVA) followed by Newman-Keul's multiple comparison test where, a: $p<0.001$, b: $p<0.01$, c: $p<0.05$, d: $p>0.05$.

Table 9 showed the effect of HMEVT and AFR on brain neurotransmitter level in $AlCl_3$ induced Alzheimer's disease in rats. The quantity of dopamine, noradrenaline and serotonin were reduced in disease control group, but they were more significantly ($p<0.001$) raised in rats which were treated with 300mg/kg of HMEVT and 1ml/kg of AFR ($p<0.01$) whereas 150mg/kg of HMEVT was not restored the dopamine level after twenty days of treatment moreover it showed less significant ($p<0.01$) effect on normalization of serotonin and noradrenaline level.

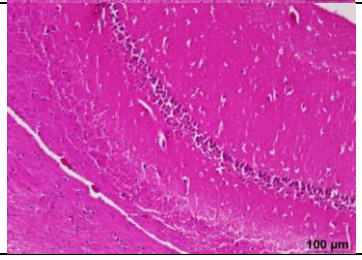
Table 9 Effect of HMEVT and AFR on brain neurotransmitter level in $AlCl_3$ induced Alzheimer's disease in rats.

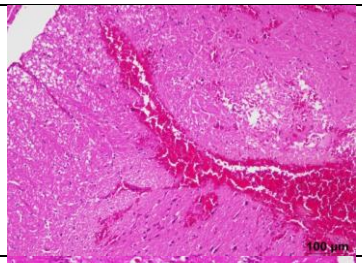
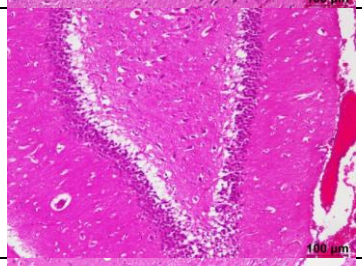
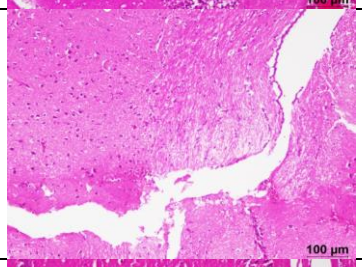
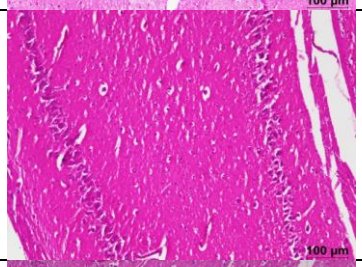
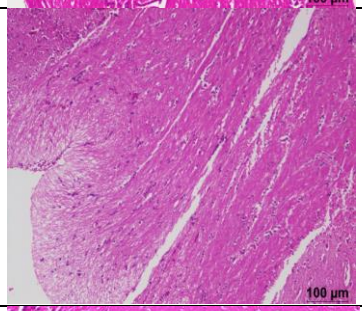
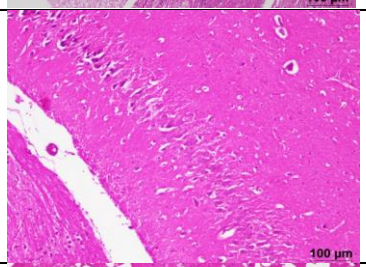
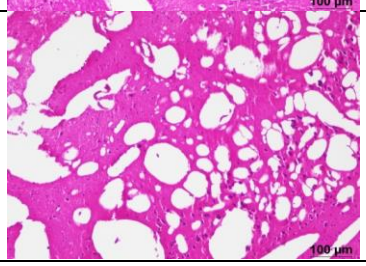
Parameters & Treatment	Dopamine (ngm/gm tissue)	Noradrenaline (ngm/gm tissue)	Serotonin (ngm/gm tissue)
Normal Control	0.974±0.001	0.615 ±0.0006	0.475 ± 0.0009
Disease Control ($AlCl_3$ 300mg/kg)	0.627±0.0009	0.378±0.0007	0.253±0.0010
Rivastigmine (0.3mg/kg) + $AlCl_3$ (300mg/kg)	0.954±0.001	0.601±0.001	0.459±0.005
HMEVT (150mg/kg) + $AlCl_3$ (300mg/kg)	0.708±0.0004[d]	0.499±0.0005[c]	0.367±0.0007[b]
HMEVT (300mg/kg) + $AlCl_3$ (300mg/kg)	0.763±0.0005[a]	0.538±0.0007[a]	0.403±0.0006[a]
AFR (1ml/kg) + $AlCl_3$ (300mg/kg)	0.936±0.0008[b]	0.586±0.0012[b]	0.423±0.0007[b]

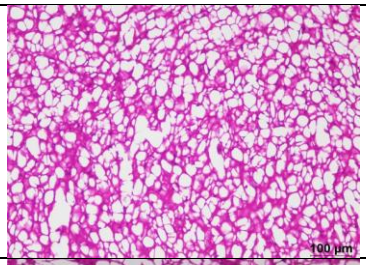
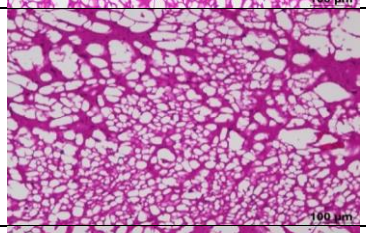
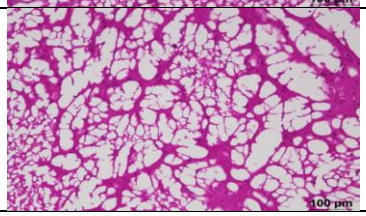
The data were expressed as mean ± SEM, n = 6. The data were analysed by One Way Analysis of Variance (ANOVA) followed by Newman-Keul's multiple comparison test where, a: $p<0.001$, b: $p<0.01$, c: $p<0.05$, d: $p>0.05$.

Table 10 showed histopathological reports of the cortex and hippocampus of the brain of Aluminum Chloride induced Alzheimer's disease with different treatment groups (n=6).

Table 10 Histopathology reports of cerebral cortex and hippocampus of brain of AD-induced rats and treated groups with HMEVT and AFR.

Groups	Parts	Histopathology image	Report (n=6)
Normal Control	Cortex		Section showed that all the cellular functional units were within normal limits
	Hippocampus		Section showed that all the cellular functional units were within normal limits

Disease Control ($AlCl_3$ 300mg/kg)	Cortex		Section showed vacuolation, neuropil, multifocal, marked vacuolation, neuronal, hippocampus, diffuse, marked congestion/ haemorrhage, multifocal, marked
	Hippocampus		Section showed vacuolation, neuropil, multifocal, moderate congestion / haemorrhage, multifocal, moderate
Rivastigmine (0.3mg/kg) + $AlCl_3$ (300mg/kg)	Cortex		Section showed vacuolation, neuropil, multifocal, minimal vacuolation, neuronal, hippocampus, diffuse, minimal congestion / haemorrhage, multifocal, minimal
	Hippocampus		Section showed vacuolation, neuropil, multifocal, minimal vacuolation, neuronal, hippocampus, diffuse, minimal congestion / haemorrhage, multifocal, minimal
HMEVT (150mg/kg) + $AlCl_3$ (300mg/kg)	Cortex		Section showed vacuolation, neuropil, multifocal, minimal vacuolation, neuronal, hippocampus, diffuse, minimal congestion / haemorrhage, multifocal, minimal
	Hippocampus		Section showed vacuolation, neuropil, multifocal, minimal vacuolation, neuronal, hippocampus, diffuse, minimal congestion / haemorrhage, multifocal, minimal
HMEVT (300mg/kg) + $AlCl_3$ (300mg/kg)	Cortex		Section showed vacuolation, neuropil, diffuse, severe, congestion / haemorrhage, multifocal, minimal

AFR (1ml/kg) + $AlCl_3$ (300mg/kg)	Hippocampus		Section showed vacuolation, neuropil, diffuse, severe, congestion / haemorrhage, multifocal, minimal
	Cortex		Section showed vacuolation, neuropil, diffuse, severe, congestion / haemorrhage, multifocal, minimal
	Hippocampus		Section showed vacuolation, neuropil, diffuse, severe, congestion / haemorrhage, multifocal, minimal

DISCUSSION

"Rasna" is one of the most significant medicinal plants in the indigenous system of medicine. It is a controversial medicinal plant and has a wide application in the health care system. There are 13 plants which are currently being identified and used as Rasna in different parts of India. VT is called as Rasna in West bengal and also in many parts of India and are traditionally used for diseases of the nervous system. It is also used in gastro-intestinal complications like dyspepsia, flatulence and neurological disorders **(Palash *et al*., 2013)**. Many chemicals are used for the induction of Alzheimer's disease including aluminium, scopolamine, colchicine, streptozotocin, sodium azide and ethanol. Aluminum is considered as one of the frequently used heavy metals for induction of cognitive impairment **(Akram and Nawaz, 2017)**. It also causes anemia, osteomalacia, and hepatic and neurological disorders. Oral administration of high amount of 300 mg/kg body weight of aluminium has been reported that, induction of AD and associated oxidative stress, cholinergic deficit and accumulation of Aβ & NFTs in the brain of rats **(Mahdi *et al*., 2019)**.

This neurotoxicity effect is due to free radical productions which make damage to lipids and proteins in brain. aluminium crosses the blood-brain barrier through a specific transferrin receptor and induces profound memory loss via disruption of various normal neuronal functions. It also causes progressive apoptotic neuronal loss, ultrastructural alterations of neurons present in cortex and hippocampus region of brain, protein misfolding, plaques depositions and biochemical modifications followed by changes in genes expression in the brain **(Zaky *et al*., 2017; Balgoon *et al*., 2015)**

Aggressive behaviour was more frequent in patients with AD. Therefore behavioural and psychological symptoms were more likely to be observed closer to the time of diagnosis AD because it has severe dementia, neurodegeneration and loss of functional independence which leads to early death. Different neuropsychiatric instruments were used to assess the behavioural change in AD. Long term administration of aluminium has been reported to change in behavioural pattern **(Kangtao and Souravh, 2018; Li *et al*., 2014).** Hence in the present study, behavioural changes also investigated on aluminum exposure for 20 days and the possible effect of HMEVT and AFR were assessed on restoration of behavioural changes. Actophotometer test, elevated plus maze test and pole climbing apparatus were used to monitor the behavioural changes.

By using actophotometer, locomotor activity was assessed to check the CNS stimulant or depressant effect on rats. Administration of aluminum for 35 days, there was a decline in locomotor activity in aluminium treated rats which was indicated that the CNS depressant effect on chronic Aluminium exposure **(Lakshmi *et al*., 2015)**. In the present study also treatment with HMEVT and AFR corrected the locomotor incoordination caused by $AlCl_3$.

EPM is extensively employed for assessing the learning, retaining memory ability and anxiety in rodents. Animals which were received only Aluminium decreased in the number of entries in open arm, decreased percentage of time spent in open arms and increased in the number of entries in closed arm **(Murugaiyan and Bhargavan, 2020; Rabiei *et al*., 2018)**. Researchers reported that the percentage of time spent in open arms and the number of entry to open arms are related the anxiety indicator parameters in the EPM and are related to $GABA_A$ receptor complex **(Jafarian *et al*., 2019)**. However the treatment with HMEVT and AFR reverse effects on $AlCl_3$induced Alzheimer's disease in rats.

From Cooks pole climbing apparatus, memory retrieval capacity was determined as the ability of an animal to retention the acquire memory process. It was indicated by increasing number of avoidance response **(Reddy *et al*., 2020)**.**Ganga Raju *et al*., 2020** reported that the time taken to climb the pole was increased in the $AlCl_3$ exposure group and was due to dementia. In the present study, taken to climb the pole was noted where the time taken to escape from the electric shock field was reduced as that of normal trained rats after continuous 20 days treatment of HMEVT and AFR.

Weight loss is the common problem found in AD which leads to weaker in muscle mass, hard to maintain physical balance and more susceptible to get systemic infection. The reason behind in weight loss is change in olfactory system which contains neurotransmitter such as acetylcholine which was deficient in AD. Changes in food consumption and behavioral disturbances are also occur in AD leading to decreased energy intake but increased energy expenditure is not the cause of weight loss in AD **(Tamura, 2007)**. In the present study, $AlCl_3$ administration significantly diminished the body weight in the disease control group of animals. It was because of less desired to water and food intake, transient diarrhoea and reduced efficacy in converting feed which leads to reduction in body mass **(Mathiyazahan and Arokiasamy, 2019)**. Gain in body weight was observed among other groups treated with HMEVT and AFR.

Toxic beta-amyloid plaques proteins are formed in Alzheimer's and collects between neurons which affect the cell function. Similarly, neurofibrillary tangles (tau) are formed and get accumulated inside neurons. In brain, one type of glial cell called microglia which engulfs and remove waste and toxins from the healthy brain. In AD, microglia fails to clear the waste debris and protein including beta-amyloid plaques. Sometimes oxidative modified proteins (carbonyl protein) also formed in brain (hippocampus) due to oxidative stress in AD **(Aksenov, MY., 2001)**. The declined level of protein was found in of Aluminium treated group and was reported by **Yokel and McNamara, 1989**. This was due to less intake of food, increased catabolism of proteins and formation of reactive oxygen species (ROS) in which hydroxyl radicals responsible for the oxidation of the side chains of some amino acids resulting in proteins hydrolysis **(Mæhre *et al*., 2018)**. However, in the present study also decreased level of protein content was found with after the administration of HMEVT and AFR.

Crapper *et al*., 1973 stated that long term exposure of $AlCl_3$ is associated with high aluminum concentration in brain. It enters into the brain via the specific high affinity receptors for transferrin (TfR) expressed in the blood brain barrier (BBB) and get accumulated in all the regions of rats with AD. In the present study also high Al concentration was found with disease control group rats, but treatment with HMEVT and AFR reduced the aluminum level in rats.

Neurotoxicity caused by aluminum is mediated mainly by increasing cellular oxidative stress which gets accumulate and enhance reactive oxygen species (ROS) formation, which depletes the normal antioxidant defense mechanism, thereby further enhancing oxidative stress and lipid peroxidation processes. It also causes changes in iron homeostasis, causing excessive free iron ions leading to oxidative damage, finally culminating in neurodegeneration **(Lakshmi *et al*., 2015)**. Long term exposure with $AlCl_3$ resulted in marked oxidative stress, which is indicated by increased lipid peroxidation result in increased level of MDA as well as decreased in reduced GSH, CAT and SOD activity. This activity may be due to the reduced axonal mitochondria turnover, disruption of the golgi or reduction of synaptic vesicles induced by aluminium exposure **(Prema *et al*., 2017).** The present study also found that decreased level of CAT, GSH and SOD as well as

increased level of MDA content was found with disease control rats but re-established in rats which were treated with HMEVT and AFR.

Neurotransmitters play an important role in maintaining synaptic and cognitive functions by sending signals across synapses. They also have a major role in causing oxidative stress, which is known to be involved in AD pathogenesis **(Reddy, 2017)**. Neurotoxic effect of aluminium significantly increases AChE activity, the key enzyme which is responsible for acetylcholine hydrolysis thereby reduction in acetylcholine level found in brain **(Ramachandran *et al*., 2019)**. Similarly $AlCl_3$ causes depletion of dopaminergic transmitters in the central nervous system and induced neurotoxicity **(Zheng and Liang, 1998)**. Moreover it also causes neuronal loss in the brain region. This neuronal loss and the resultant compensatory mechanisms lead to changes in the level of norepinephrine available in the brain, which consequently affect cognitive functions **(Gannon *et al*., 2015)**. Reynolds et al., 1995 also reported that in Alzheimers disease, significant decline of serotonin occurs which is consistent with cognitive processing. In present study, reduced level of acetylcholine, dopamine, noradrenaline and serotonin levels were observed in only $AlCl_3$ treated animals. However, the treatment with HMEVT and AFR raised the neurotransmitters level as that of normal rats.

Histopathology of hippocampus and cortex samples were analysed from neuroprotective study inorder to know the structural changes and organ toxicity. The three main structural changes occurring in the brain including neuronal loss, formation and accumulation of hyperphosphorylated tau protein called (NFTs) and aggregation of β-amyloid (Aβ) peptides termed senile or amyloid plaques. These changes are most prominent in the cholinergic system, particularly in hippocampus and cortex, which is closely associated with memory loss and cognitive dysfunction in AD. So cortex and hippocampus of brain were selected for the histopathology **(Vecchio *et al*., 2018)**. Researchers found that the $AlCl_3$ induced cellular damage in organs such as brain were analysed by histopathology. In the present study also it was confirmed by the pathologist. It's being a comparative neuroprotective study, the order of potency was HMEVT > AFR.

CONCLUSION

The present study was focused on few traditional uses of Rasna, were experimentally proven with animal models. The neuroprotective effect and the mechanism of VT were studied by using PPAR-γ and AChE receptor with β-sitosterol and gigantiol. Oral administration of HMEVT and AFR reverses the effects given by $AlCl_3$ in a dose dependent manner. Therefore VT can be used as a remedy for the treatment of AD and neurotoxicity.

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