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Neuroprotective Studies of *Rubia cordifolia* Linn. on β-amyloid Induced Cognitive Dysfunction in Mice

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Abstract: Alzheimer's disease (AD) is neurodegenerative disorder linked to pathological aggregation of misfolded proteins that accumulate fibrillary amyloid deposits in selective regions of central nervous system. AD is neuro fibrillary tangles (NFTs) and senile plaques formed by neuro accumulation of abnormal tau filaments and extra cellular deposits of β- amyloid fibrils. Amyloid plaques correlate with progressive deficits in cognitive, memory dysfunction and selectively cholinergic deficits in neurocortex, hippocampus and basal forebrain. In the present study animals were pretreated with ethanolic extract of *Rubia cordifolia* (collected from western ghats in south India) for a period of 4 weeks dose dependently(200 and 400 mg/ kg b.w) and kept In light/dark cycle. During this period the animals were trained in water-maze, Y-maze, exploratory behaviour and passive avoidance apparatus for memory. On 21st day of the treatment β- amyloid peptide was administered by IntraCerebroVentricular (i.c.v) route using Hamilton 701 RN micro syringe 26 gauze needle with 10µl volume of injection and animals were tested for cognitive and memory dysfunction. *Rubia cordifolia* extract administration significantly ($P<0.01$) reduced the β-amyloid induced cognitive and memory dysfunction. The extract decreases the neurodegeneration and helps in memory retention activity. The extract showed significant effects ($P<0.05$) in short term retention and increases long term retention of memory in step-down inhibitory avoidance task and an increase($P<0.05$) in number of head dippings, line crossings and rearings in the open field, and the water-maze test the extract treated showed the escape latency equal to the normal levels. Analysis was done by using ANOVA followed by Dunnet's "t" test. *Rubia cordifolia*t extract shows significant protective effect on neurodegeneration and shows improvement in memory retention activity when compared with Aβ 25-35 induced group, further study is needed to determine the underlying mechanism involved.

Keywords: Alzheimer's disease; Neuro fibrillary tangles; β- amyloid; IntraCerebroVentricular; *Rubia cordifolia*

Introduction

Alzheimer's disease is a progressive neurodegenerative disorder primarily manifesting as a loss of memory, senile dementia, intraneuronal neurofibrillary tangle formation and cerebral parenchyma deposition of the ß-amyloid protein in the form of amyloid plaques is the most stereotypic cognitive and neuropathological hallmarks of AD. The earliest striking symptom is loss of short term memory (amnesia). Dementia occurs in a number of brain diseases, where the impairment in cognitive abilities represents a decline from prior levels of function and interferes with the ability to perform routine daily activities, as the disease progresses memory of remote events and over learned information declines together with other cognitive ability. Behavioral disturbances include agitation, aggression, depressive mood, sleep disorder and anxiety.

Prevalence rates for dementia increase exponentially with advancing age, ranging from 10% in the age group of 60-65 years to 36% in the age group of 90 years[1]. This disease may be prese nile or senile onset and the occurrence of disease is before or after the age group of 60 years, but now it is challenging the median age of population[2]. AD was the 7th leading cause of death in 2004 with 65,829 numbers of deaths[3]. There are an estimated 24million people with dementia world wide[4].

Aß protein is a potent neurotoxin both invitro and invivo[5]. It has been substantiated that the 11 amino acid sequence (25-35) of beta amyloid is neurotoxic for primary neurons[6]. Aß is secreted by

normal cells in cultures and are detected as circulating peptide in the plasma and the cerebrospinal fluid of healthy humans and other mammals[7]. Formation of Aß involves cleavage at two different points, including one in the transmembrane domain of APP by ß and γ-secretases. Gamma secretase is a clumsy enzyme that lacks precision and cuts APP at different points in the same victinity, generating Aß fragments of different lengths including Aß 40 and Aß 42, mutations favour the formation of Aß42.

Amyloid hypothesis points to cytotoxicity of mature aggregated amyloid fibrils, which are believed to be the toxic form of protein responsible for disturbing the cells calcium ion homeostasis and thus including apatosis[8]. The calcium permeable channels in the membranes resulting in excessive calcium influx and cause the induction of neurotoxic cascades[9].

Aβ fragments are derived from Proteolytic processing of amyloid precursor protein (APP) in neurons, and the subsequent release of fragments into the extra cellular space. APP in the ER is cleaved at residue 17 by alpha-secretase protease activity encoded by ADAM-10 and TACE. Beta-secretase activity, recently identified as BACE, cleaves in the N-terminus of the Aβ fragment. Gamma-secretase cuts APP within the transmembrane domain at amino acids 40 and 42, releasing the Aβ fragment containing residues 1-40/42 as well as shorter products such as p3 (residues 17-40/42) that requires alpha-secretase cleavage. The gamma-secretase activity requires the transmembrane protein Presenilin-1 that is itself cleaved into an N-terminal and C-terminal fragment that both are required for gamma-secretase activity. Mutations in presenilin-1 have been genetically associated with familial forms of Alzheimer's disease, further supporting the role of APP processing the development of the disease. Inhibition of the beta or gamma-secretase may provide a mechanism to treat this disease. Other mechanisms may include altering degradation of Aβ, and the use of vaccines against beta-amyloid to remove aggregates.

Materials and methods

Chemicals

Pyrogallol and hydrogen peroxide were obtained from S.D. Fine Chemicals Ltd., India. Thiobarbituric acid (TBA), trichloroacetic acid (TCA), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), phosphate buffer and Tris buffer were obtained from Sigma, USA. All other reagents used were of analytical grade and obtained from commercial sources.

Alcoholic extract of roots of *Rubia cordifolia* Linn[10]

The plant of RC were collected and authenticated by the Dr.S.Rajan, field botanist, survey of medicinal plants & collection unit. The powder of shade-dried plant was extracted with ethanol by using soxhlet extraction method. The suspension of solvent dried extract (11% w/w yield) in gum acacia was administered orally for four weeks at a dose of 200 & 400 mg/kg/day.

Animals

Colony inbred strains of Swiss albino mice weighing 22-25g at the age of 5-6 weeks, obtained from king institute was used for the pharmacological studies. The animals were kept under standard conditions maintained at 23-25^{o}C, 12 hr light/dark cycle and given standard pellet diet. The animals were acclimatized to the laboratory conditions for a week prior to the experimentation and randomly divided into four groups of each six animals. Principles of animal handling were strictly adhered to and the handling of animals was made under the supervision of animal ethics committee of the institute. The experimental protocol was approved by animal ethics committee (IAEC), the IAEC proposal number is IAEC 38/2008.

Animals were divided into four groups of each six mice. Group-I: animals injected by phosphate buffered saline, group-II, III, & IV animals injected by Aβ peptide and group-III,IV treated with 200 & 400 mg/kg(p.o) EERC respectively.

Intra Cerebro Ventricular injection of Aβ peptide

The administration of a Aβ25-35 was performed by identifying the bregma pointing the skull[11], each mouse was injected at bregma with a 50µl Hamilton micro syringe fitted with a 26-gauge needle that was inserted to a depth of 2.4 mm. in brief, the needle was inserted unilaterally 1 mm to the right of the middle point equidistant from each eye, at an equal distance between the eyes and ears and perpendicular to the plane of the skull. Mice should exhibit normal behaviour with in 1 min after injection.

The injection volume was 10µl, the memory impairment due to the injection was examined on the second day of I.C.V. injection by step down inhibitory avoidance.

Step down inhibitory avoidance

The apparatus was 50 cm x 25 cm x 25 cm acrylic box, whose floor consisted of parallel 1.0 cm apart. A 7.0 cm wide, 2.5 cm high, 25.0 cm long platform occupied the centre floor. In the training session, immediately after stepping down placing their four paws on the grid the animals received a 0.4 ma, 2.0 s scrambled foot shock. In test session

no foot shock was given and step-down latency was used as a measure of retention (to a ceiling of 300 s) one-trial step-down inhibitory avoidance in rats and mice involves the activation of two separate memory types, a short-term memory (STEP) system, and a long-term memory (LTM) system. Therefore, retention tests were carried out 90 min after training to evaluate STM and 7 days after training to evaluate long-term memory[12]. The same mice were used for both tests, as testing for STM has been found not to affect LTM retention scores in previous studies.

Open field habituation

In order to control for possible effects on locomotor activity, animals were explored twice, with a 24 h interval, to a 40 cmx50 cmx60 open field whose brown linoleum floor was divided into 16 equal squares by white lines. In both sessions, animals were placed in the rear left square and left to explore it freely for 5 min during which time the number of lines crossing and rearings were counted.

Water maze task

The Morris water maze was performed as described previously[13]. The experimental apparatus consisted of a circular water tank (diameter-100 cm; height=35 cm), containing water at 28˚C to a depth of 15 cm and rendered opaque by adding powdered milk. A platform (diameter 4.5 cm; height 14.5 cm) was submerged 0.5 cm below the water surface and placed at the midpoint of one quadrant. After several trials, the test was conducted on the 14th day injection of β amyloid peptide. In each training trial, the time required to escape on to the platform was recorded.

Y-maze task

Y-maze task is used to measure the spatial working through the spontaneous alternation of behaviour[14]. The maze is made of black painted wood. Each arm is 40 cm long, 13 cm high, 3 cm wide at the bottom, 10 cm wide at the top, and converges at an equal angle. Each mouse is placed at the end of one arm and allowed to move freely through the maze during an 8-min session. 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, are recorded visually. Alternation is defined as the number of successive entries into the three arms, on overlapping triplet sets. The percentage of alternation is calculated as the ratio of actual alternations, defined as the total number of arm entries minus two, and multiplied by 100.

Acetylcholineserase (AChE) enzyme determination[15]

20 mg of brain tissue per ml of phosphate buffer (ph 8, 0.1 m) was homogenized in a homogenizer. A 0.4 ml aliquot of brain homogenate was added to a cuvette containing 2.6 ml of 0.1m phosphate buffer (ph 8). 100µl of the DTNB reagent was added to the photo cell. The absorbance was measured at 412 nm. 20µl of the acetylthiocholine iodide was added. Changes in absorbance were recorded and the change in absorbance per minute was calculated.

The enzyme activity is expressed as µmoles/minute/mg tissue.

MAO Assay

Mouse brain mitochondrial fraction are prepared by cutting the brain sample in to small pieces and rinsed in 0.25m sucrose 0.1 m tris 0.02m EDTA (ph 7.41) to remove blood. The pieces were homogenized for 45 sec in a potter-elvehjem homogenizer with 400 ml of the same medium. The homogenate was centrifuged at 800rpm for 10min and the pellets were discarded. The supernatant was then centrifuged at 12,000rpm for 20 min in the same medium. The precipitate was washed twice more with 100ml of sucrose tris EDTA and resuspended in 50ml of the medium. The protein concentration was adjusted to 1 mg/ml.

MAO activity was assessed spectrophotometrically[16]. The assay mixture contains 4mm of serotonin as specific substrates for MAO-A, 250µl solution of the mitochondrial fraction and 100mm sodium phosphate buffer (ph7.4) up to the final volume of 1ml, the reaction was allowed to proceed at 37 º c for 20 minutes and stopped by adding 1 m HCl (200µl), the reaction product was extracted with 5ml of butyl acetate, the organic phase was measured at wavelength of 280 nm in a spectrometer. Blank samples were prepared by adding 1m HCl (200µl) prior to the reaction and worked subsequently in the same manner.

Estimation of protein[17]

Total protein was estimated in brain homogenate by using total protein kit. From Erba diagnostics, Germany using semi Autoanalyser in the department.

Assay of super oxide dismutase (SOD)[18]

The assay mixture contained 1ml of pyrogallol-tris-DEPTA, 0.2ml of suitably diluted tissue and 0.8 ml of water. The rate of pyrogallol autoxidation is taken from the increase in absorbance at 420 nm. The activity of SOD was expressed as units / minutes / mg protein. One unit of the enzyme is defined as the amount of enzyme, which inhibits the rate of pyrogallol auto oxidation by 50%. The SOD is expressed as units/min/mg protein.

Assay of glutathione peroxidase (GPx)[19]

The reaction mixture consisting of 0.2 ml each of EDTA, sodium azide and H_2O_2, 0.4 ml of phosphate buffer, 0.1 ml of suitably diluted tissue was incubated at 37°C at different time intervals. The reaction was arrested by the addition of 0.5 ml of TCA and the tubes were centrifuged at 2000 rpm. To 0.5 ml of supernatant, 4 ml of Disodium hydrogen phosphate and 0.5 ml DTNB were added and the color developed was read at 420 nm Spectrophotometrically. The activity of GPx is expressed as µmoles of glutathione oxidized / minutes / mg protein.

Assay of glutathione reductase (GRD) [20]

The reaction mixture containing 1 ml of phosphate buffer, 0.5 ml of EDTA. 0.5 ml of oxidized glutathione and 0.2 ml of NADPH was made up to 3 ml with water. After the addition of 0.1 ml of suitably diluted tissue, the change in optical density at 340 nm was monitored for 2 minutes at 30 sec intervals. The activity of GR is expressed as nmoles of NADPH oxidized / minute / mg protein.

Estimation of Ascorbic acid[20] (Vit-C)

To 0.5 ml of suitably diluted tissue, 0.5 ml of water and 1 ml of TCA were added, mixed thoroughly and centrifuged. To 1 ml of the supernatant, 0.2 ml of DTC reagent was added and incubated at 37° C for 3 hrs. Then 1.5 ml of sulfuric acid was added, mixed well and the solutions were allowed to stand at room temperature for another 30 minutes. The color developed was read at 520 nm. Spectrophotometrically the level of ascorbic acid is expressed as µg /mg protein.

Statistical Analysis

The statistical analysis was carried out using Analysis of Variance (ANOVA) followed by Dunnet's test. P values <0.05 were considered as significant.

Results

EERC improves the cognitive defects in Aβ 25-35 treated mice.

Effect of EERC on Step down Inhibitory Avoidance

There was a significant difference between the control group (I) and the negative control group (II) in the step down latency in the STM and LTM. The EERC treated groups show significant tendency to increase the retention of STM and LTM. The low dose and high dose shown significance (P<0.05) when compared with the negative control was shown **Table No: 1 and Fig: 1.**

Effect of EERC on Water Maze

There is an increase in escape latency in negative control group when compared with the control group (P<0.01) of the two groups of amnesia induced animals, both showed decreased time to escape on to the escape platform. The group treated with 200 & 400 mg/kg EERC treated showed the significance of (P<0.01 and P<0.001) respectively as shown in **Table no: 1 and Fig no: 2.**

Effect of EERC on Exploratory Behavior

The amnesia induced group (negative control) indicated decrease in the exploratory behaviuor i.e. head dippings, rearings, line crossings by the (P<0.01) respectively in comparison with the control group I. The results presented by the treated groups shows significance (P<0.05) increase in the head dippings and line crossings in respect of 200 mg/kg when compare with the negative control group. The 400 mg/kg treated group showed significance of (P<0.05,) head dippings, rearings and line crossings respectively as shown in **Table no: 1 and Fig no: 3.**

Effect of EERC on Y-Maze

The amnesia induced group (negative control) indicated decrease in the alternation of behaviour by the (P<0.01) in comparison with the control group I. The results presented by the treatment groups shows significance by (P<0.01) increase in the alternation of behaviour in respect of 200 mg/kg of EERC and 400 mg/kg of EERC when compared with that of the negative control group as shown in **Table no: 1 and Fig no: 4**.

Effect of EERC on AChE Activity

Injection of Aβ 25-35 significantly (P<0.01) increased the AChE activity when compared with control group. In the treated group there was a significant (P<0.05) reduction in enzyme levels on both 200 and 400 mg/kg of EERC treated mice as shown in **Table no: 2 and Fig no: 5**.

Effect of EERC on MAO-A

The enzyme activity in Aβ 25-35 induced group had significantly increased (P<0.01) when compared with control group I. The activity of MAO-A in 200 mg/kg treated animals showed a significant reduction (P<0.05). The activity of MAO-A in 400 mg/kg treated animals show significant reduction (P<0.01) in comparison with negative control group as shown in **Table no: 2 and Fig no: 6.**

Effect of EERC on Protein Level

In the present study, the protein level of Aβ 25-35 induced group show significant (P<0.05) increase when compared with control group. A significant decrease (P<0.05) in the level of protein was observed for 200 mg/kg of EERC treated group

as shown by the results in **Table no: 2 and Fig no: 7.**

Effect of EERC on Super Oxide Dismutase

SOD levels in the brain was significantly reduced (P<0.01) in Aβ 25-35 induced group when compared to control group. Treatment with EERC at 200 and 400 mg/kg dose level showed a significant increase (P<0.01 and P<0.01) respectively, when compared with negative control group as the results were shown in **Table no: 2 and Fig no: 8**.

Effect of EERC on Glutathione Peroxidase

The GPx in the dementia induced mice (group II) showed significant (P<0.01) reduction in the enzyme activity when compared with the control group. The treatment with EERC at 200 and 400 mg/kg showed the significance (P<0.05 and P<0.01) respectively when compared with negative control group as shown in Table **no: 2 and Fig no: 9.**

Effect of EERC on Glutathione Reductase

The glutathione reductase activity of Aβ 25-35 induced group showed significant (P<0.01) decrease in the activity when compared with control group. The two groups with 200 and 400 mg/kg doses of EERC treated animals showed significant increase (P<0.01) when compared with negative control group. The results are shown in **Table no: 2 and Fig no: 10.**

Effect of EERC on Vitamin-C

The amount of vitamin-C present in Aβ 25-35 induced group shows the significant (P<0.01) decrease the activity when compared with control group. The treatment with 200 mg/kg dose showed significance (P<0.05) when compared with negative control group as shown in **Table no: 2 and Fig no: 11**

Discussion

The present study has revealed the neuroprotective effect of EERC on Aβ 25-35 induced cognitive deficits in mice. *Rubia cordifolia* is a medicinal plant with antioxidant properties. IntraCerebroVentricular injection of Aβ 25-35 induced impairment of memory assessed by passive avoidance and Morris water tests. Aβ 25-35 has the potential to induced oxidative stress in the brain cholinergic hypo function, elevation of AChE and MAO[21, 22, 23]. Moreover, it has been reported that it induces the production of hydrogen peroxide and lipid peroxide in hippocampal neurons of the rat brain[24,] and the induction of 4-hydroxy-2-nonenal and 8-hydroxy-2-deoxyguanosine (a marker of oxidative damage to DNA) immunoreactivities following infusion of Aβ 1-42 in mice brain. In the present study, we have found a significant decrease in the level of antioxidant enzymes and the elevate levels of AChE and MAO in mice brain after a single injection with Aβ 25-35. Furthermore in balance, each antioxidant enzyme were also observed and demonstrated that continuous i.c.v infusion of Aβ 1-42 in mice resulted in a significant decrease in protein expression of SOD, GPx, and glutathione-s-transferase in rat brain. Glutathione[25] (GSH) is the major non-protein thiols antioxidant in mammalian cells and it is considered to be the main intracellular redox buffer. GSH protects cellular protein-thiols against irreversible loss, thus preserving protein function. The position that GSH takes part in the redox-sensitive signaling cascade has been receiving growing support[26]. One of the most importance GSH-dependent detoxifying processes involved is glutathione peroxidase (GPx), which plays a central role in the removal of hydrogen and organic peroxides and leads to the formation of oxidized glutathione (GSSG). GSSG is reduced back to its thiols form (GSH) by the ancillary enzyme glutathione reductase (GRD), leading to the consumption of NADPH, which is mainly produced in the pentose phosphate pathway. GSH also takes part in xenobiotic conjugation with the assistance of several glutathione S-transferase isoenzymes. GSH conjugates or GSSG can be eliminated from the cell by the family of ATP dependent transporter pumps. The inhibition GSH synthesis leads to an increase in Aβ induced cell death and intracellular Aβ accumulation[27].

It's been suggested that antioxidant might contribute to the prevention of alzheimer's disease. Antioxidant such as beta carotene and vitamins C, E and A may protect cells from the type of damage that leads to aging in the brain and tissues. Both vitamin C and E are antioxidants which are likely to reduce oxidative stress and injury in the central nervous system, this may reduce the Aβ plaque deposition in the neuronal cells[28].

Presently our study, with regards of non enzymatic antioxidant we have estimated the amount of vitamin C present in the whole brain homogenate. We found an increase in the activity of vitamin C in the EERC treated group. We found that treatment with EERC ameliorated cognitive deficits in Aβ 25-35 injected mice, especially shows increase in step down inhibitory avoidance and in the exploratory behaviour. Step-down inhibitory avoidance task is a classic task of memory with a strong aversive component. EERC exerted significant effects in short-term retention and increase in long-term retention of this task. However, since the time used in our study was relatively short, it is possible that higher rates of memory improvement might be found with longer periods of treatment. We also examined the effect of EERC on the open-feld test as it is presented which allowed us to study the effect of the treatment on the general locomotor

activity of the animals. It was observed that animals treated with EERC had an increase in the number of head dippings, line crossings and rearings in the open filed. These results of our experiments have been consistent with its favorable effect on locomotor activity. In the water-maze test, consumption of EERC decreases the escape latency almost to normal levels in a dose dependent manner. It is possible that neuroprotection plays a role in favorable effect of EERC on Aβ 25-35 induced cognitive deficits.

The AChE activity has been shown to be increased around Aβ plaques in alzheimer's brain. The calcium influx followed by oxidative stress is involved in the increase in activity of AChE induced by Aβ 25-35 peptide, decreasing cell membrane order and ultimately leading to the exposure of more active enzyme. The observation that Aβ peptide increases AChE activity indicates that it can be possible to ameliorate cholinergic function, by inhibiting Aβ induce increase in AChE activity. The AChE activity in the brain was increased in mice treated with Aβ 25-35 when compared with the normal. In addition, the Aβ 25-35 induced increase in AChE was attenuated by EERC treatment.

MAO is an important enzyme involved in the metabolism of a wide range of monoamine neurotransmitters, including noradrenalin, dopamine, and 5-hydroxytryptamine. MAO exists in two forms, A and B. MAO-A in the metabolism of the major neurotransmitter monoamines. MAO-A inhibitors have been accepted to treat depression. MAO-A and MAO-B in the brain have been implicated in the etiology of Alzheimer's disease. Elevations in MAO-A in Alzheimer neurons have been linked to increase in neurotoxic metabolites and neuronal loss. Free radical generation by Aβ or other noxious stimuli could contribute to an imbalance between the production of nitric oxide and oxygen radicals and precipitate in oxidative stress. In conclusion, we suggest that markedly EERC improves cognitive deficits induced by Aβ 25-35, and this effect is mediated by the antioxidant properties of EERC. Future studies should determine the specific components in EERC responsible for preventing cognitive impairment.

Conclusion

Rubia cordifolia Linn. is a well known plant which is being used in Indian traditional medicine, as a antioxidant of CNS associated disorders, still there are some scientific evaluation to been made. Hence this research is emphasized to make the evident effect of the whole plant on memory disorder representing alzheimer's disease.

The investigation was carried out on cognitive impairment with relevance of the hypothesis, on Aβ induced AChE and MAO, oxidative stress signaling and impaired behavioural performance.

- In step down inhibitory avoidance performance the EERC at both 200 and 400 mg/kg exhibited significant retention of STM and LTM task.
- The spatial learning in water maze task showed the significant memory retention indicated by the decrease in escape latency at both dose levels of EERC in 200 and 400 mg/kg respectively.
- EERC had shown the exploratory behavioural activities and it is assessed by head dipping, rearing and line crossings. The treatment of EERC had shown the behavioural improvement on both doses.
- EERC at 200 and 400 mg/kg had shown the significant reduction in the elevated enzyme level of acetylcholine esterase which indicates the potential to increase cognitive function through the decreased degradation of acetyl choline.
- In mono amino oxidase enzyme activity, the EERC indicated a decrease in the levels of MAO-A. This depicts the regulative property of the extract on biogenic amines through the inhibitory action on MAO.
- The oxidative stress involved by the administration of Aβ produced neurotoxicity indicated the decreased levels of super oxide dismutase, glutathione peroxidase, glutathione reductase and vitamin C. Treatment of EERC shows the protection of these antioxidant enzymes on both 200 mg/kg and 400 mg/kg dose level respectively due to the rejuvenating property of the extract.

In conclusion, the neuroprotective activity of the plant *Rubia cordifolia* on alzheimer's type of dementia may be due to the inhibiting activity against AChE, MAO, free radical scavenging activity and they can be expected to be a pivot sense in neurotoxicity.

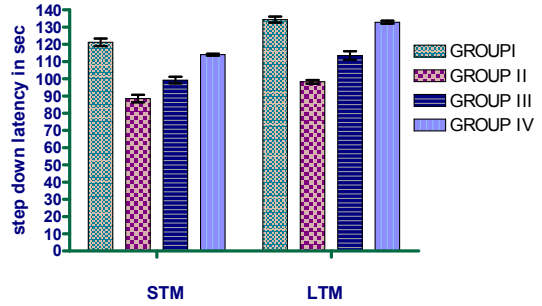
Table no: 1

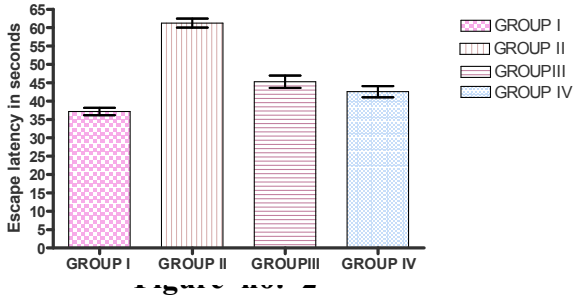
GROUP	Step down inhibitory avoidance		water maze	Expolatory Behaviour			Y-Maze
	STM	LTM	Escape latency (sec.)	Head dippings	Rearing	Line crossings	PERCENTAGE ALTERNATION
I	121.16±5.35	134.33±4.25	37.166±0.98	10.4±2.27	23±2.5	36±.85	70.52±1.5
II	* 88.52±5.4	* 98.23±2.7	* 61.25±1.24	* 4.1±0.4	* 10.16±1.98	* 14±0.92	* 55.25±2.0
III	* 99.21±4.69	* 113.52±6.	* 45.16±1.69	* 7.5±0.94	* 15.5±1.09	** 29.3±1.13	* 59.46±1.32
IV	* 114±1.36	* 132.83±2.16	* 42.17±1.54	** 9.8±0.8	** 21.1±0.76	** 34.1±1.83	* 68.60±1.89

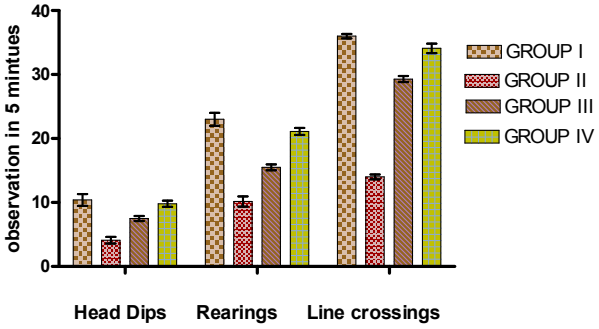
Table no: 2

GROUP		I	II	III	IV
ACETYLCHOLINE ESTERASE	Micro mole/min/mg Protein (seconds)	15.95±0.99	* 18.33±0.72	# 15.55±1.89	** 16.36±.93
MAO	MONO A	23.57±1.23	* 28.29±1.05	** 27.56±1.47	* 24.16±1.03
TOTAL PROTEIN	Mg/dl tissue	6.98±2.40	* 5.12±1.73	# 5.45±2.5	* 6.15±2.34
SOD	Units/min/mg protein	8.88±0.57	* 5.96±0.18	* 7.37±0.36	* 8.01±0.37
Glutathion peroxidase	Units/min/mg protein	35.15±1.34	* 24.26±1.20	** 28.46±0.65	* 33.28±1.36
Glutathion reductase	Units/min/mg protein	32.40±0.61	* 24.53±0.49	* 28.59±0.53	* 31.51±0.85
Vit-c	Micro gm/mg protein	0.921±0.045	* 0.432±0.069	# 0.441±0.019	* 0.789±0.023

Values are expressed as mean± SEM of 6 animals.
Symbol represents the statistical significance done by ANOVA, followed by Dunnet's "t" test. *P<0.05, **P<0.01, #P>0.05 non significant.







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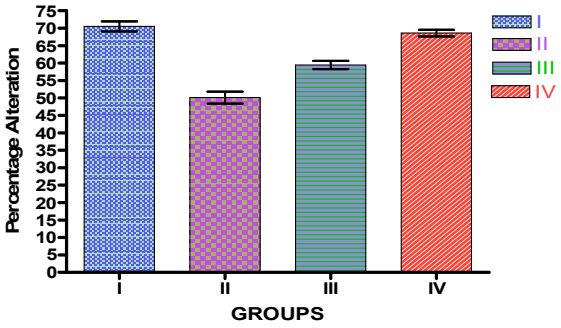

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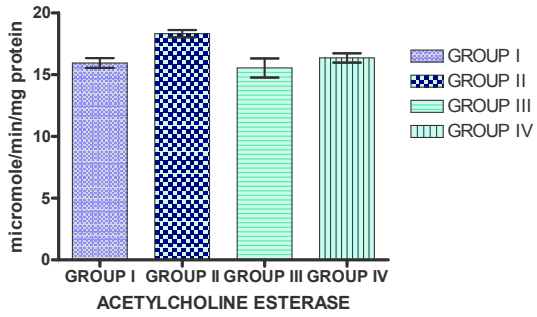

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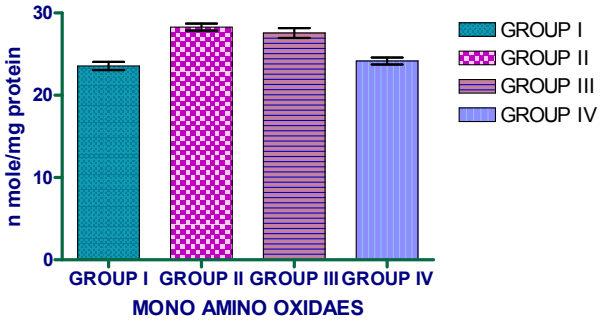

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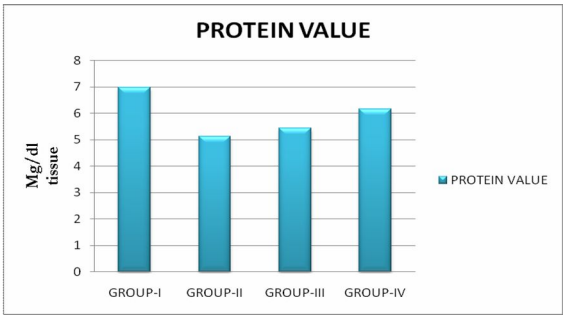

Figure no: 7

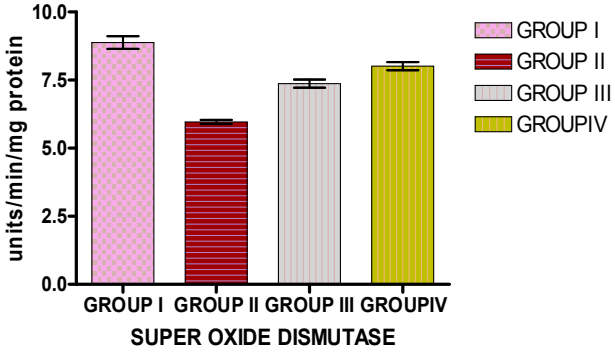

Figure no: 8

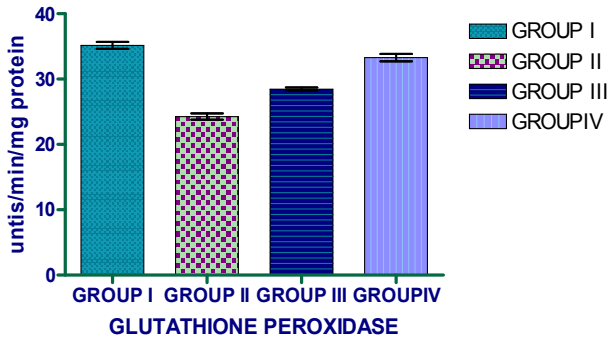

Figure no: 9

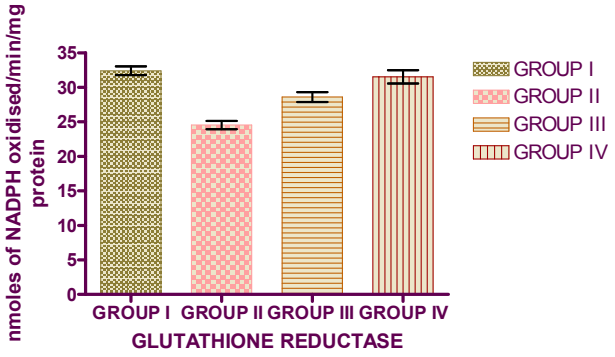

Figure no: 10

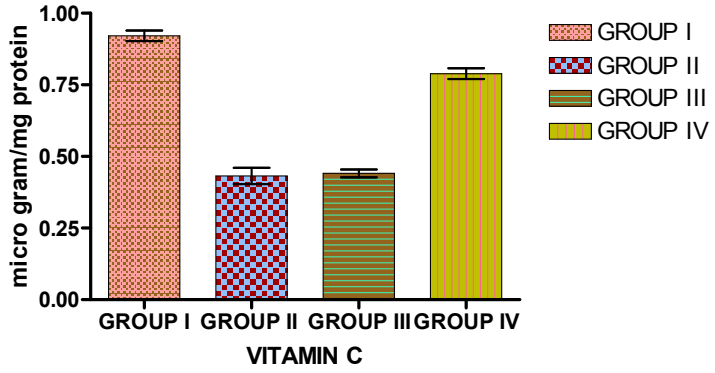

Figure no: 11

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