EFFECT OF METHANOLIC LEAF EXTRACT AND ETHYL ACETATE FRACTION OF *BUTEA MONOSPERMA* ON β-AMYLOID (25-35) INDUCED AMNESIA IN LABORATORY ANIMALS

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ABSTRACT

Alzheimer's disease (AD), mainly caused by the accumulation of β-amyloid (Aβ), increased acetylcholinesterase enzyme (AChE) and monoamino oxidase (MAO) with reduced turnover of biogenic amines. Oxidative stress in AD induces elevation of corticosterone and produce subsequent neuroimmune changes. Main objective of this study was to assess the pharmacological activity of B. monosperma leaf (methanolic extract and ethyl acetate fraction) on β-amyloid peptide induced amnesia in mice. Currently, available drugs to treat AD like AChE inhibitors exert symptomatic relief but do not reduce progression of disease and have side effects. Hence, herbal medicines may provide potential effect as compared to conventional available synthetic drugs with less or no side effects. The methanolic extract and its fraction are subjected to jumping test, rectangular maze test, Y-maze test and biochemical estimations such as AChE, peroxidase, MDA and DPPH radical scavenging activity. From the above results, it was found that both methanol extract and ethyl acetate fraction of B. monosperma are having significant anti-amnesic activity and anti-oxidant activity. Ethyl acetate fraction is having more significant activity when compared to methanol extract. By identifying and isolating the chemical constituents responsible for anti-amnesic and anti-oxidant activities, we may find a breakthrough in the treatment of other neurodegenerative disorders which occur due to oxidative stress.

KEY WORDS

Alzheimer's disease, B.monosperma, β-amyloid peptide, Y-maze test and biochemical estimations

Introduction

Dementia is a syndrome characterized by disturbance of multiple brain functions, including memory, thinking, orientation, comprehension, calculation, learning capacity, language and judgement. The impairments of cognitive function are commonly accompanied and occasionally preceded by deterioration in emotional control, social behavior or motivation (Alan, 1996).

Herbal medicines employed in traditional folklore treatment strategies provide a wide remarkable application in human ailments. Traditional therapeutic techniques still play a potential role in covering the essential health benefits in developing parts of the world. The World Health Organization (WHO) reported that the world's 80% of population relies on conventional medication. In the present scenario, 25% prescription written contains at least one active ingredient of plant origin (Lin *et al*., 2003).

Ethnopharmacological advancement and bioassay-guided fractionation and isolation had been provided the best lead for identifying the potential AChE inhibitors from herbs, as well as those for memory related disorders. A wide variety of medicinal plants had been implicated to possess AChE inhibitory effect and may be appropriate to treat the neurodegenerative disease such as AD (Rao *et al*., 2005). To validate their folklore use for the treatment of different diseases. As CNS active agents, identification of *Rawolfia serpentina, Mucuna pruriens, Ocimum santum Withania somnifera, Centella asiatica* and *Bacopa monneria* indicated the importance for treatment of brain related disease and disorders (Bhattacharya *et al*., 2000). Especially *Centella*

asiatica and *Bacopa monneria, Curcuma longa* and *Gingo biloba* are used to treat loss of memory including Alzheimer's types of dementia (Gertz and Kiefer, 2004). There were significant evidences to sustain the conception that abnormal production of reactive oxygen species (ROS) called as free radicals along with amyloid beta (Aβ) protein causes neuronal vulnerability and leads to neuropathological disease, such as AD. Apart from the production of ROS and Aβ, the involvement of AChE, MAO, neurotransmitters and neurohormonal changes were consistent during the neuropathology of AD.

One of the folklore claims of the plant *Butea monosperma* is used as rejuvenator in the treatment of neurodegeneration and in memory. Experimentally the present study was designed to determine the folklore effect of the selected plant extracts and the bioactive molecule in the involvement of AChE, ROS, neuroimmune and neuroendocrine pathways for the learning and memory process (Kasture *et al,,* 2002; Soman *et al*., 2004) evaluated the effect of leaf extract on stress, cognition, anxiety in rats.

Main objective of the study is to assess the pharmacological activity of *B. monosperma* leaf extract on β-amyloid peptide induced amnesia in mice. Currently available drugs to treat AD like AChE inhibitors exert symptomatic relief but do not reduce progression of disease and have side effects. Herbal medicines may provide potential effect as compared to conventional available synthetic drugs with less or no side effects.

Materials and Methods

Chemicals

Acetylthiocholine iodide, dithiobisnitro benzoic acid, reduced glutathione, β-amyloid $_{(25-35)}$ were purchased from Sigma Aldrich,USA. Other reagent solvents including ethyl acetate, methanol used were analytical grade purchased from Himedia laboratories, Hyderabad, India. DPPH, ascorbic acid, thiobarbituric acid, sodiumdodecyl sulphate, tetra ethoxy propane, trichloro acetic acid, donepezil were purchased from Himedia laboratories, Hyderabad, India.

Plant material and extraction

The leaves of *Butea Monosperma* were collected from the Kakatiya University, Warangal, Telangana, India, during month of May and authenticated by botanist Prof. Mustafa, department of Botany, Kakatiya University, Warangal, Telangana, India. The collected leaves were shade dried, powdered and extracted with Methanolic solvent by using soxhlet apparatus and fractionated with Ethyl acetate solvent.

Experimental animals

Swiss albino mice (male) weighing 22-25 g at the age of 5-6 weeks, obtained from Sainath agencies, Hyderabad was used for the pharmacological studies. The animals were kept under standard conditions maintained at 23-25°C, 12h light /dark cycle and given standard pellet diet (Vyas Labs, Hyderabad) and water *ad libitum.* The animals were acclimatized to the laboratory conditions for a week prior to the experimentation and randomly divided into five groups of six animals each. Handling and experimentation were conducted in accordance with the approved guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi and the experimental protocol was approved by Institutional Animal Ethical Committee (IAEC/05/UCPSc/KU/2016).

Grouping and induction of neurotoxicity

Neurotoxicity was induced by intra cerebroventricular (i.c.v.) injection of A β $_{(25-35)}$ peptide by identifying bregma point in the skull. Each animal was injected with 10μl which contain 10μg of β-amyloid peptide (Laursen and Belknap,1986). Except group 1-positive control, received with i.c.v. injection of phosphate buffered saline (PBS), group 2-negative control received only with i.c.v. injection of β-amyloid peptide, group 3-animals injected with β-amyloid peptide and treated with Donepezil (p.o.) 5mg/kg which is used as standard drug, group 4-animals are injected with β-amyloid peptide and treated with 200 mg/kg of ethyl acetate fraction, group 5-animals are injected with β-amyloid peptide and treated with 200 mg/kg of methanol extract. Drug treatment started on 14th day of the β-amyloid peptide treatment and continued for six days. On the 7th day of the drug treatment or 21st day after β-amyloid peptide treatment behavioral studies and biochemical parameters were estimated (Hanish *et al*., 2011; Singh *et al*., 2011 and Hanish *et al*., 2011).

Jumping box (conditioned avoidance test):

It is done by using medicraft jumping box. Box divided into 2 equal chambers by plexiglass partition, with a gate providing access to adjacent compartment through 14 x17 cm space. In each trial animal is subjected to light for 30 seconds followed by a sound stimulus for 10 seconds. Immediately after sound stimulus, mice receive a single low intensity foot shock (0.5 mA, 3 sec). Each animal received a daily session of 15 trials with an inter trial duration of 15 seconds for 5 days (Barros *et al.*, 2000).

Rectangular Maze test:

Assessment of memory was done using medicraft rectangular maze. The apparatus consisted of three interconnected chambers A, B and C. Chamber B constituted the maze. Food deprived mice were placed in chamber A and challenged to learn and remember the location of C, after travelling through B. Their presence in chamber C was indicated by a pilot light. Chamber C contained the reward which was food for the hungry animal. The animals were trained for consecutive daily sessions, and the time required to traverse the maze was noted. They were considered trained when the maze completion time for 3 consecutive days was more or less constant. Maze traverse time was then recorded for each animal before and after drug treatment (Parle *et al.*, 2004).

Y-maze test:

The Y-maze task was used to measure the spatial working memory in mice. The maze is made of gray plastic. Each arm is 40 cm long, 13 cm high, 3 cmwide at the bottom, 10 cm wide at the top, and converged at an equal angle. Each mouse was placed at the end of one arm and allowed to move freely through the maze for 8 min. Mice tend to explore the maze systematically, entering each arm in turn. The ability to alternate requires that the mice know which arm they have already visited. The series of arm entries, including possible returns into the same arm, is recorded by a video-tracking system (VJ Instruments, Washim, Maharastra, India). Alteration is defined as the successive entries into the three arms, on overlapping triplet sets. The percentage of alteration is calculated as the ratio of actual alterations to possible alterations, defined as the total number of arm entries minus two, and multiplied by hundred. Typically, mice exhibit an alteration percentage of 60–70%, and perform 25–35 arm entries within the 8-min session (Reddy, 1997).

BIOCHEMICAL ESTIMATIONS

Acetylcholinesterase (AChE) enzyme:

Acetylcholinesterase (AChE) enzyme levels are estimated by as per the method described by Ellman *et al.*, 1961. In a Potter-Elvehjem homogenizer 20 mg of brain tissue/ml with Phosphate buffer (pH 8, 0.1 M) was homogenized. 0.4 ml aliquot was added to cuvette contain 2.6 ml of 0.1M phosphate buffer (pH 8). To the photocell 100 µl DTNB solution was added and absorbance was read at 412 nm. Then change of absorbance was recorded after adding 20 µl of the acetylthiocholine iodide and change in absorbance/minute was calculated and activity of enzyme is expressed as µmoles/min/g tissue

Assay of glutathione peroxidase:

GSH was determined by its reaction with 5,5-dithiobis (2-nitrobenzoic acid) DTNB to yield a yellow chromophore which was measured spectrophotometrically (Lawrence and Burk, 1976). The brain homogenate was mixed with an equal amount of 10% trichloro acetic acid and centrifuged at 2000 g for 10 minutes. The supernatant was used for GSH estimation. To 0.1 ml of processed tissue sample, 2 ml of phosphate buffer (ph 8.4), 0.5ml of DTNB and 0.4 ml of double distilled water were added and the mixture was shaken vigorously. The intensity of color developed was read at 420 nm immediately in spectrophotometer. The activity of GPx is expressed as µmoles/ minutes/ mg protein.

Estimation of MDA:

MDA which is a measure of lipid peroxidation was described by Ohkawa *et al.*, 1979. Briefly, brain tissues were homogenized with 10 times (w/v) 0.1 sodium phosphate buffer (pH 7.4). the reagents acetic acid 1.5 ml (20%) pH 3.5, 1.5 ml thiobarbituric acid (0.8%) and 0.2 ml sodium dodecyl sulfate (8.1%) were added to 0.1 ml of processed tissue sample. Mixture was then heated at 100 c for 60 minutes. Mixture was then cooled with tap water and 5 ml of n-butanol:pyridine (15:1 % v/v), 1ml of distilled water was added. Mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 minutes, organic layer was withdrawn and absorbance was measured at 532 nm using spectrophotometer. 1,1, 3, 3-tetra ethoxy propane is used as standard.

DPPH radical scavenging activity:

The capacity to scavenge the ''stable'' free radical DPPH by methanol and ethyl acetate fraction was measured which is based on the reduction of methanolic solution of the coloured free radical of 1, 1- diphenyl-2-picryl hydrazyl (DPPH) (Harikiran and Narsimha Reddy, 2017). A methanol DPPH solution (0.1 mM, 1 ml) was mixed with serial dilutions (10, 20, 40, 60, 80 µg/ml) of the methanol extract and ethyl acetate fraction and incubated for 30 min at room temperature. For each concentration, the assay was run in triplicate and the absorbance was read at 517 nm. Ascorbic acid was used as standard to compare with extract and fraction. IC_{50} (the antiradical dose required to cause a 50% inhibition) for ascorbic acid, methanolic extract and ethyl acetate fraction was determined.

Results and discussion

The leaves of *Butea monosperma* were shade dried, powdered and extracted with methanolic solvent and the ethyl acetate fraction was prepared from the methanolic extract. Yield is noted in terms of w/w of dry material and yield was found to be 12.5% for methanol extract and 6.5% for ethyl acetate fraction. The test extract and fraction were considered as nontoxic because it did not show any toxic signs or symptoms and mortality in the oral dose of 2000 mg/kg of the both methanol extract and ethyl acetate fraction in mice. According to OECD-423 guidelines, the LD50 of 2000 mg/kg and above is mentioned as unclassified. So further pharmacological screening is carried out. Hence two doses (1/10th and 1/5th of 2000 mg/kg) methanolic extract and their fraction with ethyl acetate were selected for the neuroprotective study.

Jumping box (conditioned avoidance test):

The open field habituation memory was depicted in the Table 1. In jumping box test there was an in increase in latency period in negative control group (24.6±2.07) when compared to positive control (2.5±0.22), and there is decrease in latency period in groups treated with M. extract (19.5±0.67) and EA fraction (11±1.09).

Table 1: The latency period of positive control, negative control, standard, methanol extract (M extract), ethyl acetate fraction (EA fraction) groups in jumping box test.

Group	Mean ± SD
Positive control	2.5 ± 0.22
Negative control (βA)	24.6 ± 2.07
Standard(dpz)	7 ± 1.21***
EA fraction	19.5 ± 0.6708*
M extract	11 ± 1.0954***

Values are expressed as mean±SEM (n=6). *p<0.05, **p<0.01,***p<0.001 when compared to negative control group. ANOVA (one-way) followed by Bonferroni's test

Table 2. Effect of methanol extract (M extract) and ethyl acetate fraction (EA fraction) on behavioral activity by Y-maze test.

Group	Mean ± SD
Positive control	2.5 ± 0.22
Negative control (βA)	24.6 ± 2.076
Standard (dpz)	7 ± 1.2***
EA Fraction	44.42±1.71***
M extract	32.19±2.18*

Values are expressed as mean±SEM (n=6). *p<0.05,**p<0.01,***p<0.001 when compared to negative control group. ANOVA (one-way) followed by Bonferroni's test.
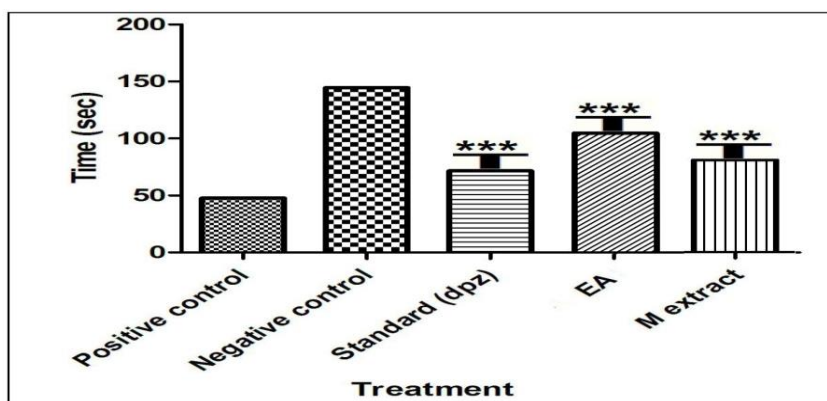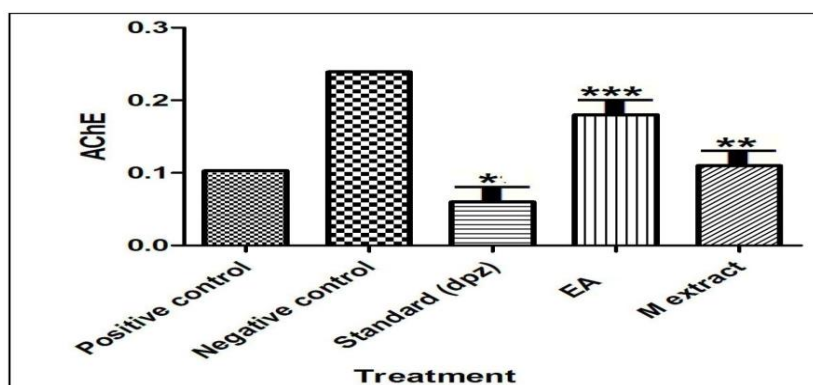
Figure 1: Maze traversing period of positive control, negative control, standard (dpz), methanol extract (M extract) and ethyl acetate fraction (EA) treated groups. All values are expressed as mean±S.E.M (n=6). *p<0.05, **p<0.01, ***p<0.001 when compared to negative control group. ANOVA (one-way) followed by Bonferroni's test

Figure 2: Acetylcholinesterase levels of positive control, negative control, standard (dpz), methanol extract (M extract) and ethyl acetate fraction (EA) treated groups. All values are expressed as mean±S.E.M (n=6). *p<0.05, **p<0.01,*p<0.001 when compared to negative control group. ANOVA (one-way) followed by Bonferroni's test.**
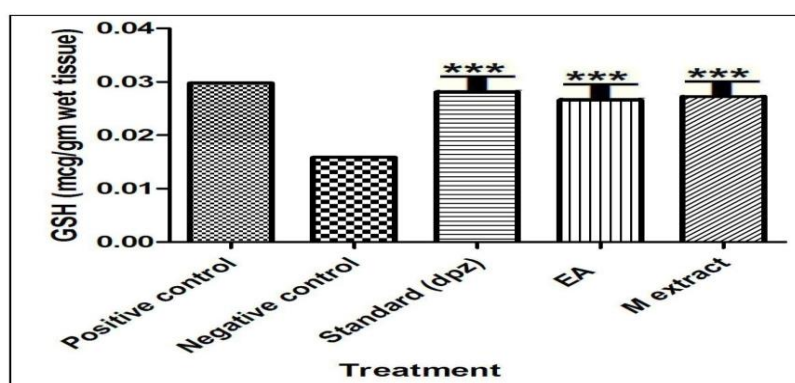

Figure 3: Glutathione levels of positive control, negative control, standard (dpz), methanol extract (M extract) and ethyl acetate fraction (EA) treated groups. All values are expressed as mean±S.E.M (n=6). *p<0.05, **p<0.01, ***p<0.001 when compared to negative control group. ANOVA (one-way) followed by Bonferroni's test.
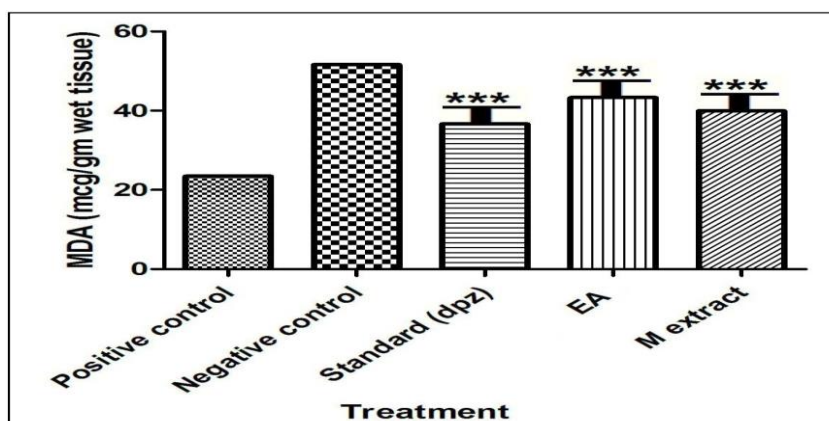
Figure 4: MDA levels of positive control, negative control, standard (dpz), methanol extract treated (M extract), ethyl acetate fraction (EA) treated groups. All values are expressed as mean±S.E.M (n=6). $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ **when compared to negative control group. ANOVA (one-way) followed by Bonferroni's test.**

Rectangular maze test:

The hippocampal learning of Aβ induced group (negative control) was declined and shown a significant ($p<0.001$) increase in escape latency while comparing the positive control group. In the treatment groups, the low dose of EA fraction (200 mg/kg) depicted a significant ($p<0.05$) decrease in escape latency duration, the high dose does not significantly ($p>0.05$) reduced the duration of time taken to escape onto the escape platform but there was a significant ($p<0.05$) change in high dose treated animals when compared to the low dose treated animals. The readings were showed in Figure 1. From the results, in rectangular maze test there was an increase in maze traverse period in negative control group (144.66±10.05) when compared to positive control (47.86±2.37), and there is a decrease in traverse period in groups treated with ethyl acetate fraction (104.83) and methanol extract (81.16±2.21).

Y-maze test

The percentage alteration in the neurotoxicity-induced group (II) was significantly ($p<0.001$) reduced when compared with the positive control group. In the treatment groups, the ethyl acetate fraction exhibited significant improvement when compared with methonalic extract (Table 2).

BIOCHEMICAL ESTIMATIONS

The difference in biochemical estimations in treatment groups were summarised in the Figure 2, 3 and 4. The i.c.v injection of Aβ peptide in negative control animals showed an extremely significant ($P<0.001$) increase in brain AChE levels. The AChE enzyme levels were significantly decreased in the treatment groups and indicated a difference with $p<0.01$, $p<0.05$, $p<0.001$ and $p<0.05$, respectively when compared with the amnesia induced group.

From biochemical parameters, it was found that acetyl cholinesterase levels are increased in negative (0.2394±0.015) when compared to positive control (0.1031±0.0012), and decreased levels are observed in ethyl acetate fraction (0.1804±0.0026) and methanol extract (0.1144±0.0026).

Glutathione levels are decreased in negative (0.0159±0.00023) when compared to positive control (0.0298±0.00014), and levels are increased in ethyl acetate fraction (0.0293±0.00708) and methanol extract (0.0266±0.00186).

MDA levels are increased in negative (51.63±0.5464) when compared to positive control (23.46±0.305), and decreased levels are observed in ethyl acetate fraction (43.31±0.2212) and methanol extract (39.98±0.3012).

The evaluation of antioxidant property on AD induced mice expressed the prospective effect of M.extract and EA.fraction from *Butea monosperma*. In the negative control animals, all antioxidant parameters of brain were significantly ($P<0.001$) decreased when compared to phosphate buffered saline treated animals (positive control group). There was a significant ($P<0.001$) increase in all the antioxidant levels were observed in the standard drug treated group. From DPPH scavenging assay the IC50 of M.extract was found to be (38.53μg/mL), IC50 of EA.fraction was found to be

(26.42µg/mL), IC50 ascorbic acid was found to be (2.92µg/mL).

Conclusion:

The pharmacological evaluations on methanolic extract and ethyl acetate fraction of *Butea monosperma* indicated the anti-amnesic effect. The Neuroprotective and anti-amnesic effect of these drugs were evidently supported by decrement of neurotransmitter metabolic enzyme (AChE and glutathione) with escalation in antioxidants. Thus, to conclude the extracts and bioactive molecule from the ethyl acetate fraction expressed a prospective turnover of biogenic amine (serotonin and dopamine) for learning and memory process associated with neuroendocrine and neuroimmune bidirectional pathways.

Acknowledgements

Declaration of interest

The authors declare that no conflict of interest.

References

1. Alan M.P., Neurochemical Studies of Alzheimer's disease. Neurodegeneration, 5:381-391 (1996).
2. Lin R.D., Hou W.C., Yen K.Y., Lee M.H., Inhibition of monoamine oxidase B (MAO-B) by Chinese herbal medicines. Phytomed, 10:650-656, (2003).
3. Rao Sulochana B., Chetana M., UmaDevi P., Centella asiatica treatment during postnatal period enhances learning andmemory in mice. Physiol Behav, 86: 449-457, (2005).
4. Bhattacharya S.K., Bhattacharya A., Kumar A., Ghosal S., Antioxidant activity of Bacopa monniera in rat frontal cortex, striatum and Hippocampus, Phytother Res, 14:174-179. (2009).
5. Gertz H.J., Kiefer M., Review about Ginkgo Biloba Special Extract EGb 761 (Ginkgo). Cur Pharma des, 10:261-264, (2004).
6. Kasture V.S., Kasture S.B., Chopde C.T., Anti convulsive activity of *Butea monosperma* flowers in laboratory animals. Pharmacol Biochem Behav, 72: 965-972, (2002).
7. Soman I., Mengi S.A., Kasture S.B., Effect of leaves of *Butea frondosa* on stress, anxiety, cognition in rats. Pharmacol Biochem Behav, 79: 11-16, (2004).
8. Laursen S.E., Belknap J.K., Intra cerebro ventricular injections in mice. Some methodological refinements. J Pharmacol Methods, 16: 355–357, (1986).
9. Hanish Singh J.C., Alagarasamy V., Prakash Diwan V., Sateesh Kumar S., Narsimha Reddy Y., Neuroprotective effect of Alpinia galanga (L.) fractions on Aβ$_{(25-35)}$ induced amnesia in mice. J Ethnopharmacol, 138: 85- 91, (2011).
10. Hanish Singh J.C., Alagarasamy V., Sateesh Kumar S., Narsimha Reddy Y., Neurotransmitter Metabolic Enzymes and Antioxidant Status on Alzheimer's Disease Induced Mice Treated with Alpinia galanga (L.) Willd. Phytother Res, 25: 1061-1067 (2011).
11. Singh H.J.C., Alagarasamy V., Sateesh Kumar S., Narsimha R.Y., Neuroprotective potential of ethanolic extract of *Pseudarthria viscida* (L) Wight and Arn against β-amyloid $_{(25-35)}$ induced amnesia in mice. Ind J Biochemy Biophys, 48: 197-201, (2011).
12. Barros D.M., Izquierdo L.A., Mello S.T., Ardenghi P., Pereira P., Medina J.H., Izquierdo I., Molecular signaling pathways in the cerebral cortex are required for retrieval of one-trial inhibitory avoidance learning in rats. Behav Brain Res,114:183-192, (2000).
13. Parle M, Singh N., Animal models for testing memory, Asia Pac J Pharmacol, 16:101–120, (2004).
14. Reddy D.S., Assessment of notropic and amnestic activity of centrally acting agents. Indian J Pharmacol 29:208-221, (1997).
15. Ellman G.L., Courtney K.D., Anders V., Featherstone R.M., A new and rapid colorimetric determination of acetyl cholinesterase activity. Biochemic pharmacol, 7:88-95, (1961).
16. Lawrence R.A., Burk R.F., Glutathione peroxidase activity in selenium deficient rat liver. Biochemical and Biophysical Research Communications, 71,952–958, (1976).
17. Ohkawa H., Ohishi N., Yagi K., Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem, 95(2): 351–8, (1979).
18. Harikiran L, Narsimha Reddy Y. Evaluation of oxidative stress induction in rats following exposure silver nanorods. Tox mech meth, 27(4): 272-278, (2017).

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