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Anti-alzheimer activity of citrus *maxima* (J. Burm.) Merr fruit peel extract in mice with scopolamine induced alzheimer's disease

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Abstract
Alzheimer's disease is a progressive, neurodegenerative disorder that primarily affects the elderly population responsible for 60% of all dementia in people aged 65 or older. The present study deals with the pharmacological activity of fruit peels of *Citrus maxima* (J. Burm.) Merr. in Scopolamine induced Alzheimer's disease. Animals were maintained as per ethical guidelines for animal protection and welfare, bearing the Resolution No. 05/243/CPCSEA. Dt: 10/08/2015. The mice were divided into four groups and changes in behavioral aspects were analyzed by using Morris maze and Y Maze method. The studies were performed using Rivastigmine as standard. The whole brain after the studies were homogenized and examined for acetyl cholinesterase level. The results revealed the Anti Alzheimer activity of ethanolic extract *Citrus maxima* peel, with decrease in the brain acetyl cholinesterase level. Which could be considered as an effective tool for the treatment of Alzheimer's.

Keywords: *Citrus maxima,* Scopolamine, acetylcholinesterase level

Introduction

Alzheimer's disease (AD) is a progressive, degenerative and fatal brain disease, in which cell to cell connections in the brain are lost. Alzheimer's disease is the most common form of dementia [1]. Globally approximately 1-5% of the population is affected by Alzheimer's disease [2]. Women are disproportionately the victims of Alzheimer's disease, with evidence suggesting that women with AD display more severe cognitive impairment relative to age-matched males with AD, as well as a more rapid rate of cognitive decline [3].

Alzheimer is a peculiar disease of cerebral cortex identified by Dr. Alios Alzheimer in 1906 and was later named as Alzheimer's disease [4].

Alzheimer's disease is characterized by increasing loss of memory and cognitive function and at an anatomical level, by plaques of a protein called amyloid and by neurofibrillary tangles [5]. It is described as thin cortex speckled with usual brown clumps and irregular knots that was found to be growing inside the brain cells and are now known as amyloid plaques and neurofibrillary tangles.

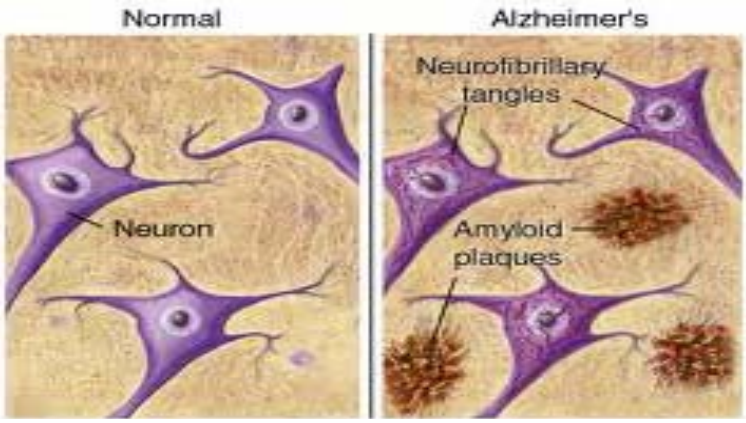


Fig 1: Difference in Normal and Alzheimer's brain.

Due to various side effects that are observed in the synthetic drugs the human population is now moving towards the natural source in search of remedies for Alzheimer's disease.

There are over 1.5 million practitioners of traditional medicinal system using medicinal plants in preventive, promotional and curative applications.

Citrus maxima commonly known as Papanus, distributed throughout India. Bark and root of *Citrus maxima* contain β-sitosterol, acridone alkaloid. Essential oil from the leaves and unripe fruits contain limonin, nerolol, nerolyl acetate and geraniol [6]. Like other citrus plant pommelos are rich in Vitamin C. They are generally used eaten as fruit. The present study is aimed to investigate the therapeutic effects of Ethanolic extract of *Citrus maxima* peels on Scopalamine induced mice.

Materials and Methods

Plant Collection and Authentication

Fresh fruits were collected from Cuddalore, Tamil Nadu, authenticated by Dr. G.V.S. Murthy, Scientist "F" and Head of Office, Botanical Survey of India, Southern Regional Centre, T.N.A.U campus, Lawley road Coimbatore- 641003. A specimen copy of the Herbarium is placed in the Department of Pharmacognosy with Voucher No: 18/PCOG/2015. The Peels of fruit *Citrus maxima* were separated and shade dried, cut into pieces, powdered and was stored in the air tight container.

Extraction

The dried coarsely powdered sample of *Citrus maxima*. (J. Burm.) Merr peels (500gm) was first extracted with n- Hexane (60-80 °C) in Soxhlet apparatus and then with solvents of increasing polarity like ethyl acetate and ethanol at 60 – 70 ºC. They were then followed with maceration with chloroform water. Each extract was concentrated using rotary vacuum evaporator. Ethanolic extract of *Citrus maxima* has been reported earlier to have significant *in vitro* anti-oxidant and *in vitro* acetyl cholinesterase activity than other extract. Thus Ethanolic extract of *Citrus maxima* was subjected for *in vivo* analysis.

Plant extract

Ethanolic extract of peels of *Citrus maxima.* Burm.

Animal selection and procurement

Healthy young Swiss-Albino mice (weighing about 20-30gm) were procured from the Madras Medical College animal house. The animals used for the entire study was approved by the Institutional Animal Ethical Committee which is certified by the Committee for the purpose of control and supervision of experiments on animals, India.

Approved CPCSEA Registration No: 05/243/CPCSEA Dated: 10/08/2015.

In Vivo Evaluation of Anti-Alzheimer Activity

Scopalamine induced memory impairment [7]

The reversible cholinergic receptor antagonist scopolamine induces memory loss by impairing memory acquisition possess of short term memory in rodents and humans. Scopolamine also interferes in CNS cholinergic neurotransmission resulting in memory impairment.

Grouping of animals

Animals were divided into 4 groups of six animals each.

Group I: Scopalamine (0.3mg/kg b.wt) was injected after training. TL (Transfer Latency) was recorded after 1 hour of injection.

Group II: Rivastigmine (0.3 mg/kg b.wt) was injected for 14 days and on the 14th day after 30min of drug administration; scopolamine (0.3mg/kg b.wt) was given i.p. TL was recorded after 1 hour of injection.

Group III: Test drug I (200mg/kg) was given orally for 14 days and on 14th day after 30min of drug administration; Scopalamine (0.3mg/kg b.wt) was given i.p. TL was recorded after 1 hour of injection.

Group IV: Test drug II (400mg/kg b.wt) was given orally for 7 days and on 7th day after 90min of drug administration; Scopalamine (0.3mg/kg b.wt) was given i.p. TL was recorded after 1 hour of injection.

Morris Water Maze

The Morris water maze was developed by Richard Morris in 1984. It is the most popular task in behavioral neuroscience; and in its most basic form, it assesses spatial learning and memory along with nonspatial discrimination learning. Performance in the Morris Water Maze is acutely sensitive to manipulations of the hippocampus.

In this model the animals are placed into a large circular pool of water and they can escape on to a hidden platform. The platform is hidden by its placement just below the water surface which is made opaque by mixing Titanium dioxide in the water. Therefore the platform offers no local cues to guide the escape behavior. The animal can escape from swimming by climbing on to the platform and with time the animal apparently learns the spatial location of the platform from any starting position at the circumference of the pool. Morris water maze consists of a large circular tank made of black opaque polyvinyl chloride or hardboard coated with fiber glass and resin and then surface painted white (1.8-2m in diameter and 0.4-0.6m high). The pool is filled upto a height of 30cm with water until the top of the platform is submerged 1cm below the water surface and maintained at around 25 °C and rendered opaque by addition of small quantity of milk or non-toxic white color. The pool is provided with filling and draining facilities and is mounted at waist level. The tank is hypothetically divided into 4 equal quadrants the platform (11cm^2) of 29 cm height is located in the centre of one of these 4 quadrants.

Before beginning acquisition training, mice are given a pertaining acclimatization session during which they are allowed to swim in the pool for 5min without the platform.

Fig 2: Morris water maze

The platform remains fixed in the position during the training session. Each animal is subjected to four consecutive trials for four days during which they are allowed to escape on the hidden platform and allowed to remain for 20seconds. Mice are released from the four points along the perimeter of the maze arbitrarily designated as N, S, W and E. Escape latency

time to locate the hidden platform in water maze is noted as an index of acquisition or learning. In case the animal is unable to locate the hidden platform within 120seconds, it is gently guided by hand to the platform and allowed to remain there for 20seconds. In each training session, the escape latency was recorded. After 4 days trail and 14 days treatment the animals were treated with amnesia inducing agent, 30 min after last oral dose of test compounds. After 1 hour mice were allowed to swim and the escape latency were recorded.

Y Maze Test

The Y-maze is a simple two-trial recognition test for measuring spatial recognition memory, it does not require learning of a rule, and thus is useful for studying memory in rodents, and in particular for the study of genetic influences on the response to novelty and recognition processes. Immediate working memory performance was assessed by recording spontaneous alternation behavior using Y maze made up of black painted wood. Each arm was 40cm long, 12cm high, 3cm wide at the bottom and 10cm wide at the top and converged in an equilateral triangular central area.

Fig 3: Y Maze.

Each mice were placed at the end of one arm and allowed to move freely through the maze during an 8 minute session. The series of arm entries was recorded visually. Entry was considered to be complicated when the hind paws of the mouse had completely entered the arm. Alternation was defined as successive entries into the three different arms (A, B and C) on overlapping triplet sets. The percentage of triads in which all three arms were represented i.e, ABC, CAB or BCA but not BAB was not recorded as an alternation to estimate short term memory. Percentage alternation was calculated as the ratio of actual to possible alternation (defined as the total number of arm entries minus two), multiplied by hundred as shown

%alternation = {(No. of alternations) / (Total arm entries - 2)} x 100

On the 14th day, 60 minutes after the treatment of last dose and 30 minutes after scopolamine dose arm entries were recorded visually and percentage alternation was calculated.

Estimation of Brain Cholinesterase (*In Vivo*) [8-10]

Acetylcholinesterase enzyme activity was estimated by Elman method.

Reagents

1. 0.1M phosphate buffer

- Solution A: 5.22gm of potassium hydrogen phosphate and 4.68gm of sodium hydrogen phosphate are dissolved in 150ml of distilled water.
- Solution B: 6.2gm of sodium hydroxide dissolved in 150ml of distilled water.

Solution B is added to solution A to get the deserved pH (8.0 or 7.0) and then finally the volume is made upto 300ml with distilled water.

2. DTNB Reagent

39.6mg of DTNB with 15mg of sodium bicarbonate is dissolved in 10ml of 0.1M phosphate buffer (pH 7.0)

3. Acetylthiocholine (ATC)

21.67mg of acetylthiocholine was dissolved in 1ml of distilled water.

Preparation of Brain Homogenate

The animals were scarified by anaesthetized using thiopentone and brains of the animals were removed quickly and placed in ice cold saline. The tissues were weighed and homogenized in 0.1M phosphate buffer (pH-8) and the brain homogenate was used for the estimation of Brain AchE level.

Estimation of Brain Acetyl cholinesterase level

The esterase activity was measured by providing an artificial substrate, acetylthiocholine (ATC). Thiocholine released because of the cleavage of ATC by AchE was allowed to react with the –SH reagent 5, 5'-dithiobis nitro benzoic acid, which is reduced to thio nitro benzoic acid, a yellow coloured anion with an absorption maxima at 412nm.

Assay Procedure

- ✓ 0.4ml of aliquot of brain homogenate was added to a cuvette containing 2.6ml of phosphate buffer (0.1M) and to this 100µl of DTNB was added.
- ✓ The contents of the cuvette were mixed thoroughly by bubbling air and absorbance was measured at 412nm in spectrophotometer, when absorbance reaches a stable value, it was recorded as the basal reading.
- ✓ 20µl of substrate (ATC) was added and change in absorbance was recorded for a period of 10minutess at intervals of 2minutes. Change in the absorbance per minute was determined.

Reagent	Sample	Blank
Phosphate buffer solution	2.6ml	2.7ml
Supernatant	0.4ml	0.4ml
DTNB	0.1ml	---

The enzyme activity was calculated using the following formula

$R = 5.74 (10^{-4}) \Delta A/Co$,

R = Rate in moles substrate hydrolyzed per min per gm of tissue,

ΔA= Change in absorbance per min,

Co = Original concentration of tissue (mg/ml).

Histopathology

The mice from each group were anaesthetized using intraperitoneal injection of thiopentone sodium. The brain was carefully removed without any injury after opening the skull. The collected brain was washed with ice cold normal saline

and fixed in 10% formalin saline. Paraffin embedded sections were taken 100µm thickness and processed in alcohol-xylene series and stained with Haematoxyli-Eosin dye. The sections were examined microscopically for histopathological changes in the hippocampal zone.

Statistical Analysis

The statistical analysis was carried by one way ANOVA followed by Dunnet's t test. P values <0.05 (95% confidence limit)

Result and Discussion

***In Vivo* Anti Alzheimer Activity**

The Ethanolic extract of *Citrus maxima* (EECM) were selected based on the above *in vitro* studies. It was given on the Mice in the dose of 200 and 400 mg selected as 1/10 and 1/5th of the dose which was proved to be non-toxic in acute toxicity studies.

Mice were studied for transfer latency using Morris Water Maze and Y maze. The transfer latency are tabulated in table 22 & 23 and plotted in Fig 51& 52.

Table 1: Effect of transfer latency using Morris Water Maze

S. No.	Group	Treatment	Acquisition Memory (Sec)	Retention Memory Day 15 (Sec)
1	I	Negative control Scopalamine (0.3 mg/kg)	19.66 ±1.632^{b}	18.88±1.602 ^{a}
2	II	EECM 200 mg/kg	18.66 ± 1.96^{a}	15.5 ± 1.51^{b}
3	III	EECM 400 mg/kg	14.16 ± 1.60^{a}	10.66 ± 0.80^{a}
4	IV	Rivastigmine (0.3 mg/kg)	14.5 ± 1.37^{a}	10.16 ± 1.47^{a}

Mean ±SD, n=6.

All values are expressed as Mean ±SD and datas were analysed by One Way Annova followed by Dunnett's test a-*P*<0.05: b-P<0.01 when compared with control group

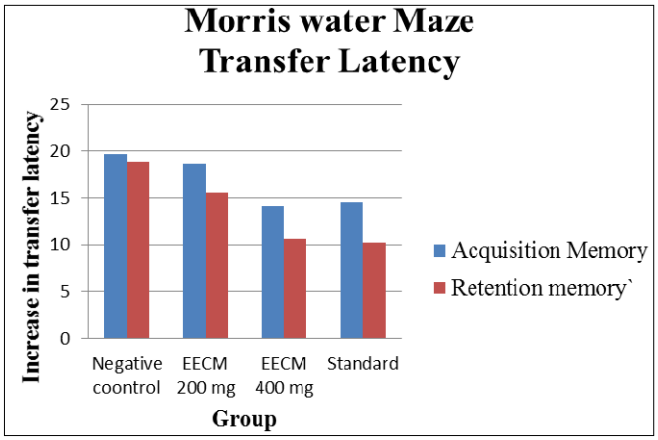


Fig 4: Morris water Maze Method.

Y Maze

Table 2: Effect on Transfer Latency using Y Maze

Group	Treatment	% alteration of mice
I	Negative control Scopalamine (0.3 mg/kg)	26.125± 0.63*
II	EECM 200 mg/kg	35.23 ± 0.75%**
III	EECM 400 mg/kg	38.54 ± 0.56%**
IV	Rivastigmine (0.3 mg/kg)	45.26 ± 0.43% *

Mean ±SD, n=6

$$\%\text{alternation} = \frac{(\text{No. of alternations})}{(\text{Total arm entries } -2)} \times 100$$

All values are expressed as Mean ±SD and datas were analysed by One Way Annova followed by Dunnett's test P*<0.05: P**<0.01 when compared with control group

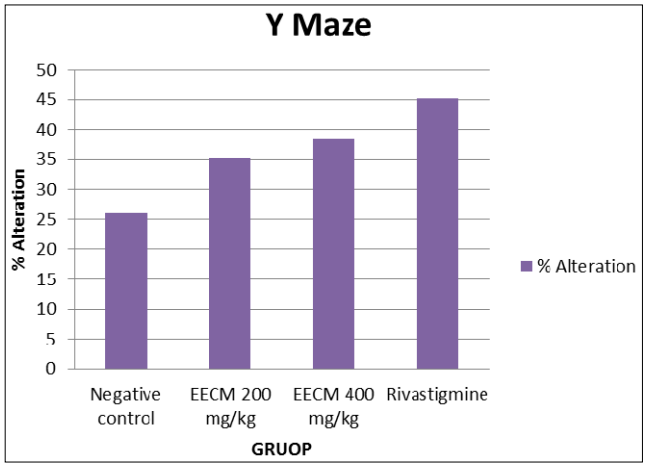


Fig 5: Effect on Transfer Latency using Y Maze

Study was done on Day 14 after the last dose of daily dose. Number of alterations and total arm entries were measured and Percentage alteration were calculated.

Estimation of brain acetyl cholinesterase activity

Brain is homogenized after the treatment and measured for the acetyl cholinesterase level using Elman's method.

Table 3: Brain Acetyl cholinesterase level

Group	Treatment	AChE Level Mmoles/Mg Protein
I	Negative control Scopalamine (0.3 mg/kg)	32.00±0.522^{a}
II	EECM 200 mg/kg	27.00±0732^{a}
III	EECM 400 mg/kg	22.00±0.593^{b}
IV	Rivastigmine (0.3 mg/kg)	19.00±1.06^{b}

Mean ±SD, n=6.

All values are expressed as Mean ±SD and datas were analysed by One Way Annova followed by Dunnett's test a-P<0.05: b-P<0.01 when compared with control group

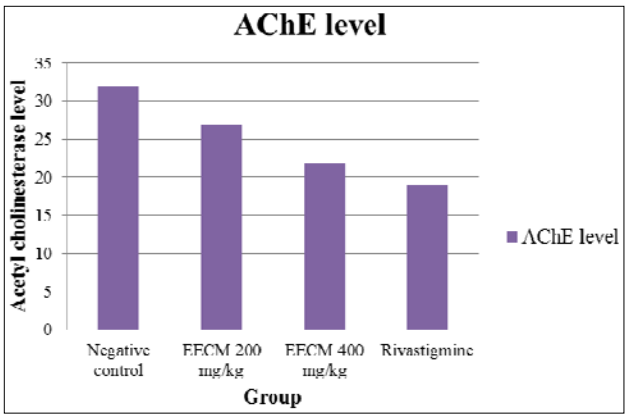
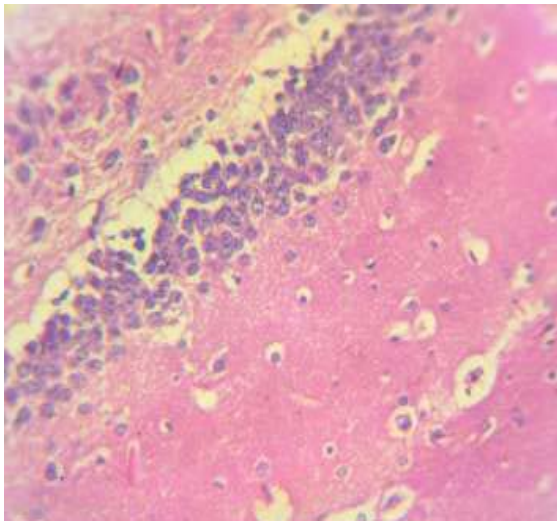


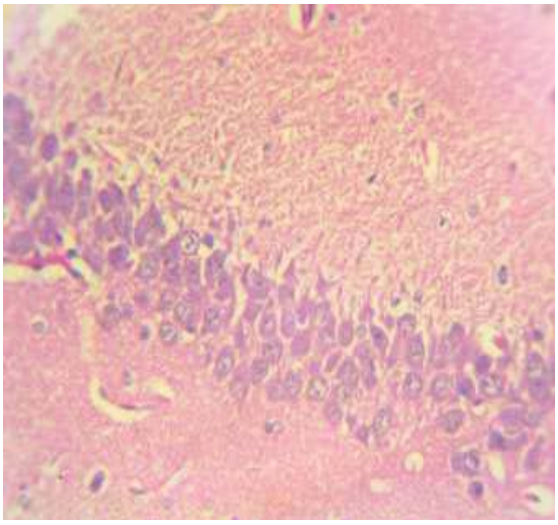
Fig 6: Brain acetyl cholinesterase level

Histopathology

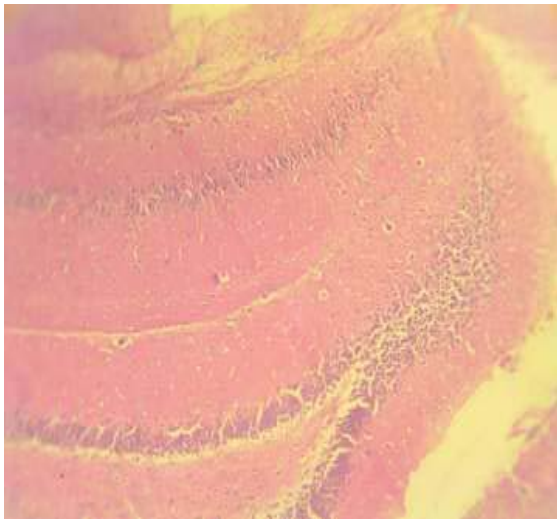
Histopathology of the brain of various rats were analyzed and figured out.



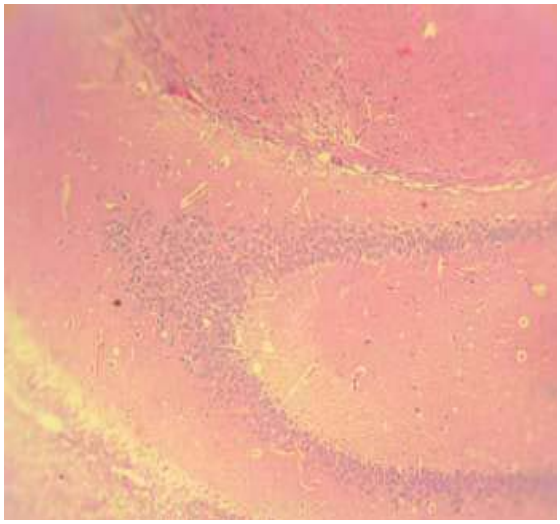
Histopathology of Group I (Scopalamine Induced)
Slight increase in density of the neuronal cells



Histopathology of Group II (200mg/kg)
Significantly decrease in density of the neuronal cells



Histopathology of Group III (400mg/kg)
Significantly increase in density of the neuronal cell



Histopathology of Group IV (Standard)
Normal density of neuronal cells.

Fig 7: Histopathology of various groups

Conclusion

The ethanolic extract of the peels of *Citrus maxima* was administered orally for fourteen days showed a dose dependent and significant improvement in memory of young mice and it successfully reversed the memory deficits induced by Scopalamine. Furthermore a significant decreased in acetyl cholinesterase level in mice brain thereby increasing the Acetyl choline (Neurotransmitter) level in the body.

These finding suggest that ethanolic extract of the peels of *Citrus maxima* has Anti Alzheimer activity. This study paves the way for the researchers to perform clinical studies.

Reference

1. http://www.human-emory.net/disorders_alzheimers.html
2. The World health report, Mental health new understanding, new hope (pdf).World Health Organization, 2001.
3. Dunkin JJ. The Neuropsychology of Women. Springer New York, 2009, 209-223.

4. Alzheimer's disease and Dementia. A Guide For Adult Protective Services Workers. available at http://www.dcf.state.fl.us/programs/aps/docs/ Dementia Pamphlet. Pdf.
5. ZoharaYaniv, Uriel Bachrach. Hand book of Medicinal plants; CBS publisher, 2007, 358-361.
6. Xu G, Liu D, Chen J, Ye X, Ma Y, Shi J. Juice Components And Antioxidant Capacity Of Citrus Varieties Cultivated In China, Food Chemistry 2008; 106(2):545-551.
7. Gupta SK. Drug screening methods (Pre-clinical evaluation of New Drugs), Jaypee Bross Medical Publishers, Ed-2, 2005, 433-436.
8. Khadabadi SS, Baviskar BA, Deore SL. Pharmacognosy and Phytochemistry, Pharma Med Press/BSP books, 2014, 523-524.
9. Ellman GL, Courtney DK, Andres V, Feathestone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology, 1961; 7:88-95.
10. Bhattacharya SK, Upadhyay SN, Jaiswal AK. Effect of Piracetam on electroshock induced amnesia and decrease in brain AchE in rats. Indian journal of Experimental Biology. 1993; 31:822-824.