Evaluation of Nootropic Activity of *Limonia acidissima* Against Scopolamine-induced Amnesia in Rats

*Limonia acidissima*’nın Sıçanlarda Skopolamin ile İndüklenen Amneziye Karşı Nootropik Aktivitesinin Değerlendirilmesi

**Amaç:** Bu çalışmada, sıçanlarda *Limonia acidissima*’nın nootropik aktivitesinin değerlendirilmesi amaçlanmıştır.

**Gereç ve Yöntemler:** Nootropik aktiviteyi değerlendirmek için *Limonia acidissima*’nin metanol ekstresi, standart olarak piracetam (200 mg/kg, i.p.) kullanıldı. İlaçların sıçanlarda öğrenme ve hafıza üzerindeki etkisi, metanol ekstresi ile indüklenen amnezi modelinde Y-labirent testi ve yükseltilmiş artı labirent testi kullanılarak değerlendirildi. Lokomotor aktivite, bir aktofotometre kullanılarak gerçekleştirdi. Ayrıca, sıçan beyinlerinin asetilkolinestreaz aktivitesi ve histopatolojik incelemesi yapıldı.

**Bulgular:** Metanol ekstresi ile *Limonia acidissima*’nın metanol ekstresi, Y-labirent testi ve yükseltilmiş artı labirent testi hizmet vermesi ile öğrenme ve hafıza üzerindeki etkisi, skopolamin ile indüklenen amnezi modelinde görüldü. Metanol ekstresi, sıçanlarda lokomotor aktiviteyi artırdı ve asetilkolinestreaz enzim aktivitesini önemli ölçüde düşürdü. Hem düşük hem de yüksek dozda ekstrelde yapılan histopatolojik çalışmada, normal kontrol, negatif kontrol ve standart tedaviye kıyasla efektif rezervatif skorlar elde edildi.

**Sonuç:** Sonuçlar, *Limonia acidissima*’nin metanol ekstresinin uygulanmasının farklı deney modellerinde öğrenme ve hafıza geliştirdiğini göstermiştir. Histopatolojik çalışma, ekstreinin nöroprotektil özellikte olduğunu ve ekstreinin Alzheimer hastalığının tedavisinde kullanılabileceğini göstermiştir.

**Anahtar kelimeler:** Nootropik aktivite, *Limonia acidissima*, Alzheimer hastalığı, piracetam, skopolamin

**ABSTRACT**

**Objectives:** The present study aimed to evaluate the nootropic activity of *Limonia acidissima* in rats.

**Materials and Methods:** Methanolic extract of *Limonia acidissima* was used to evaluate nootropic activity, piracetam (200 mg/kg, i.p.) was used as a standard, and scopolamine (1 mg/kg, i.p.) was used to induce amnesia. The effect of drugs on learning and memory in rats was evaluated by using the Y-maze task and elevated plus maze on scopolamine-induced amnesia models. Locomotor activity was performed using an actophotometer. Also, levels of acetylcholinestrease, including histopathological examination of rat brains, were assessed.

**Results:** Methanolic extract of *Limonia acidissima* showed increased alteration of the behavior response and percentage spontaneous alteration with the Y-maze task. In the elevated plus maze scopolamine-induced amnesia model, methanolic extract of *Limonia acidissima* showed a decrease in transfer latency, which is indicative of cognition improvement. Methanolic extract increased locomotor activity in rats and decreased the levels of acetylcholinestrease enzyme significantly. A histopathological study with both low and high doses of extract showed effective regenerative scores as compared to normal control, negative control and standard treatment.

**Conclusion:** The results suggested that the administration of methanolic extract of *Limonia acidissima* enhances learning and memory in different experimental models. The histopathological study revealed the neuroprotective property of the extract. The study indicates that the extract may be used in the treatment of Alzheimer’s disease.

**Key words:** Nootropic activity, *Limonia acidissima*, Alzheimer’s disease, piracetam, scopolamine
INTRODUCTION

According to the World Health Organization, approximately 450 million people suffer from a mental or behavioral disorder.1 Dementia (age-related mental disorder) is a characteristic symptom of Alzheimer’s disease (AD).2,3 AD is a progressive, neurodegenerative, and cerebrovascular disease.2,5 It destroys cells in the brain, causing problems with memory, unusual behavior6, difficulty thinking, personality changes,7 and ultimately death.8-10 AD is characterized by the loss of neuronal cells and is primarily linked to neurofibrillary tangles and neuritic plaques.7,11 The cholinergic system in the brain plays an important role in learning and memory,12 which involves acetylcholine (Ach).13 Dementia is produced due to reduction of Ach in the brains of patients with AD.7 In rodents and human beings, drugs like scopolamine impair learning and memory.4,13 Memory loss, amnesia, dementia, anxiety, schizophrenia, and AD may be produced due to certain conditions like age, stress, and emotion.14-15

There are a few nootropic medicines used in the treatment of AD, called nootropic drugs, belonging to the class of psychotropic agents.16 The term nootropic was coined by Giurgea in 1972, from the Greek noon (mind) and tropos (turn).17 Nootropics are also referred to as smart drugs, as they improve mental functions such as memory, increase blood circulation to the brain, and improve the oxygen supply to the brain.18 Synthetic medicines like tacrine, dopnezil,19 aniracetam, piracetam, and rivastigmine are used for the treatment of cognitive dysfunction and memory loss associated with AD.20 However, these drugs pose some adverse effects and bioavailability issues.21 To overcome these problems, researchers are seeking herbal formulations that can overcome the adverse effects of synthetic drugs.22

Many herbs have been studied extensively and reported to have memory-enhancing properties.22 The plant Limonia acidissima is a herbal drug used in Ayurvedic systems of medicine.23 It is also used in a variety of conditions for its antimicrobial, hepatoprotective, antidiarrhoeal, anticancer, diuretic, hepatoprotective, antispermatogenic, antioxidant, antiabetic, and wound-healing activities.24,25

The present study seeks to determine whether the methanolic extract of leaves and fruit pulp of Limonia acidissima shows nootropic activity in an animal model.

MATERIALS AND METHODS

Experimental animals

Wistar rats of either sex weighing between 150 and 200 g were used for the present study. They were housed under standard laboratory conditions (temperature 25°C±1°C), relative humidity 55%±5% and 12.00:12.00 h dark: light cycle) with a standard pellet diet and water ad libitum.

The experiment was conducted as per the standard procedure prescribed by CPCSEA, India. The study protocol was approved by the Ethical Committee of IAEC of Yashoda Technical Campus, Satara (YSPM/YTC/PHARMA/20/2017).

Drugs and chemicals

Piracetam (Dr. Reddy’s, India), scopolamine hydrobromide (APP Pharmaceuticals, India), and normal saline were used for the study. All other reagents and chemicals were of analytical grade and procured from Loba Chemie, Mumbai, India.

Plant material and preparation of extracts

Plant material was collected from Phaltan, identified, and authenticated by the Department of Botany, Yashwantrao Chavan Institute of Science, Satara, Maharashtra, India. A voucher specimen (no: 57) of plant material was kept in the Department of Pharmacognosy, Yashoda Technical Campus, Satara. The fully ripe fruits and leaves of the plant were dried in the shade for 1 week. Then, fruits were cracked open, and the rind, seed, and pulp were separated and shade dried with the leaves at room temperature for more than 2 weeks. After drying, the rind, pulp, and seeds were ground separately into a coarse powder, and the leaves was also separately ground into a coarse powder for further extraction. The coarsely powdered material was extracted with methanol by using the Soxhlet extraction method for 4 days at 45°C, at a ratio of 1:1 (seeds, pulp, and leaves). The obtained extract was filtered and concentrated (yield 8.8%).

Preliminary phytochemical investigation

The methanolic extract was subjected to phytochemical tests for alkaloids, flavonoids, glycosides, saponins, carbohydrates, and tannins.26

Acute toxicity study

An acute toxicity study was performed according to OECD guideline 423. Six swiss albino female mice, weighing in the range of 20-25 g, were administered the test solution at a dose of 2,000 mg/kg. After administration of the test formulation at intervals of 30 min, 1 h, 2 h, 4 h, 24 h, 48 h, and 72 h up to a period of 14 days, mice were observed for clinical signs, gross behavioral changes, and mortality.27

Behavioral study

Rats were trained for the behavioral study by conducting 1 week of training during which they did not receive any plant extract or drug. The completely trained rats were selected for the study. An experiment was carried out in the light period between 8.00 am and 03:00 pm in a sound-proof room.

Y-maze task

Five groups of animals were formed, each group comprising six animals as follows. Group 1: Control group (normal saline), group 2: Negative group (scopolamine 1 mg/kg i.p.), group 3: Standard treatment (piracetam 200 mg/kg i.p. + scopolamine 1 mg/kg i.p.), group 4: Low dose of extract + scopolamine (200 mg/kg p.o + 1 mg/kg i.p.), group 5: High dose of extract + scopolamine (400 mg/kg p.o + 1 mg/kg i.p.).

The Y-maze task is a simple method to evaluate memory-enhancing activity in laboratory animals. It is generally used to check behavioral patterns in animals. The wooden Y-maze was used for the study. It consists of three arms with an angle of 120° between each of the two arms. Each arm was 8 cm wide,
30 cm long, and 15 cm deep. The arms were designated as the start arm (A), novel arm with food stimuli (B), and other arm (C). In the first trial, the rat was placed just inside the arm and allowed to move freely through the apparatus for 5-10 min. In the second trial, the rat was placed in the maze and explored arms A, B, and C, systematically entering each arm. The ability to alternate requires that the rat know which arm they have already visited. A total of 13 entries were recorded for each rat visually, and finally, the percentage of spontaneous alternation was calculated by using the following formula:

\[
\text{Percentage alternation} = \frac{\text{Number of positive entries}}{\text{(Total number of arm entries - 2)}} \times 100
\]

### Scopolamine-induced amnesia in rats

Animals were grouped as per the Y-maze task. All animals were treated for 14 days, and at the end of the treatment period, all the extract-treated animals were subjected to scopolamine (1 mg/kg i.p.) 60 minutes after administration of extract, except the first group, which served as a vehicle control.

In this method, scopolamine is used as an inducer of memory impairments in rats, which is calculated by using the elevated plus maze (EPM) apparatus. The EPM is made up of wooden material. It consists of two arms, a closed arm and an open arm. In the EPM task, transfer latency was recorded. The rat was placed in the arm and allowed to explore the maze for 1 min. If the rat was not transferred into the other arm within 90 sec, it was gently pushed into the other arm, and the transfer latency was considered as 90 sec. All groups of animals were treated for 14 days, and at the end of the treatment, transfer latency was recorded and served as the parameter transfer latency on the 14th day (acquisition day) and 15th day (retention day).

### Locomotor activity

Animals were grouped as per the Y-maze task. Before the test, all rats were fasted for 4 h and treated as per the grouping. One hour after treatment, each rat was placed individually in an actophotometer for a period of 10 minutes, and locomotor activity was scored. The difference in locomotor activity of all groups was recorded.

### Estimation of acetylcholinesterase enzyme

Animals were grouped as per the Y-maze task. All rats were treated as per the groups with saline, scopolamine, piracetam, and extract. After 60 minutes of treatment, rats were decapitated, and the brains were removed quickly and stored in ice-cold saline. The frontal cortex, hippocampus, and septum were quickly dissected out on a petri dish chilled on crushed ice. The tissues were weighed and homogenized in 0.05 M phosphate buffer (pH 7.2). The homogenate (0.4 mL) was added to a test tube containing 2.6 mL phosphate buffer and 100 μL of 5,5-dithiobisnitrobenzoic acid and mixed. The absorbance of the resulting mixture was measured at 412 nm using a spectrophotometer. The stable value of absorbance was recorded. Then, acetylthiocholine iodide (20 μL) was added, and the change in absorbance per minute was determined. The mean change in absorbance was considered for calculation using the following formula, and acetylcholinesterase activity was measured as μM/L/min/g of tissue:

\[
R = \frac{OD_{\text{final}} - OD_{\text{initial}}}{E \times \text{volume of assay}} \times \text{mg of protein}
\]

Where R is the rate of enzyme activity in “n” mole of Ach iodide hydrolyzed per minute per mg of protein, OD is the change in absorbance per minute, and E is the extinction coefficient (1.36x10^4 M^-1cm^-1).

### Brain histopathology

After the treatment and behavioral studies, two animals from each group were sacrificed by excessive CO₂ anesthesia, and the brains were isolated and kept in 10% formaldehyde solution. The brain was stained with cresyl violet, and the cerebellum and basal ganglia were studied under a light microscope.

### Statistical analysis

The statistical analysis was carried out by using GraphPad Prism software version 5.0, and the results were compared by One-Way ANOVA followed by Tukey’s multiple comparison test. A p value less than 0.05 was considered as statistically significant.

### RESULTS

#### Preliminary phytochemical investigation

The results of preliminary phytochemical screening of methanolic extract of Limonia acidissima are given in Table 1. The Limonia acidissima extract gave positive results for alkaloids, flavonoids, carbohydrates, glycosides, and saponin. Proteins, steroids, and phenols were absent.

#### Acute toxicity test

As female mice are more sensitive to drugs than males, female mice were used in the acute oral toxicity study as per OECD guideline 423. Methanolic extract of Limonia acidissima did not showed any toxic effects up to 2.000 mg/kg oral dose. Central nervous system (CNS) stimulation parameters such as hyperactivity, irritability, tremors, and convulsions were found to be negative in mice. CNS depressant parameters such as hypoactivity, narcosis, and ataxia were found to be negative in mice.

### Table 1. Phytochemical analysis of Limonia acidissima extract

| Serial number | Phytochemical test | Result |
|---------------|--------------------|--------|
| 1             | Test for alkaloids | + Ve   |
| 2             | Test flavonoids    | + Ve   |
| 3             | Test for carbohydrates | + Ve |
| 4             | Test for glycosides | + Ve   |
| 5             | Test for saponin   | + Ve   |
| 6             | Test for proteins  | - Ve   |
| 7             | Test for steroids  | - Ve   |
| 8             | Test for phenols   | - Ve   |

+ Ve: Indicates the presence of compounds, - Ve: Indicates the absence of compounds
Y-maze task
The Y-maze model proved to be a sensitive measure of spatial recognition memory. The effect on alteration behavior was studied on the parameter % alteration (Table 2). The negative control group showed a significant (p<0.001) decrease in the alternation of behavior when compared with the normal control. The results of the standard treatment groups showed a significant (p<0.001) increase in the alternation of behavior with respect to methanolic extract of *Limonia acidissima* 400 mg/kg.

Scopolamine-induced amnesia in rats
Scopolamine-induced amnesia in a rat model was carried out by using the EPM. The scopolamine-treated group showed a significant (p<0.001) increase in transfer latency and memory retention on the 14th acquisition and 15th retention days when compared with the normal control, standard treatment, and high-dose extract groups, respectively. The piracetam standard treatment group, when compared with methanolic extract of *Limonia acidissima* at a dose of 200 mg/kg, showed a significant (p<0.01) decrease in transfer latency and memory retention on the 14th acquisition day and 15th retention day, respectively. The result is given in Table 2.

Locomotor activity
Locomotor activity in rats was assessed using an actopotometer. The methanolic extract of *Limonia acidissima* showed nootropic activity by increasing locomotor activity (Table 3). The negative group showed a decrease in locomotor activity as compared with the normal control, while the extract-treated groups showed significantly (p<0.001) increased locomotor activity as compared with negative control group.

| Group | Treatment | Alterations (%) | Acquisition day 14 (sec) | Retention day 15 (sec) |
|-------|-----------|-----------------|--------------------------|-----------------------|
| 1     | Normal control: (Normal saline, p.o.) | 66.31±2.45       | 41.00±1.67               | 38.33±1.22            |
| 2     | Negative control: Scopolamine (1 mg/kg, p.o.) | 35.80±3.19*       | 74.14±3.37#               | 71.50±3.50#           |
| 3     | Standard treatment: Piracetam (200 mg/kg, i.p.) + scopolamine (1 mg/kg, i.p.) | 80.50±3.61**       | 25.67±1.22##              | 21.33±1.22##          |
| 4     | Low dose of extract (100 mg/kg, p.o.) + scopolamine (1 mg/kg, i.p.) | 46.45±2.71**##   | 34.83±1.24**##             | 31.50±1.35**##        |
| 5     | High dose of extract (400 mg/kg, p.o.) + scopolamine (1 mg/kg, i.p.) | 70.27±2.29**##   | 27.50±1.17**##            | 23.67±1.17**##        |

Values represent mean ± standard error of the mean; n=6, analysis was performed using One-Way ANOVA followed by Tukey’s multiple comparison test, p value less than 0.05 was considered as statistically significant. *: p<0.05, #: p<0.01, †: p<0.001, #: Data compared with normal control, †: Data compared with negative control, ††: Data compared with standard treatment

Estimation of acetyl cholinesterase (AChE) enzyme
The results for levels of AChE are given in Table 3. In the normal control, the level of AChE was very low, while in the negative control it was found to be high. In the case of the piracetam-treated group, the level of AChE was significantly reduced as compared with the negative control group. The methanolic extract of *Limonia acidissima* (200 and 400 mg/kg) significantly (p<0.001) lowered AChE activity as compared with the negative control. The significant decrease in the level of AChE indicated that *Limonia acidissima* is a potential anti cholinesterase agent and possesses nootropic activity.

Histopathological analysis of scopolamine-induced amnesia in rats
The results of histopathological analysis are given in Figure 1 and Table 4. Figure 1A shows the histopathological section of normal control rat showed neuronal degeneration without vascular degeneration and gliosis, while the negative control group (Figure 1B) showed vascular degeneration, neuronal degeneration, and gliosis. In the case of the standard treatment group (Figure 1C), low-dose group (Figure 1D) and high-dose group (Figure 1E), vascular degeneration, neuronal degeneration, and gliosis were found to be lower as compared with the negative control group. Group 2, treated with scopolamine, showed maximum pathological changes as compared with the rest of the groups. Both a low dose and a high dose of methanolic extract of *Limonia acidissima* showed good regenerative scores as compared with the normal control, negative control and standard treatment.

| Group | Treatment | After treatment | AChE (μM/L/min/mg Protein) |
|-------|-----------|-----------------|-----------------|
| 1     | Normal control: (Normal saline, p.o.) | 562±30.12   | 13.03±1.55 |
| 2     | Negative control: Scopolamine (1 mg/kg, p.o.) | 251.2 42.66* | 24.09±1.56* |
| 3     | Standard treatment: Piracetam (200 mg/kg, i.p.) + scopolamine (1 mg/kg, i.p.) | 324.5±48.5** | 20.97±1.20** |
| 4     | Low dose of extract (100 mg/kg, p.o.) + scopolamine (1 mg/kg, i.p.) | 451.3±48.18** | 18.00±1.19** |
| 5     | High dose of extract (400 mg/kg, p.o.) + scopolamine (1 mg/kg, i.p.) | 515±52.65* | 15.52±1.13* |

Values represent mean ± standard error of the mean; n=6, analysis was performed using One-Way ANOVA followed by Tukey’s multiple comparison test, p value less than 0.05 was considered as statistically significant. *: p<0.05, #: p<0.01, †: p<0.001, #: Data compared with normal control, †: Data compared with negative control, ††: Data compared with standard treatment, AChE: Acetyl cholinesterase
DISCUSSION

The number of patients suffering from AD is rising steadily day by day all over the world. AD is characterized by degenerative changes in the brain resulting in memory loss. The main cause of AD is death of cholinergic neurons in the basal forebrain area, which results in a deficit of Ach. Scopolamine is an anti-muscarinic agent; after administration, it produces memory deficit. Scopolamine-induced memory loss is a well-reported animal model for screening anti-amnestic molecules. It is a non-selective muscarinic receptor antagonist that competitively inhibits muscarinic receptors for Ach and reduces the level of Ach. Furthermore, it causes impairment of learning acquisition short-term memory as it produces depression of the cerebral cortex, especially in the motor areas.

Piracetam is a standard drug used to treat amnesia, dementia, and other health problems like brain stroke, AD, vascular dementia, D LB, and Huntington’s disease. Piracetam has the ability to bind to receptors and increases also as an antioxidant/neurotonic. It also enhances the number of Ach receptors; thus, it might be effective at enhancing learning and memory.

In this study, we investigated the effect of methanolic extract of Limonia acidissima on spatial memory and neurodegeneration in an animal model of AD. The results clearly demonstrated that spatial memory and neurodegeneration were significantly improved by Limonia acidissima. Methanolic extract of Limonia acidissima at doses of 200 mg/kg and 400 mg/kg was administered orally for 7 days and improved learning and memory significantly in rats in the Y-maze task. When the negative control group was compared with the standard treatment and extract groups, both standard and extract-treated groups showed a significant (p<0.001) increase in percentage alteration behavior.

In the EPM model, methanolic extract of Limonia acidissima possessed nootropic activity on scopolamine-induced amnesia. The extract-treated group showed a significant decrease in transfer latency and memory retention with p<0.001 when compared with the negative and standard group at the 14th acquisition and 15th retention day of the study, which is an indicative of cognition improvement.

Locomotor activity in patients suffering from AD is absent. The actophometer is the model used to observe the locomotor behavior of animals. So, in the present study the actophometer model was used to know about the locomotor activity of animals treated with methanolic extracts. The methanolic extract of Limonia acidissima passes nootropic activity by increasing locomotor activity. The significant (p<0.001) locomotor activity was observed in rats treated with low-dose and high-dose treated groups.

Table 4. Histopathology of the brain tissues in scopolamine-induced amnesia in rats

| Group                                      | Vascular degeneration | Neuronal degeneration | Gliosis |
|--------------------------------------------|-----------------------|-----------------------|---------|
| Normal control: (Normal saline, p.o.)     | 0                     | +                     | 0       |
| Negative control: Scopolamine (1 mg/kg, p.o.) | +++                   | +++                   | ++      |
| Standard treatment: Piracetam (200 mg/kg, i.p.) + scopolamine (1 mg/kg, i.p.) | ++                   | ++                    | +       |
| Low dose of extract (100 mg/kg, p.o.) + scopolamine (1 mg/kg i.p.) | ++                   | ++                    | +       |
| High dose of extract (400 mg/kg, p.o.) + scopolamine (1 mg/kg, i.p.) | +                    | ++                    | +       |

No abnormality detected (0), damage/active changes up to less than 25% (+), damage/active changes up to less than 50% (++), damage/active changes up to less 75% (+++), damage/active changes up to more than 75 (++++)
Ach is considered as a major neurotransmitter involved the regulation of cognitive functions. Changes in the cholinergic system lead to impairment in learning, memory, and behavior in patients with dementia. In the present study, *Limonia acidissima* inhibited acetylcholinesterase, thereby elevating the Ach concentration in the brain. The findings suggested a possible neuroprotective role of *Limonia acidissima*. Thus, it seems that *Limonia acidissima* may prove to be useful in the treatment of AD. However, further investigations are necessary to support these results.

The histopathological study also showed that both a low dose and a high dose of methanolic extract of *Limonia acidissima* showed good regenerative scores as compared with other group, which is good indicator of the neuro-protective property with the potential to treat AD.

In a few studies, the use of antiinflammatory drugs showed a reduction in symptoms of AD. Epidemiological studies also confirmed the suitability of non-steroidal antiinflammatory drugs to reduce the incidence of AD. *Limonia acidissima* has been reported to produce antiinflammatory action in rodents. This antiinflammatory property of *Limonia acidissima* will likely help in the treatment Alzheimer’s patients.

**CONCLUSION**

The present study suggests that methanolic extract of *Limonia acidissima* provided significant protection against AD. The methanolic extract of *Limonia acidissima* increased locomotor activity in rats and inhibited acetylcholinesterase, thereby elevating the Ach concentration in the brain. The histopathology of the brain also showed a good regenerative score; therefore, methanolic extract of *Limonia acidissima* can be used in the management of AD. An extensive study along these lines is required in the future to support the methanolic extract of *Limonia acidissima* as a novel and natural nootropic/memory-enhancing agent.

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