EVALUATION OF PUNICA GRANATUM LEAVES EXTRACT IN SCOPOLAMINE INDUCED LEARNING AND MEMORY IMPAIRMENT IN MICE

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ABSTRACT

Objective: To evaluate the effect of Punica granatum (Pomegranate) leaves extract on learning and memory functions in scopolamine induced learning and memory impairment in mice. Memory enhancing activity of Punica granatum in scopolamine induced amnesic mice was investigated by using elevated plus maze and Morris water maze. AchE activity, SOD, lipid peroxidation, glutathione level of brain homogenate was performed in control/ scopolamine/ standard / Punica granatum leaves extract treated mice. Material and methods: A total of 36 mice weighing about 25-30 g were randomized into 6 equal groups as follows: received normal saline, Scopolamine treated group (0.4 mg/kg i.p.) on 21th day, Piracetam treated group (200 mg/kg i.p.) for 21 day + scopolamine on 21th day, Punica granatum leaves extract treated groups (100, 200 and 400 mg/kg) for 21 days + scopolamine on 21th day. Result: Administration of Punica granatum leaves extract significantly restored learning and memory impairment induced by scopolamine in the Morris water maze and Elevated plus maze, reduction in AchE activity, increased activity of brain antioxidant enzyme such as SOD, glutathione and also reduced the increased activity of lipid peroxidation. Conclusion: Punica granatum leaves extract has improving effect on learning and memory impairment produced by scopolamine and may have beneficial effect in the treatment of Alzheimer’s disease and amnesia.

KEYWORD: Punica granatum, AchE, Piracetam, scopolamine and antioxidant.
INTRODUCTION

1.1 Learning And Memory

Learning is a part of memory responsible for capturing data or translating information about one's environment.\(^1\) Learning is defined as the act of obtaining new or making partial changes to existing knowledge, behavior and skill. Learning is a long term change in mental representation (mental imagery of things that are latest or old seen or sensed by sensory organ) and enhance due to experience.\(^2\)

Memory is the process in which captured information is encoded, stored and retrieved.

Three main stages in forming and retrieval of memory

- Encoding or registration (receiving, processing and combining of received information)
- Storage (creation of a lasting/permanent record of the encoded information)
- Retrieval, recall or recollection (calling back the stored information in response to some cue for use in a process or activity)\(^3\)

Types of memory
Types of human memory
Disorders of Learning And Memory

Disorders of learning and memory

1.2 RACETAMS (NOOTROPICS)

Racetam are not naturally occurring, they are chemically synthesized. All racetams have 2-pyrrolidone nucleus and these all racetams are derivatives of gamma aminobutyric acid (GABA).

The first Racetam (Piracetam) was discovered in the late 1960s. Since then, more than twenty piracetam-like substances have been synthesized and proposed for cognitive improvement or treatment of cognitive impairment and central nervous system disorders. They have broadly been defined as nootropic (from the Greek words noos for mind and tropein for towards).\(^4\)

1.2.1 PIRACETAM

- Synonyms: 2-pyrrolidoneacetamide, 2-pyrrolidinoneacetamide(2).
- Molecular formula: C\(_6\)H\(_{12}\)N\(_2\)O\(_2\)
- Mol. Wt. 142.15 g/mol
- First reported: for the treatment of motion sickness (1966 & 1967)\(^5\)

After p.o administration Piracetam is absorbed very well. Also having almost 100% bioavailability.\(^6\) Piracetam is excreted practically unchanged through urine and completely eliminated after 30-32 h. It is highly hydrophilic drug that’s why they cross the blood-brain barrier slowly.

Piracetam is effective in patient with mild to moderate dementia, and seem to be effective in patients suffering from Alzheimer's disease.\(^7,8\) It has no significant effect on GABA receptors, does not affect the synaptosomal uptake of GABA and does not affect GABA
levels in either plasma or brain. Piracetam attenuates amnesia, Piracetam is a positive allosteric modulator of AMPA receptor and also have effect on NMDA receptor. Piracetam induced increase in the ACh content without change in the choline levels in hippocampus and improves neurotransmitter acetylcholine function via muscarinic receptor which are implicated in learning and memory process. Piracetam did not change the steady state levels of ACh.\[^9\]

**METHODOLOGY**

1.1 **Plant material**
The leaves of shrub *Punica granatum* were collected from my farm house Rudrapur, Uttarakhand.

1.2 **Animals**
Albino mice weighing 25-30g were procured from departmental animal house of Division of pharmaceutical sciences of Shri Guru Ram Rai Institute of Technology and Science, Patel Nagar, Dehradun.

Animals were acclimatized in the departmental animal house facility and housed in polypropylene cages with husk bedding (renewed every 48 hrs.), under 12:12 hrs light dark cycle at 25° C ± 5° C. and were fed with standard Commercial pellet diet and water *ad libitum*.

The protocol were approved by Institutional animal ethics committee (Registration No.M.PH./IAEC/O1/2015/ECC-7) and were carried out in accordance with the CPCSEA.

1.3 **Collection and authentication of plant**
The leaves of shrub *Punica granatum* were collected from our farm house Rudrapur, Uttarakhand.

The authentication of *Punica granatum* was confirmed by Botanical Servey of India (BSI) Dehradun after submission of herbarium.

1.4 **Extraction**
Ethanolic extract was prepared by using the souxhlet apparatus (soaking the leaves of *Punica granatum* in 75% ethanol) at 30° C. After that filtered and kept in water bath to obtain the
extract and collected. Finally obtained extract was weighted and percent yield of extract was calculated.\cite{10}

1.5 Experimental induction of learning and memory impairment
Scopolamine (0.4 mg/kg) was used in the present study for induction of Amnesia.

1.6 Experimental protocol
Six groups to be employed in present study for both EPM & MWM and each group contain six animals, as follows
- Group 1- Normal control group- normal saline was administered
- Group 2- Disease induced group- Scopolamine (0.4mg/kg i.p.) was administered on 21\textsuperscript{th} day
- Group 3- Standard group treated with Piracetam (200mg/kg i.p.) for 21 days + scopolamine (0.4 mg/kg i.p.) on 21\textsuperscript{th} day
- Group 4- Test group treated with low dose of \textit{Punica granatum} (100mg/kg p.o.) for 21 days + scopolamine (0.4 mg/kg i.p.) on 21\textsuperscript{th} day
- Group 5- Test group treated with medium dose of \textit{Punica granatum} (200mg/kg p.o.) for 21\textsuperscript{th} days + scopolamine (0.4 mg/kg i.p.) on 21\textsuperscript{th} day
- Group 6- Test group treated with higher dose of \textit{Punica granatum} (400mg/kg p.o.) for 21\textsuperscript{th} days + scopolamine (0.4 mg/kg i.p.) on 21\textsuperscript{th} day

BEHAVIORAL MODELS
- **Morris water maze**

MWM is a circular pool (60 cm in diameter and 25 cm in height), mainly used for mice. MWM was filled to be a depth of 20 cm with opaque water which is made up from milk or non toxic white dye and temperature should be maintained at 25°C. The pool was divided into four equal quadrants (Q1, Q2, Q3 and Q4) with the help of thread. A hidden platform, painted white with top surface (6 cm × 6 cm) was placed 1 cm below the surface of water in any one quadrant (Q4 in present study) of the pool. Each and every mice subjected to four consecutive time trials each day, a gap is between trial was 5 min.\cite{11}

Data is collected on basis of two trials
1. **Acquisition Trial**
4 trials (one from each starting position) per session was given for four days and each trial having ceiling time of 0 sec. Once the animal climb on to platform. The animals remain there
for 30s before the commencement of next trial. The time to reach the hidden platform is recorded as escape latency (within 120 sec). If animal failed to find platform within 120 sec then allowed to remain there for 20 sec.

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 2</td>
<td>Q2</td>
<td>Q3</td>
<td>Q4</td>
<td>Q1</td>
</tr>
<tr>
<td>Day 3</td>
<td>Q3</td>
<td>Q4</td>
<td>Q1</td>
<td>Q2</td>
</tr>
<tr>
<td>Day 4</td>
<td>Q4</td>
<td>Q1</td>
<td>Q2</td>
<td>Q3</td>
</tr>
</tbody>
</table>

2. Retrieval Trial
The determination of retention memory was carried out on the next day (5th day). The time spent in Quadrant which is previously hidden is recorded. The time spent in the target quadrant was noted down and it indicates the degree of memory consolidation.\[^{12}\]

Morris water maze day 1 to day 4 (Acquisition)

Morris water maze day 5 (Retrieval)
- Elevated plus maze
It is an exteroceptive behavioral model for evaluating learning and memory. The EPM apparatus for mice contains two open arms i.e 16 cm × 5 cm and two enclosed arm i.e 16 cm × 5 cm × 15 cm. The elevated plus maze is elevated at 25 cm height from the floor.

Mice was placed at one end of the open arm, facing toward opposite from the centre of EPM (an first day of experiment). For each mice of all groups, transfer latency (TL) was recorded at first day of experiment. On second day (after 24 hrs) same procedure was followed for TL.
(Retrieval). If the animal was not able enter into covered arms with in 90 sec then pushed the animal into one of the enclosed arm and the TL was recorded as 90 sec.\cite{11,13}

BIOCHEMICAL PARAMETERS
All biochemical parameters were performed in the brain homogenate after the MWM & EPM evaluation.

Preparation of brain homogenate
All mice were sacrificed by cervical dislocation and brain were removed through breakdown of temporal bone and rinsed with ice cold saline (isotonic). Brain tissue samples were homogenized with ice cold phosphate buffer (0.1 M & pH 7.4) and the volume of phosphate buffer is 10 time the weight of the brain tissue.\cite{14}

Following are the biochemical parameters which were used in present study.

- Estimation of brain cholinesterase activity according to the Ellman G F \textit{et al.}\cite{15,16}
- Estimation of lipid peroxidation or MDA according to the Ohkawa \textit{et al.}
- Estimation of superoxide dismutase according to the Mishra \textit{et al.}
- Estimation of reduced glutathione according to the Ellman GL method
- Estimation of total protein was measured in all brain samples by the method of Lowry OH using bovine serum albumin (BSA) (1 mg/ml) as a standard.

Estimation of brain cholinesterase activity
According to the Ellman G F \textit{et al.}\cite{15,16}
Principle

The brain cholinesterase activity is assessed by providing an artificial chemical, acetylthiocholine (ATC). After the cleavage of ATC, thiocholine is formed with acetic acid allowing to react with 5, 5’-dithiobis-(2 nitrobenzoic acid) (DTNB), yellow colour anion is formed (Thionitrobenzoic acid). The concentration of thionitrobenzoic acid is detected using UV spectrophotometer at 412 nm.

![Chemical Reaction Diagram]

Reagents

- Phosphate buffer (0.1 M)
- Solution A (5.22 g K$_2$HPO$_4$ + 4.6 g NaH$_2$PO$_4$ were dissolved in 150 ml of distilled water)
- Solution B (6.2 g NaOH + 150 ml distilled water)
- Solution A + solution B
- DTNB reagent (10 mg DTNB + 50 ml 0.9% NaCl solution + 50 ml phosphate buffer)
- ATC (75 mg of acetylthiocholine + 50 ml distilled water)

Procedure

Cuvette containing 2.6 ml phosphate buffer, add 0.4 ml of brain homogenate and 100 µl of DTNB. The content of cuvette was mixed thoroughly and measured the absorbance at 412 nm in the spectrophotometer i.e basal reading. After this 20 µl of ATC was added and change in absorbance was noted down.

CALCULATION

\[
\text{AChE activity (M/ml)} = \frac{A/\text{min} \times Vt}{\text{Extinction Coefficient} \times L \times Vs}
\]

Where,

- \(A/\text{min}\) = change in absorbance per min
- Extinction coefficient = 1.361 \times 10^4/M/cm
- L = Light path (1cm)
- Vt = Total volume (3.1 ml)
- Vs = Sample volume (4ml)
Unit = \( \mu M/min/mg \) of tissue

**Estimation of lipid peroxidation**
Lipid peroxidation levels was estimated by the Ohkawa et al method\(^{[17]}\).

Thiobarbituric acid (TBA) reaction is done in this method. 2- thiobarbituric acid reactive substance (TBARS) are naturally present in biological livings which increase in concentration as a response to oxidative stress.

**Principle**
One molecule of MDA reacts with two molecule of TBA under acidic condition and form MDA – TBA adduct i.e pink colored. Absorbance was measured at 535 nm in spectrophotometer.

\[
\text{H}^+ + 2 \text{thiobarbituric acid} + 1 \text{MDA} \rightarrow \text{MDA – TBA adduct (pink color)}
\]

**Reagent**
- Trichloroacetic acid – 30 \%
- Thiobarbituric acid – 0.8 \%

Blank solution – 2 ml distilled water, 2 ml trichloroacetic acid and 2 ml of thiobarbituric acid.

**Procedure**
Taken 2 ml of brain homogenate, add 2 ml of 30\% of trichloroacetic acid then add 0.8\% thiobarbituric acid reagent in a test tube. Keep test tube in cold water for half an hour. After this process homogenate was centrifuged at 3000 RPM for 15 min, supernatant was separated out and absorbance was noted down at 535 nm against blank.

Malondialdehyde = n moles formed per mg of protein in the body

Concentration = \( A \times (V/E) \times P \)

Where,
- A= Absorbance at 535 nm
- V= Volume of solution
- E= Extinction coefficient \( (1.56 \times 10^5 \text{ m}^{-1}\text{ cm}^{-1}) \)
- P= mg of protein per g of tissue

All values was indicates in nM of MDA/mg of protein
Estimation of reduced glutathione

Reduced glutathione estimation was done by the method of Ellman GL.\textsuperscript{[16,18]}

Principle

DTNB, phosphate buffer when reacts with reduced glutathione form yellow colour. Absorbance at 412 nm.

\[
\text{GR} \quad \text{NADPH} + \text{H}^+ + \text{GSSG} \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow 2 \text{GSH} + \text{NADP}^+
\]

This above reaction takes place when oxidative stress is found, involving the oxidation of NADPH. This reduced gluthione (GSH) works in the principle of estimation. GSH reacts with DTNB and phosphate buffer and gives yellow colour.

\[
\text{DTNB} + \text{Phosphate buffer} + \text{GSH} \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \text{yellow colored}
\]

Reagent

- Reaction buffer (0.1 M sodium phosphate buffer)
  Stock A – 2.78 g sodium phosphate monobasic + 100 ml water
  Stock B – 2.84 g anhydrous phosphate dibasic + 100 ml water
  Stock A and stock B were mixed together then adjust volume upto 100 ml with the help of distilled water. Add 37.2 mg of EDTA to above solution.

- Ellman’s reagent – 4 mg DTNB (Ellman’s reagent) were dissolved in 1 ml of reaction buffer.

Procedure

Add 10% Trichloroacetic acid + brain homogenate and centrifuged. Formed mixture was mixed with DTNB and phosphate buffer. Mix well and incubate at room temperature for 15 min and then absorbance was noted at 412 nm.

CALCULATION

\[
\text{GSH} = \frac{\text{Absorbance}}{1.4550 \times 10^2} \times 11.2 \times 10^2
\]

Unit expressed as nM/mg of protein

Estimation of superoxide dismutase

SOD was estimated according to Kono method.\textsuperscript{[19]}
Principle
This method follow the basic principle of the inhibitory effect of SOD on the reduction of nitroblue tetrazolium (NBT) dye by superoxide radical which are formed by the auto oxidation of hydroxylamine hydrochloride.

Procedure
Cuvette containing 1.3 ml of NaOH buffer + 500 µl NBT and the reaction was started by the addition of 100 µl hydroxylamine hydrochloride. After 2 or 3 min 70 µl enzyme extract was added. Increase in absorbance at 540 nm was noted after % inhibition in the rate of NBT reduction.

Calculation
\[
\% \text{inhibition} = \frac{\text{Change in ab/min (blank)} - \text{change in ab/min (test)}}{\text{change in ab/min (blank)}} \times 100
\]
\[
\text{Unit/ml enzyme} = \frac{\% \text{inhibition} \times V_t}{50 \% \times V_s} \times 100
\]
Where,
\(V_t = \) Total volume i.e 1 ml
\(V_s = \) Volume of the sample i.e 0.1 ml
Unit expressed in unit/mg of protein

Estimation of total protein
Total protein was done according to the method of Lowry OH, et al 1951. Total amount of brain total protein was represent in mg.

STATISTICAL ANALYSIS
The statistical analysis was carried out using prism graph pad 5 software. All values was represent as mean ± SEM. Multiple comparision between different groups was performed using one way analysis of variance followed by Tukey’s test for all behavioral test and biochemical evaluation except escape latency in Morris water maze. In Morris water maze two way analysis of variance was used followed by Bonferroni’s test. Difference level for statistical significant result, P<0.05 was considered.

RESULT
1.1 Percentage yield of ethanolic extract and physical characteristics
Weight of dried leaves of *Punica granatum* = 980 g
Weight of extract obtained = 60.5g

\[
\% \text{ yield} = \frac{\text{wt. of extract obtained}}{\text{wt. of dried leaves taken}} \times 100
\]

% yield of ethanolic extract = 6.17%

### Physical characteristics of ethanolic extract of *Punica granatum* leaves

<table>
<thead>
<tr>
<th>EXTRACT</th>
<th>COLOUR</th>
<th>ODOUR</th>
<th>% EXTRACTIVE VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>75 % Ethyl alcohol</td>
<td>Brown</td>
<td>Characteristics</td>
<td>60.5 %</td>
</tr>
</tbody>
</table>

#### 1.3 Pharmacological study

- **Behavioral estimation**

*Effect of *Punica granatum* leaves extract (PGLE) on transfer latency (TL) of scopolamine treated mice in Elevated plus maze (EPM)*

Treatment with scopolamine showed significant (P<0.001) increase in TL time when compared to control group. Ethanolic extract of *Punica granatum* leaves and (100 mg/kg, 200 mg/kg and 400 mg/kg) and standard significantly (P<0.001) reduce TL time when compared to disease control group. The effect of PGLE 400 mg/kg was noticed to be comparable to standard treated group. Therefore, PGLE treated groups was not effective as standard treated group.

*Effect of *Punica granatum* leaves extract (PGLE) on escape latency and time spend in target quadrant of scopolamine treated mice in Morris water maze (MWM)*

Treatment with ethanolic leaves extract of *Punica granatum* (100, 200 and 400 mg/kg p.o) significantly (P<0.001) decrease EL time and TSTQ when compared to disease control group. The effect of PGLE 400 mg/kg was noticed to be comparable to standard treated group.

- **Biochemical estimation**

*Effect of *Punica granatum* leaves extract on brain AchE activity of scopolamine treated mice*

Treatment with scopolamine showed significant (P<0.001) increase in brain AchE activity when compared to control group (vehicle treated). The mice treated with standard showed significant (P<0.001, 0.05) decrease in AchE activity when compared to scopolamine treated group whereas a significant (P<0.001, 0.01) decrease in AchE activity was observed in the PGLE treated group.
Effect of *Punica granatum* leaves extract on lipid peroxidation of scopolamine treated mice

Lipid peroxidation study revealed that scopolamine treated group showed significant (P<0.001) increase in MDA level when compared to control group. Standard and PGLE treated group were able to prevent the elevated MDA level, showed significant (P<0.001, 0.01 and 0.05) decrease in MDA level when compared to Scopolamine treated group.

Effect of *Punica granatum* leaves extract on level of glutathione scopolamine treated mice

Scopolamine treated group showed significant (P<0.001) decrease in glutathione level when compared with control group. Whereas, standard treated showed significant (P<0.001) increase in activity of glutathione when compared to scopolamine treated group. In other hand PGLE treated group showed significant (P<0.001) increase in glutathione level when compared to standard treated group but results are not better than standard treated group.

Effect of *Punica granatum* leaves extract on level of SOD scopolamine treated mice

Scopolamine treated group showed significant (P<0.001) decrease in SOD level when compared with control group. Whereas, standard treated showed significant (P<0.001) increase in activity of SOD level when compared to scopolamine treated group. In other hand PGLE treated group showed significant (P<0.001) increase in SOD level when compared to standard treated group but results are not better than standard treated group.

RESULTS WITH THE HELP OF TABLE AND GRAPH -

<p>| Table: 1. Transfer latency of mice on elevated plus maze (Acquisition and retrieval in sec) |</p>
<table>
<thead>
<tr>
<th>S.no.</th>
<th>Treatments</th>
<th>Acquisition (in sec)</th>
<th>Retrieval (in sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control</td>
<td>48.33 ± 1.10</td>
<td>35 ± 2.12</td>
</tr>
<tr>
<td>2.</td>
<td>Disease control (SCO)</td>
<td>54.33 ± 1.27</td>
<td>60 ± 1.69&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3.</td>
<td>Standard (PIR+SCO)</td>
<td>51.0 ± 0.97</td>
<td>35.67 ± 1.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.</td>
<td>PGLE 100 + SCO</td>
<td>54.81 ± 1.61</td>
<td>48.67 ± 1.25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5.</td>
<td>PGLE 200 + SCO</td>
<td>54.16 ± 2.61</td>
<td>45 ± 1.59&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>6.</td>
<td>PGLE 400 + SCO</td>
<td>53.33 ± 1.63</td>
<td>39.17 ± 1.49&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> represent significant versus control group (P<0.001)

<sup>b</sup> represent significant versus disease control group (P<0.001)

<sup>c</sup> represent significant versus PGLE 100 + SCO group (P<0.001)
The statistical analysis was carried out using prism graph pad 5software. All values was represent as mean ± SEM. Multiple comparision between different groups was performed using two way analysis of variance followed by Bonferroni’s test.

Graph 1- Transfer latency of mice on elevated plus maze (Acquisition and retrieval in sec)

*** represent significant (P<0.001) versus Acquisition

Control indicates administration of normal saline (10 ml/kg) for 21 days, SCO indicates administration of scopolamine (0.4 mg/kg i.p) on 21st day, PIR + SCO indicates administration of piracetam (200 mg/kg i.p) for 21 days + scopolamine 30 min after Piracetam administration on 21st day, PGLE 100 + SCO indicates administration of Punica granatum leaves extract (100 mg/kg i.p) for 21 days + scopolamine 30 min after Punica granatum leaves extract administration on 21st day, PGLE 200 + SCO indicates administration of Punica granatum leaves extract (200 mg/kg i.p) for 21 days + scopolamine 30 min after Punica granatum leaves extract administration on 21st day, PGLE 400 + SCO indicates administration of Punica granatum leaves extract (400 mg/kg i.p) for 21 days + scopolamine 30 min after Punica granatum leaves extract administration on 21st day

Table 2: Transfer latency of mice on elevated plus maze (Retrieval in sec)

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Treatment groups</th>
<th>Transfer latency in sec (Retrieval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>35 ± 2.12</td>
</tr>
<tr>
<td>2</td>
<td>Disease control (SCO)</td>
<td>60 ± 1.69</td>
</tr>
<tr>
<td>3</td>
<td>Standard (PIR + SCO)</td>
<td>35.67 ± 1.02</td>
</tr>
<tr>
<td>4</td>
<td>PGLE 100 + SCO</td>
<td>48.67 ± 1.25</td>
</tr>
<tr>
<td>5</td>
<td>PGLE 200 + SCO</td>
<td>45 ± 1.59(^a)</td>
</tr>
<tr>
<td>----</td>
<td>-------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>6</td>
<td>PGLE 400 + SCO</td>
<td>39.17 ± 1.49(^b,c)</td>
</tr>
</tbody>
</table>

\(^a\) represent significant versus control group (P<0.001)
\(^b\) represent significant versus disease control group (P<0.001)
\(^c\) represent significant versus PGLE 100 + SCO group (P<0.001)

The statistical analysis was carried out using prism graph pad 5 software. All values were represent as mean ± SEM. Multiple comparision between different groups was performed using one way analysis of variance followed by Tukey’s test.

**Graph 2- Transfer latency of mice on elevated plus maze (Retrieval in sec)**

**Control** indicates administration of normal saline (10 ml/kg) for 21 days, **SCO** indicates administration of scopolamine (0.4 mg/kg i.p) on 21\(^{st}\) day, **PIR + SCO** indicates administration of piracetam (200 mg/kg i.p) for 21 days + scopolamine 30 min after Piracetam administration on 21\(^{st}\) day, **PGLE 100 + SCO** indicates administration of *Punica granatum* leaves extract (100 mg/kg i.p) for 21 days + scopolamine 30 min after *Punica granatum* leaves extract administration on 21\(^{st}\) day, **PGLE 200 + SCO** indicates administration of *Punica granatum* leaves extract (200 mg/kg i.p) for 21 days + scopolamine 30 min after *Punica granatum* leaves extract administration on 21\(^{st}\) day, **PGLE 400 + SCO** indicates administration of *Punica granatum* leaves extract (400 mg/kg i.p) for 21 days + scopolamine 30 min after *Punica granatum* leaves extract administration on 21\(^{st}\) day.
a represent significant versus control group, b represent significant versus disease control group, c represent significant versus PGLE 100 + SCO group. *** represent very significant at p<0.001. All values are expressed as Mean ± SEM.

**Table 3: Escape latency of mice using Morris water maze (1 – 4 days) in sec**

<table>
<thead>
<tr>
<th>S.no.</th>
<th>Group treatments</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>102.7 ± 1.02</td>
<td>91 ± 1.81</td>
<td>72.66 ± 1.26</td>
<td>45 ± 1.57</td>
</tr>
<tr>
<td>2</td>
<td>Disease control (SCO)</td>
<td>103.8 ± 1.13</td>
<td>101.8 ± 2.79a</td>
<td>103.3 ± 2.23a</td>
<td>96.83 ± 1.10a</td>
</tr>
<tr>
<td>3</td>
<td>Standard (PIR + SCO)</td>
<td>103 ± 1.50</td>
<td>91.33 ± 1.78b</td>
<td>79 ± 1.94b</td>
<td>59.67 ± 2.42b</td>
</tr>
<tr>
<td>4</td>
<td>PGLE 100 + SCO</td>
<td>104.3 ± 1.56</td>
<td>97 ± 1.15b</td>
<td>92.33 ± 2.76b</td>
<td>85.67 ± 1.49b</td>
</tr>
<tr>
<td>5</td>
<td>PGLE 200 + SCO</td>
<td>104.7 ± 0.99</td>
<td>96 ± 2.49b</td>
<td>87.50 ± 1.38b</td>
<td>74 ± 1.36b</td>
</tr>
<tr>
<td>6</td>
<td>PGLE 400 + SCO</td>
<td>107.3 ± 1.08</td>
<td>95.33 ± 2.84b</td>
<td>82.50 ± 1.057b</td>
<td>66.17 ± 2.83b</td>
</tr>
</tbody>
</table>

a represent significant versus control group (P<0.001)

b represent significant versus disease control group (P<0.001)

The statistical analysis was carried out using prism graph pad 5 software. All values was represent as mean ± SEM. Multiple comparision between different groups was performed using two way analysis of variance followed by Bonferroni’s test.

**Graph 3- Escape latency of mice using Morris water maze (1 – 4 days) in sec**

**Control** indicates administration of normal saline (10 ml/kg) for 21 days, **SCO** indicates administration of scopolamine (0.4 mg/kg i.p) on 21st day, **PIR + SCO** indicates administration of piracetam (200 mg/kg i.p) for 21 days + scopolamine 30 min after Piracetam administration on 21st day, **PGLE 100 + SCO** indicates administration of *Punica granatum* leaves extract (100 mg/kg i.p) for 21 days + scopolamine 30 min after *Punica granatum* leaves extract administration on 21st day, **PGLE 200 + SCO** indicates...
administration of *Punica granatum* leaves extract (200 mg/kg i.p) for 21 days + scopolamine 30 min after *Punica granatum* leaves extract administration on 21st day, **PGLE 400 + SCO** indicates administration of *Punica granatum* leaves extract (400 mg/kg i.p) for 21 days + scopolamine 30 min after *Punica granatum* leaves extract administration on 21st day.

a represent significant versus control group, b represent significant versus disease control group.

*** represent very significant at p<0.001, ** represent highly significant at p<0.01 and * represent significant at p<0.05. All values are expressed as Mean ± SEM.

**Table 4: Effect of *Punica granatum* leaves extract on time spent in target quadrant in scopolamine treated mice in Morris water maze**

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Treatment groups</th>
<th>Time spent in target quadrant (in sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>79.50 ± 0.94</td>
</tr>
<tr>
<td>2</td>
<td>Disease control (SCO)</td>
<td>46.17 ± 2.47 a</td>
</tr>
<tr>
<td>3</td>
<td>Standard (PIR + SCO)</td>
<td>84 ± 1.78 b</td>
</tr>
<tr>
<td>4</td>
<td>PGLE 100 + SCO</td>
<td>65 ± 2.73 b</td>
</tr>
<tr>
<td>5</td>
<td>PGLE 200 + SCO</td>
<td>67 ± 1.77 b, c</td>
</tr>
<tr>
<td>6</td>
<td>PGLE 400 + SCO</td>
<td>74 ± 1.05 b, c</td>
</tr>
</tbody>
</table>

a represent significant versus control group (P<0.001)
b represent significant versus disease control group (P<0.001)
c represent significant versus PGLE 100 + SCO group (P<0.001 and 0.05)

The statistical analysis was carried out using prism graph pad 5 software. All values was represent as mean ± SEM. Multiple comparision between different groups was performed using one way analysis of variance followed by Tukey’s test.

**Graph 4: Time spent in target quadrant in scopolamine treated mice in Morris water maze**
Control indicates administration of normal saline (10 ml/kg) for 21 days, SCO indicates administration of scopolamine (0.4 mg/kg i.p) on 21st day, PIR + SCO indicates administration of piracetam (200 mg/kg i.p) for 21 days + scopolamine 30 min after Piracetam administration on 21st day, PGLE 100 + SCO indicates administration of Punica granatum leaves extract (100 mg/kg i.p) for 21 days + scopolamine 30 min after Punica granatum leaves extract administration on 21st day, PGLE 200 + SCO indicates administration of Punica granatum leaves extract (200 mg/kg i.p) for 21 days + scopolamine 30 min after Punica granatum leaves extract administration on 21st day, PGLE 400 + SCO indicates administration of Punica granatum leaves extract (400 mg/kg i.p) for 21 days + scopolamine 30 min after Punica granatum leaves extract administration on 21st day.

a represent significant versus control group, b represent significant versus disease control group, c represent significant versus PGLE 100 + SCO group.

*** represent very significant at p<0.001 and * represent significant at p<0.05. All values are expressed as Mean ± SEM.

Table 5: Effect of Punica granatum leaves extract on brain cholinesterase activity in scopolamine treated mice.

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Treatment groups</th>
<th>Enzyme activity (µM/min/mg of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>15.67 ± 1.71</td>
</tr>
<tr>
<td>2</td>
<td>Disease control (SCO)</td>
<td>45.33 ± 1.61&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>Standard (PIR + SCO)</td>
<td>22.83 ± 1.19&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>PGLE 100 + SCO</td>
<td>41 ± 2.73&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>PGLE 200 + SCO</td>
<td>33.50 ± 1.36&lt;sup&gt;b, c&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>PGLE 400 + SCO</td>
<td>27.83 ± 2.60&lt;sup&gt;b, c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

a represent significant versus control group (P<0.001)
b represent significant versus disease control group (P<0.001 and 0.05)
c represent significant versus PGLE 100 + SCO group (P<0.001 and 0.01)

The statistical analysis was carried out using prism graph pad 5software. All values was represent as mean ± SEM. Multiple comparision between different groups was performed using one way analysis of variance followed by Tukey’s test.
Graph 5- Effect of *Punica granatum* leaves extract on brain cholinesterase activity in scopolamine treated mice.

**Control** indicates administration of normal saline (10 ml/kg) for 21 days, **SCO** indicates administration of scopolamine (0.4 mg/kg i.p) on 21st day, **PIR + SCO** indicates administration of piracetam (200 mg/kg i.p) for 21 days + scopolamine 30 min after Piracetam administration on 21st day, **PGLE 100 + SCO** indicates administration of *Punica granatum* leaves extract (100 mg/kg i.p) for 21 days + scopolamine 30 min after *Punica granatum* leaves extract administration on 21st day, **PGLE 200 + SCO** indicates administration of *Punica granatum* leaves extract (200 mg/kg i.p) for 21 days + scopolamine 30 min after *Punica granatum* leaves extract administration on 21st day, **PGLE 400 + SCO** indicates administration of *Punica granatum* leaves extract (400 mg/kg i.p) for 21 days + scopolamine 30 min after *Punica granatum* leaves extract administration on 21st day.

a represent significant versus control group, b represent significant versus disease control group, c represent significant versus PGLE 100 + SCO group.

*** represent very significant at p<0.001, ** represent highly significant at p<0.01 and * represent significant at p<0.05. All values are expressed as Mean ± SEM.
Table 6: Effect of *Punica granatum* leaves extract on lipid peroxidation in scopolamine treated mice

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Treatment groups</th>
<th>Level of MDA (nM/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3.83 ± 0.97</td>
</tr>
<tr>
<td>2</td>
<td>Disease control (SCO)</td>
<td>0.81 ± 0.93 ^a</td>
</tr>
<tr>
<td>3</td>
<td>Standard (PIR + SCO)</td>
<td>4.33 ± 1.05 ^b</td>
</tr>
<tr>
<td>4</td>
<td>PGLE 100 + SCO</td>
<td>6.66 ± 1.19 ^b</td>
</tr>
<tr>
<td>5</td>
<td>PGLE 200 + SCO</td>
<td>5.66 ± 1.42 ^b,^c</td>
</tr>
<tr>
<td>6</td>
<td>PGLE 400 + SCO</td>
<td>4.83 ± 1.13 ^b,^c</td>
</tr>
</tbody>
</table>

^a^ represent significant versus control group (P<0.001)
^b^ represent significant versus disease control group (P<0.001 and 0.05)
^c^ represent significant versus PGLE 100 + SCO group (P<0.01)

The statistical analysis was carried out using prism graph pad 5 software. All values was represent as mean ± SEM. Multiple comparision between different groups was performed using one way analysis of variance followed by Tukey’s test.

**Graph 6- Effect of *Punica granatum* leaves extract on lipid peroxidation in scopolamine treated mice**

**Control** indicates administration of normal saline (10 ml/kg) for 21 days, **SCO** indicates administration of scopolamine (0.4 mg/kg i.p) on 21st day, **PIR + SCO** indicates administration of piracetam (200 mg/kg i.p) for 21 days + scopolamine 30 min after Piracetam administration on 21st day, **PGLE 100 + SCO** indicates administration of *Punica granatum* leaves extract (100 mg/kg i.p) for 21 days + scopolamine 30 min after *Punica granatum* leaves extract administration on 21st day, **PGLE 200 + SCO** indicates administration of *Punica granatum* leaves extract (200 mg/kg i.p) for 21 days + scopolamine
30 min after *Punica granatum* leaves extract administration on 21st day, **PGLE 400 + SCO** indicates administration of *Punica granatum* leaves extract (400 mg/kg i.p) for 21 days + scopolamine 30 min after *Punica granatum* leaves extract administration on 21st day.

a represent significant versus control group, b represent significant versus disease control group, c represent significant versus PGLE 100 + SCO group. *** represent very significant at p<0.001, ** represent highly significant at p<0.01 and * represent significant at p<0.05.

All values are expressed as Mean ± SEM.

### Table 7: Effect of *Punica granatum* leaves extract on level of glutathione in scopolamine treated mice

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Treatment groups</th>
<th>Level of glutathione (nM/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>41.65 ± 1.55</td>
</tr>
<tr>
<td>2</td>
<td>Disease control (SCO)</td>
<td>25 ± 1.39 b</td>
</tr>
<tr>
<td>3</td>
<td>Standard (PIR + SCO)</td>
<td>42.83 ± 1.49 b</td>
</tr>
<tr>
<td>4</td>
<td>PGLE 100 + SCO</td>
<td>28.50 ± 2.56</td>
</tr>
<tr>
<td>5</td>
<td>PGLE 200 + SCO</td>
<td>29.17 ± 1.01 b</td>
</tr>
<tr>
<td>6</td>
<td>PGLE 400 + SCO</td>
<td>33.33 ± 1.17 b</td>
</tr>
</tbody>
</table>

a represent significant versus control group (P<0.001)
b represent significant versus disease control group (P<0.001 and 0.05)
c represent significant versus PGLE 100 + SCO group (P<0.001)

The statistical analysis was carried out using prism graph pad 5software. All values was represent as mean ± SEM. Multiple comparision between different groups was performed using one way analysis of variance followed by Tukey’s test.

Graph 7- Effect of *Punica granatum* leaves extract on level of glutathione in scopolamine treated mice
**Control** indicates administration of normal saline (10 ml/kg) for 21 days, **SCO** indicates administration of scopolamine (0.4 mg/kg i.p) on 21st day, **PIR + SCO** indicates administration of piracetam (200 mg/kg i.p) for 21 days + scopolamine 30 min after Piracetam administration on 21st day, **PGLE 100 + SCO** indicates administration of *Punica granatum* leaves extract (100 mg/kg i.p) for 21 days + scopolamine 30 min after *Punica granatum* leaves extract administration on 21st day, **PGLE 200 + SCO** indicates administration of *Punica granatum* leaves extract (200 mg/kg i.p) for 21 days + scopolamine 30 min after *Punica granatum* leaves extract administration on 21st day, **PGLE 400 + SCO** indicates administration of *Punica granatum* leaves extract (400 mg/kg i.p) for 21 days + scopolamine 30 min after *Punica granatum* leaves extract administration on 21st day.

a represent significant versus control group, b represent significant versus disease control group, c represent significant versus PGLE 100 + SCO group. *** represent very significant at p<0.001 and * represent significant at p<0.05. All values are expressed as Mean ± SEM.

**Table 8: Effect of *Punica granatum* leaves extract on level of superoxide dismutase in scopolamine treated mice**

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Treatment groups</th>
<th>Level of SOD (Units/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>7.66 ± 1.49</td>
</tr>
<tr>
<td>2</td>
<td>Disease control (SCO)</td>
<td>2.50 ± 2.42a</td>
</tr>
<tr>
<td>3</td>
<td>Standard (PIR + SCO)</td>
<td>8.16 ± 1.06b</td>
</tr>
<tr>
<td>4</td>
<td>PGLE 100 + SCO</td>
<td>5 ± 0.91b</td>
</tr>
<tr>
<td>5</td>
<td>PGLE 200 + SCO</td>
<td>6.33 ± 0.99b</td>
</tr>
<tr>
<td>6</td>
<td>PGLE 400 + SCO</td>
<td>7.13 ± 1.33b, c</td>
</tr>
</tbody>
</table>

a represent significant versus control group (P<0.001)  
b represent significant versus disease control group (P<0.001 and 0.05)  
c represent significant versus PGLE 100 + SCO group (P<0.01)

The statistical analysis was carried out using prism graph pad 5software. All values was represent as mean ± SEM. Multiple comparision between different groups was performed using one way analysis of variance followed by Tukey’s test.
Graph 8- Effect of *Punica granatum* leaves extract on level of superoxide dismutase in scopolamine treated mice

**Control** indicates administration of normal saline (10 ml/kg) for 21 days, **SCO** indicates administration of scopolamine (0.4 mg/kg i.p) on 21st day, **PIR + SCO** indicates administration of piracetam (200 mg/kg i.p) for 21 days + scopolamine 30 min after Piracetam administration on 21st day, **PGLE 100 + SCO** indicates administration of *Punica granatum* leaves extract (100 mg/kg i.p) for 21 days + scopolamine 30 min after *Punica granatum* leaves extract administration on 21st day, **PGLE 200 + SCO** indicates administration of *Punica granatum* leaves extract (200 mg/kg i.p) for 21 days + scopolamine 30 min after *Punica granatum* leaves extract administration on 21st day, **PGLE 400 + SCO** indicates administration of *Punica granatum* leaves extract (400 mg/kg i.p) for 21 days + scopolamine 30 min after *Punica granatum* leaves extract administration on 21st day.

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**DISCUSSION**

Alzheimer’s disease is slowly or gradually progressive dementia affecting both (1) cognition (process of acquiring knowledge and understand thought & sense), (2) behavior (conducts oneself toward others).

Learning is defined as the act of obtaining new or making partial changes to existing knowledge, behavior and skill. Learning is a long term change in mental representation
(mental imagery of things that are latest or old seen or sensed by sensory organ) and enhance due to experience.

Memory is the process in which captured information is encoded, stored and retrieved.

Learning and memory impairment is closely related to the cholinergic hypothesis. The level of Ach (messenger in the brain) decrease badly in the brain and at the same time nerve ending and brain cell also damaged and some structural changes are also found in brain.

Oxidative stress also plays an important role in the learning and memory impairment. Oxidative Formation of Aβ in AD brains leads to induces lipid peroxidation and generates reactive oxygen & nitrogen species which contains unpaired extra electron. This extra unpaired electron needs other molecule for stable configuration. During this process extra molecule binds with another molecule and form free radical (high energy electron) and this free radical alter the molecule to which they attached and finally cause cellular and molecular damage and leads to AD.

The most widely used nootropics (Racetams) drugs act as AMPA modulator. After the administration of Piracetam is believed (1) to reduce free radicals, oxidative stress due to free radicals can cause cholinergic damage, (2) modulator of central neurotransmitter like Ach and glutamate, (3) positively allosteric modulator of AMPA R.

Scopolamine significantly impaired the hippocampus dependent memory, after the administration of scopolamine on 21st day they cause cholinergic dysfunction through elevation of the level of AchE and reduce the level of Ach in brain and they competitively block the muscarinic receptor, hence scopolamine induced neurotoxicity or learning and memory impairment.

The plant Punica granatum is a rich source of flavones and flavanoids, these flavanoids and flavones are well known for antioxidant potential, hence shows significant effect on learning and memory.

Elevated plus maze and Morris water maze test are employed as most extensively accepted model for evaluation of learning and memory. Mice taken lesser seconds in transfer latency (Retrieval) as compared to transfer latency (acquisition) it means standard and PGLE showed better result in retrieval day. In morris water maze, after the administration of standard and
PGLE significant reduction in escape latency time and also increase time spent in target quadrant on 5th day.

**Determination of brain AchE activity**

The AchE rised level in Alzheimer’s disease has results in the hypothesis that learning and memory impairment is linked to cholinergic degradation. Therefore precise approach for treatment of Alzheimer’s disease is to increase Ach level in the brain region.

The present study shows that a significant increase in AchE in scopolamine administered mice. A significant decrease AchE function has been showed in the mice treated with PGLE especially in 400 mg/kg dose. Thus the leave extract of *Punica granatum* is known to inhibit the increased AchE activity.

**Antioxidant parameter**

Too much synthesis of free radicals or the reactive oxygen species can lead to the damage of the cell and tissue of the brain which leads to cell death. The class of main defence antioxidant that prevent the generation of new free radical species are SOD and glutathione. Hydrogen peroxide was covered from SOD and glutathione peroxide alters hydrogen peroxide into non harming molecules.

The further study concluded that the oxidative stress is induced by incorporating of scopolamine on 21 day. In mice scopolamine decrease the SOD and glutathione level. After the administration of PIR and PGLE showed significant increase in SOD and glutathione level.

**Lipid peroxidation**

Rised in the level of lipid peroxidation in the brain shows the neuronal damage. The decrease of antioxidant defence and/or increase in free radical generation deteriorates the prooxidant and antioxidant balance regulation which leads to oxidative stress and cell death.

The oxidative stress produced by scopolamine has been linked with the rised amount of lipid peroxidation. Infact the administration of ethanolic extract of *Punica granatum* in our experiment was potentially active in reducing the oxidative stress. This indicates that *Punica granatum* leaves extract has potent antioxidant activity to reduce the oxidative stress induced lipid peroxidation.
CONCLUSION
The aim of the present study was to evaluate the therapeutic potential of *Punica granatum* on scopolamine induced learning and memory impairment in mice. This was proved by the following parameters.

For initially the phytochemical constituents in ethanolic extract of *Punica granatum* were found to be glycoside, flavones, alkaloids, carbohydrate, tannins and phenols. Disease induced (scopolamine treated) mice shows a significant rise in the AchE activity. Whereas, increased activity is reverse by treatment of group with PGLE and Piracetam (standard). Decrease in the AchE activity by *Punica granatum* extract may directly or indirectly related to the cholinergic dependent learning and memory.

After administration of scopolamine, disease control mice showed decrease in free radical scavenging enzyme such as SOD and glutathione. In other hand after the administration of PGLE, free radical scavenging enzyme restored significantly.

In present study treatment by *Punica granatum* decrease the lipid peroxidation. Hence, it was concluded the *Punica granatum* have a potential role in management of learning and memory impairment.

REFERENCES


15. Habidur Rahman, “Inhibition of acetylcholinestrase and antioxidant activity are mostly used mechanism of Nardostacys Jata Mansi in sleep deficiency alziemer’s mice model”, IJPPR, 2011; 1809-1815.

