ABSTRACT

Objective: The objective of the present study was to evaluate the possible neuroprotective effect of ethanolic extract of *Mimosa pudica* (EEMP) in chronic Alzheimer’s model using albino wistar rats.

Methods: The dried whole part of the plant were subjected to hot continuous extraction method using ethanol as solvent and were subjected for phytochemical analysis. The parameters employed for assessing nootropic activity were transfer latency, retention latency and number of memory errors in elevated plus maze, Morris water maze and radial arm maze. These activities were tested at oral dose of extract 500 mg/kg, piracetam 50 mg/kg was used for comparison and D-galactose 100mg/kg was used as control group. Results: Phytochemical screening confirmed the presence of flavonoids, phenolic compounds and steroids. EEMP treatment had shown a significant protective effect in memory dysfunction caused by D-Galactose in various model. Conclusion: It is concluded that ethanolic extract of *M. pudica* possessed significant neuroprotective effect against D-Galactose induced Alzheimer’s model.

KEY WORDS: Alzheimers disease, D-Galactose, EEMP.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by impairment of memory and eventually by disturbances in reasoning, planning, language, and
perception. Poor learning abilities and impaired memory are the striking features accompanying with elderly population and stress exposure.\(^2\) Dementia and impairment in memory are increasing day by day in the modern society, which might be due to the life style changes or changes in food habit or the spoiling relationship prevail in the society or increased stress exposure in modern life. Hence there is an urgent need for cognitive enhancer to develop. Recent studies showed that the oxidative stress play a major role in neurodegenerative disorders, and therefore the present study focuses on *M. pudica* due to its potent antioxidant activity. The earlier literatures supported with its sedative hypnotic activity and antioxidant activity.\(^3, 4\)

The research laboratory for neuropsychopharmacology, University College of Pharmacy, Cheruvandoor Campus, Mahatma Gandhi University is currently focusing to develop some herbal medicines for neurodegenerative disorders. The main thrust area of neuropharmacology division is to invent some compounds for Alzheimer’s disease, Parkinson’s disease, depression, stress and phobic disorders. Ittiyavirah and George in 2013 had reported that ethanolic extract of *Mimosa pudica* established a significant neuroprotection in Aluminium chloride induced Alzheimers model.\(^4\) Hence in continuation, our current research was focusing the possible protective effect of EEMP on various paradigms in D-Galactose induced model for dementia.

Several naturally occurring herbs viz, Abana, *Bacopa monnieri* *Centella asiatica* and *Celastrus paniculatus* were found to have potent nootropic activities and they are considered as the herbal nootropics.\(^5,6,7\) Piracetam is the standard compound for compair0ing the nootropic activity oh herbal drugs.\(^8\)

**MATERIALS AND METHOD**

**Materials**
Ethanol (Travancore Chemicals, Trivandrum), piracetam (Nice Chemicals (P), Ltd, Kochi, Kerala), D-galactose (Travancore Chemicals, Trivandrum), CMC, hydrogen peroxide (Nice chemicals (P), Ltd, Kochi, Kerala), gallic acid (Nice Chemicals (P), Ltd, Kochi, Kerala), methanol, riboflavin, EDTA, sodium hydroxide, potassium dihydrogen phosphate, nitro blue tetrazolium (NBT), Soxhlet extractor (Glastron Laboratory Equipments, Haryana, India), UV/VIS spectrophotometer (Systronic double beam-UV-2201, systronics, India).
Plant material
The whole plant of *M. pudica* was collected from the botanical garden of University College of Pharmacy (UCP), Cheruvandoor campus, Mahatma Gandhi University, Kottayam, and authenticated from Prof. Jomy Augustine, Professor & Head, Department of Botany, St Thomas College, Palai, Kottayam, Kerala.

Extraction
The dried whole plant was grounded; the free flowing powder obtained was subjected for Soxhlet extraction with dehydrated alcohol. The extract was filtered through Whatmann filter paper and dried at 40-50°C to get a blackish green semisolid mass, which was dissolved in saline solution for final use.

Animals
Albino Wistar rats weighing 150-200g of either sex maintained under standard husbandry conditions were used for the study. They were obtained from the registered animal house of the UCP. The experiments were performed after getting the approval from the institutional animal ethical committee (IAEC/004/MPH/UCP/CVR/13), UCP Cheruvandoor Campus, MG University, Kerala, India.

IN VITRO ANTIOXIDANT ASSAY
1. Hydrogen peroxide scavenging assay
   1 ml of the EEMP and 0.6 ml hydrogen peroxide was added to the test tube and it was made upto 5ml with phosphate buffer. The absorbance was measured at 230nm using UV spectrophotometer against a blank solution containing phosphate buffer without using hydrogen peroxide. Assay was done in triplicate for all test samples and averages were counted. Percentage inhibition was determined by the given formula:
   \[
   \%\text{inhibition} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
   \]
   where, \(A_0\) was the absorbance of the control and \(A_1\) was the absorbance of sample.

2. Super oxide radical scavenging assay
   0.02ml of various concentrations of extract (12.5-200µg/ml), 0.05ml of Riboflavin solution (0.12mM), 0.2 ml of EDTA solution [0.1M], and 0.1 ml NBT (Nitro-blue tetrazolium) solution [1.5mM] were mixed in test tube and reaction mixture was diluted up to 2.64 ml with phosphate buffer [0.067M]. The absorbance of solution was measured at 560 nm using
DMSO as blank after illumination for 5 min and difference in OD was determined after 30 min incubation in fluorescent light using UV visible spectrometer.

\[
\% \text{ Inhibition} = \left[ \frac{\text{OD of control} - \text{OD of test sample}}{\text{OD of control}} \right] \times 100
\]

**IN VIVO ASSAY**

**Estimation of nootropic activity in D-Galactose induced Alzheimer’s model**

D-galactose is the one of the most commonly used model for the evaluation of chronic Alzheimer’s model. Nootropic activity was explored using D-Galactose induced model\(^{[11]}\) in various paradigms including Morris water maze, radial arm maze and elevated plus maze and recorded the retention latency, number of errors and inflexion ratio respectively.

**Experimental design**

Four groups of six rats. Each group received the following treatment schedule:

- Negative control group received 0.5\%w/v CMC p.o for a period of 42 days.
- Positive control group received D-Galactose(100mg/kg) p.o for a period of 42 days, while the test and standard group received D-Galactose for a period of 21 days each and followed by 21 day treatment of EEMP(500 mg/kg) and piracetam(50 mg/kg) respectively.

**Elevated plus maze\(^{[12]}\)**

Elevated plus maze consisted of two open arms (35 × 6 cm) crossed with two enclosed arms (35 × 6 × 15 cm). The arms were connected with a central square of 5x5 cm dimension. The rats were placed individually at the end of one arm facing away from the center of the elevated plus maze and the time taken by the rat to move from open arm to either of the closed arms with all its four legs termed as **Transfer latency (TL)**, was recorded. On the first day, the rats were allowed to explore the plus maze for 20 sec. After the measurement of TL rats were returned to their home cages after the first trial. 24 days later, the rats were placed on the elevated plus – maze individually as before and TL was recorded again. TL measured on 1\(^{st}\) and 2\(^{nd}\) day served as parameters for acquisition and retrieval respectively.

**Radial arm maze\(^{[13]}\)**

The radial arm maze used was open type which consists of a central circular arena and 8 equally sized arms. Each arm consists of 60 cm length and 20 cm breadth. The animals were placed on the central platform and allowed to move freely on each arm. At far end of each arm a small plastic dish was kept and sucrose food pellet was mounted on it. The spatial working and reference memory were evaluated by the instrument.
The animals were habituated to the environment, placed to the central platform and allowed to explore maze for 15 min. Reinforces (or baits) are scattered on the arms. For the training periods of first days all the arms were baited and followed by baiting on alternative arms. Retention latencies (time for rat to reach the reward) were recorded on 21st and 42nd day.

The spatial memory error was measured in the radial arm maze apparatus. The two types of spatial memory include spatial working and spatial reference memory. Spatial working memory error is considered as the double entries into the baited arm while the spatial reference memory error is considered as entries in never baited arm.

**Morris water maze** [14]

Morris water maze consisted of large circular pool (75 cm diameter and 30 cm height) filled with water at a depth of 20 cm. The pool was divided into four equal quadrants with the help of a thread. A circular platform was placed in one quadrant of the pool 1 cm above the water level during the acquisition phase. A similar platform was placed 1 cm below the water level during the retention phase. The position of the platform was not changed in any quadrant during assessing of both phases. Each animal was subjected to four consecutive trials with a gap of 5 min. Each animal was allowed 120 sec to locate the platform.

Retention phase: On 21st day the animals were released on the Morris water maze from one of the quadrant which should be followed throughout the experiment, and were allowed to find the hidden platform and the time taken for the finding was recorded and termed as the first retention latency. It was repeated on 42nd day; the last day of drug treatment and the time taken for finding the hidden platform were recorded and termed as the second retention latency.

**Statistical analysis:** Statistical analysis was done by using Graphpad prism, version 5.0. All the values were expressed as Mean±Standard error mean (SEM). The values were analyzed for statistical significance using one way analysis of variance (ANOVA) followed by Dunnet’s multiple comparison test.

**RESULTS**

**Phytochemical investigation**

The phytochemicals present in the ethanolic extract of *M.pudica* were alkaloids, glycosides, flavonoids, terpenoids and steroids.
Table 1: Effect of EEMP on mean Inflexion Ratio in Elevated Plus maze test:

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Groups</th>
<th>Treatment</th>
<th>Mean IR (Mean±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle</td>
<td>CMC(0.5%w/v)</td>
<td>0.0215±0.002</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>CMC(0.5%w/v) + D-galactose(100mg/kg) (p.o.)</td>
<td>0.0128±0.0009</td>
</tr>
<tr>
<td>3</td>
<td>Standard</td>
<td>piracetam(50mg/kg)+D-galactose(100mg/kg) (p.o.)</td>
<td>0.0425±0.003***</td>
</tr>
<tr>
<td>4</td>
<td>Test</td>
<td>EEMP(500mg/kg) + D-galactose(100mg/kg) (p.o.)</td>
<td>0.0378±0.008**</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM (n=6), analysed by one-way ANOVA followed by Dunnett’s post hoc test, *Represents statistical significance Vs positive control. (P<0.05), **P<0.01, ***P<0.001

DISCUSSION

The present study demonstrated that M. pudica extract prevents memory deficit against D-Galactose induced senescence. D-Galactose plays a prime role in the pathogenesis of aging. Various hypothesis have been put forward to explain the mechanism of D-galactose induced aging including glycometabolism block, formation of advanced glycation end product (AGE) and free radical injury etc. The plant M.pudica showed well antioxidant activity by in vitro antioxidant assay and the IC$_{50}$ values were comparable with that of standard.

In vitro antioxidant studies were carried out to assess the scavenging potential of M. pudica. Hydrogen peroxide scavenging assay has been used to evaluate the free radical scavenging activity of natural antioxidant. Hydrogen peroxide radicals react with suitable reducing agents as a result of which the electrons become paired off forming the neutralized product water. In hydrogen peroxide scavenging assay the IC$_{50}$ values for EEMP was 19µg/ml and that of Ascorbic acid was 5 µg /ml (Fig: 1). The phytochemical analysis confirmed the presence of phenolics, flavonoids, and tannins. So from this finding It can be predicted that the H$_2$O$_2$ Scavenging might be due to the presence of phenolic compound. Hydrogen peroxide Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol groups. H$_2$O$_2$ can cross cell membrane rapidly. Once inside the cell H$_2$O$_2$ can probably react with Fe$^{2+}$ and possibly Cu$^{2+}$ ions to form hydroxyl radical and this may be the origin of many of its toxic effects. Manisha et al., 2009 had reported that plant extract containing phenolic compounds can donate electrons to hydrogen peroxide and thus neutralizing to water.
Hence we postulated that the active principles in the EEMP have hydrogen/ electron donors and can react with free radicals to convert them to more stable products thus scavenging the free radical and terminate radical chain reactions. Hence in present study we also postulated that the presence of phenolic compounds might be contributed to similar response.

The technique in super oxide radical scavenging assay (Fig: 2) involves the extract along with riboflavin reduces NBT, so the intensities of blue color formed has been changed. Free radical scavenging activity increased with increasing the concentration of extract. This is the reason behind more intense color formation and high OD, which means less inhibition at that particular concentration, and vice versa. Manisha et al., 2009 had reported that plant extract containing phenolic compounds and flavanoids have the ability to donate electrons and this may contribute the present responsibility.

The findings of in vitro results were strongly supported by the in vivo analysis done by D-Galactose model with various paradigms viz, Radial arm maze, morris water maze and elevated plus maze method.

In the present study D-galactose induced rats’ takes longer time to find the hidden platform during the retrieval trial in morris water maze, while the EEMP treated rats show very short time to reach the goal (Fig: 3&4). Retrieval time is the time taken by the rat to reach the hidden platform. This maze respects a more specific test of spatial memory, not confounded by working memory effects.\textsuperscript{[16]} The hidden platform version of the Morris Water Maze is a test of spatial memory which is sensitive to hippocampal damage, while the visible platform version of the Morris Water Maze is a non-hippocampal task, which is disrupted by dorsal striatum lesions.\textsuperscript{[17]}

Observation has been further strengthened by the results of Radial arm maze method. In this study the EEMP treated rats showed little errors in entering the arms (Fig: 5-8) and a less time to complete the whole session in the maze (Fig: 9&10). The two parameters measured were working memory error and reference memory error. The entry into never baited arm considered as the reference memory error while the re-entry into a baited arm should considered as the working memory error.

The results has been further strengthened with the positive results given by the EPM(elevated plus maze) test, in which EEMP treated rats can overcome memory impairment induced by
D-galactose. The results showed it take EEMP treated rats take less transfer latency time. The transfer latency can be defined as the time take by the rat to enter any one of the closed arms with all its four legs.

CONCLUSION
It was concluded that EEMP possessed significant neuroprotective effect in chronic Alzheimer’s model induced by D-Galactose.

REFERENCES


