EVALUATION OF NOOTROPIC ACTIVITY OF CARICA PAPAYA IN MICE

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ABSTRACT

Alzheimer’s disease is a progressive neurodegenerative disorder primarily manifesting as a loss of memory, senile dementia, and intraneuronal neurofibrillary tangle formation. C. papaya has a very long history of medicinal use in Chinese and Indian herbal traditions. The objective of this study is to evaluate the nootropic activity of Carica papaya by using animal model mice. The dried seeds of papaya fruits were used for the extraction by cold maceration method using ethanol as solvent. Preliminary Phytochemical study was performed. Estimation of anti-oxidant enzymes like super oxide dismutase, glutathione peroxide and glutathione reductase were done in extract treated mice. Estimation of acetylcholinesterase levels was also done. The ethyl acetate extract gave positive results for alkaloids, flavanoids, carbohydrates, tannins, glycosides, and absence of proteins, saponins, steroids, terpenes, phenols, gums and mucilage. In the present study, we have found a significant decrease in the level of antioxidant enzymes and the elevated of AChE in mice brain after a single injection of Scopolamine. EECP at 200 mg/ kg and 400 mg/kg had shown the significant reduction in the elevated enzyme level of acetylcholine esterase. The oxidative stress involved by the administration of Scopolamine produced neurotoxicity indicated the decreased levels of super oxide dismutase, glutathione peroxidase, glutathione reductase. Treatment of EECP shows the protection of these antioxidant enzymes on both 200 mg/kg and 400 mg/kg dose level respectively due to the rejuvenating property of the extract.

Key Words: Alzeimer’s disease, C. papaya, acetylcholinestarase, oxidative stress.

INTRODUCTION

Prevalence rates for dementia increase exponentially with advancing age, ranging from 10% in the age group of 60-65 years to 36% in the age group of 90 years (Sharma et al., 1997). This disease may be presenile or senile onset and the occurrence of disease is before or after the age group of 60 years, but now it is challenging the median age of population (Sloane et al., 2002). AD was the 7th leading cause of death in 2004 with 65,829 numbers of deaths (Hebert et al., 2003). There are an estimated 24 million people with dementia worldwide (Wimo et al., 2003).

Dementia occurs in a number of brain diseases, where the impairment in cognitive abilities represents a decline from prior levels of function and interferes with the ability to perform routine daily activities, as the disease progresses memory of remote events and over learned information declines together with other cognitive ability. Behavioral disturbances
include agitation, aggression, depressive mood, sleep disorder and anxiety.

*Carica papaya* grows either as wild or cultivated crop throughout India ascending upto 180m in the Himalayas. *C. papaya* is now also found across Europe, in southern Russia, northern Asia Minor, southern Siberia, China, Indonesia, Japan, Burma, Sri Lanka, Australia, as well as southern Canada and the northern United States. *C. papaya* has a very long history of medicinal use in Chinese and Indian herbal traditions. It is widely employed in modern herbal medicine as its sedative, laxative, diuretic and carminative properties. It is used in Ayurveda to counter the side effects of all hallucinogens. Both roots and leaves of *C. papaya* have shown antioxidant, antimicrobial and insecticidal activities (Delbridge *et al.*, 1998). Main chemical components are papain, Chymopapain, Pectin, Carposide, Carpaine, pseudocarpaine, dehydrocarpines, Caotenoids, Cryptoglavine and antheraxanthin (Ayurvedic Formulary of India, 2003). The main objective of this study is to evaluate the nootropic activity of *C. papaya* extract.

**MATERIALS AND METHODS**

**Plant Collection:**
Plant material used in this study includes seeds of *C. papaya* L. was collected from local areas of Warangal, Andhra Pradesh, India. It was identified and authenticated by Prof. Dr. Vatsavaya S.Raju, Department of Botany, Kakatyia University, Warangal, Andhra Pradesh, India.

**Preparation of Ethanolic Extract:**
The papaya fruits were cut into small pieces and the wet seeds were separated out. These were then gently and thoroughly rinsed in water and dried at room temperature. The dried seeds were pulverized into fine powder using mixer grinder. The coarse powder was used for the extraction by cold maceration method using ethanol as solvent at room temperature.

**Preliminary Phytochemical Analysis:**
The ethyl acetate extract of the fruit of *C. papaya* L. (EECP) was subjected to preliminary phytochemical screening (Harbone, 1973).

1. **Test for Alkaloids:**
The extract were treated with diluted HCl and filtered. The filtrate was treated with various alkaloidal agents.
   - **Mayers Test:** Sample was treated with Mayers reagent, appearance of cream color indicates presence of alkaloids.
   - **Dragendroff’s Test:** Sample was treated with Dragendroff’s reagent, appearance of reddish brown precipitate indicates presence of alkaloids.
   - **Hagers Test:** Sample was treated with Hagers reagent, appearance of yellow color indicates presence of alkaloids.
   - **Wagers Test:** Sample was treated with Wagers reagent, appearance of brown precipitate indicates presence of alkaloids.

2. **Test for Carbohydrates:**
The extracts were treated with 3 ml of alpha naphthol in alcohol and Conc. Sulphuric acid was carefully added to side of the test tubes. Formation of a violet ring at the junction of two liquids indicates presence of carbohydrates.
   - **Fehling’s Test:** To the sample Fehling’s solution A and B was added and heated for two minutes. Appearance of reddish brown color indicates presence of reducing sugars.
   - **Benedicts Test:** To the sample benedicts solution was added and heated, appearance of reddish orange precipitate indicates presence of reducing sugars.
   - **Barfoed’s Test:** The sample were treated with Barfoed’s reagent and heated, appearance of reddish orange precipitate indicates presence of reducing sugars.

3. **Test for Proteins:**
   - **Biuret’s Test:** To the extracts copper sulphate solution followed by sodium hydroxide solution, a violet color precipitates indicates presence of proteins.
   - **Millions Test:** To the extracts millions reagent was added, appearance of pink color indicates presence of proteins.
4. Test for Steroids:
   • Libermann Burchard’s Test: The extracts were treated with Conc. Sulphuric acid and glacial acetic acid followed by acetic anhydride, a violet ring appears at the junction of the liquids and appearance of green color in the aqueous layer indicates presence of steroids.

5. Test for Sterols:
   • The extracts were treated with 5% KOH solution, appearance of pink color indicates the presence of sterols.

6. Test for Phenols
   • The extracts were treated with neutral ferric chloride solution, appearance of violet color indicates presence of phenols.
   • The extracts were treated with 10% sodium chloride solution, appearance of cream color indicates presence of phenols.

7. Test for Tannins:
   • The extracts were treated with 10% lead acetate solution appearance of white precipitate indicates presence of tannins.
   • The extracts were treated with aqueous bromine water; appearance of white precipitate indicates presence of tannins.

8. Test for Flavanoids
   • 5ml of the extracts solution was hydrolyzed with 10% sulphuric acid and cooled. It was then extracted with diethyl ether and divided in to 3 portions in three separate test tubes. 1ml of diluted sodium carbonate, 1ml of 0.1 N sodium hydroxide and 1 ml of diluted ammonia solutions were added to the first second and third test tube respectively. Development of yellow color in each test tube indicates presence of flavanoids.
   • Shinoda’s Test: The extracts were dissolved in alcohol, to which a piece of magnesium followed by drop wise addition of Conc. HCl and heated. Appearance of magenta color indicates presence of flavanoids.

9. Test for Gums and Mucilage:
   • The extracts were treated with 25 ml absolute alcohol and then the solution was filtered. The filtrate was examined for its swelling properties.

10. Test for Glycosides:
    • A pinch of the extract were dissolved in glacial acetic acid and few drops of ferric chloride solution was added followed by the addition of Conc. Sulphuric acid, formation of red ring at the junction of the two liquids indicates presence of glycosides.

11. Test for Saponins:
    • Foam test 1 ml of the extract was diluted to 2ml with distilled water, formation of foam in the upper part of the test tubes presence of saponins.

12. Test for Terpenes:
    • The extracts were treated with tin and thionyl chloride, appearance of pink color indicates presence of terpenes.

Animals:
Swiss albino mice (18-22gm) of either sex were obtained from the animal house in St. John college of Pharmacy, Warangal, Andhra Pradesh. The animals were maintained in a well-ventilated room with 12:12 hour light/dark cycle in polypropylene cages. Standard pellet feed (Hindustan Lever Limited, Bangalore) and drinking water was provided ad libitum through out experimentation period. Animals were acclimatized to laboratory conditions one week prior to initiation of experiments.

Ethical committee clearance was obtained from IAEC (Institutional Animal Ethics Committee) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals).

Toxicological Evaluation

Acute oral toxicity study:
The acute oral toxicity procedure was followed by using OECD 423 guidelines. The acute toxic class method is a stepwise procedure with 3 animals of a single sex per step. Depending on the mortality and/or morbidund status of the animals, on the average 2-4 steps may be
necessary to allow judgment on the acute toxicity of the test substance. This procedure results in the use of a minimal number of animals while allowing for acceptable data based scientific conclusion.

The method uses defined doses (5, 50, 500, 200 mg/kg body weight) and the results allow a substance to be ranked and classified according to the Globally Harmonized System (GHS) for the classification of chemicals which cause acute toxicity.

Albino mice weighing 20-25g were used for the study. The starting dose level of ethyl acetate extract of *C. papaya* L. was 200 mg/kg body weight p.o. as most of the crude extracts possess LD5 value more than 200 mg/kg, p.o. So the starting dose used was 200 mg/kg, p.o. Dose volume administered was 1ml/10gm body weight to the mice, which were fasted overnight with water ad libitum. Food was withheld for a further 3-4 hours after administration (p.o) of drugs and observed for the signs of toxicity.

Body weight of the mice before and after termination were noted and any changes in skin and fur, eyes and mucous membranes and also respiratory, circulatory, autonomic and central nervous system and locomotor activity and behavior pattern were observed, and also sign of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma were noted. The onset of toxicity and signs of toxicity were also noted.

**In-vivo Pharmacological Evaluation:**

**Experimental Design:**
Animals were divided into five groups of each six animals.

**GROUP I:** Animals treated with normal saline served as control group.

**GROUP II:** Animals treated with Scopolamine (1 mg/kg/i.p) served as negative control.

**GROUP III:** Animals injected by Scopolamine and treated with EECP (200 mg/ kg/p.o.)

**GROUP IV:** Animals injected by Scopolamine and treated with EECP (400 mg/ kg/p.o.)

**GROUP V:** Animals treated with Piracetam (150 mg/kg/i.p.) served as standard group.

**Behavioral Studies:**

**a. Water maze task:**
The Morris water maze was performed as described previously. The experimental apparatus consisted of a circular water tank (diameter=10cm; height=35 cm), containing water at 28°C to a depth of 15 cm and rendered opaque by adding powdered milk. A platform (diameter 4.5 cm; height 14.5 cm) was submerged 0.5 cm below the water surface and placed at the midpoint of one quadrant. After several trials, the test was conducted on the 14th day injection of amyloid peptide. In each training trial, the time required to escape on to the platform was recorded.

**b. Y-maze task:**
Y-maze task is used to measure the spatial working through the spontaneous alternation of behaviour. The maze is made of black painted wood. Each arm is 4cm long, 13 cm high, 3 cm wide at the bottom, 1cm wide at the top, and converges at an equal angle. Each mouse is placed at the end of one arm and allowed to move freely through the maze during an 8-min session. Mice tend to explore the maze systematically, entering each arm in turn. The ability to alternate requires that the mice know which arm they have already visited. The series of arm entries, including possible returns into the same arm, are recorded visually. Alternation is defined as the number of successive entries into the three arms, on overlapping triplet sets. The percentage of alternation is calculated as the ratio of actual alternations, defined as the total number of arm entries minus two, and multiplied by 100.

**c. Elevated Plus Maze:**
Nootropic activity was assessed by using the elevated plus-maze learning task, which measures spatial long-term memory. Transfer latency (TL), the time in which the animal moves from the open arm to the enclosed arm was utilized as an index of learning and memory process. The elevated plus-maze consisted of two open arms (50x10 cm) and two closed arms (50 x10 x40 cm) with an open roof. The maze was elevated to a height of 50 cm from the floor. The animals were placed individually at the end
of either of the open arms and the transfer latency was noted on the first day. If the animal did not enter an enclosed arm with in 90 s, it was gently pushed in to the enclosed arms and the TL was assigned as 90 s. To become acquainted with the maze, the animals were allowed to explore the plus-maze for 20 s after reaching the closed arm and then returned to its home cage. Retention was examined 24 h after the first day trial.

\[ \text{IR} = \frac{(L_0 - L_1)}{L_0}, \]

Where, L is the initial transfer latency (TL) in sec on first time,
L1 is the transfer latency (TL) in sec on 2nd time.

Decrease IR indicates the induction of amnesia, and increased IR indicates in improvement in cognition and memory impairment.

Biochemical evaluation:
Estimation of Neurotransmitter Metabolic Enzyme Acetylcholineserase (AChE) Enzyme, Estimation Of Antioxidant Enzymes- Super Oxide Dismutase (SOD), Glutathione Peroxidase (GPx), Glutathione Reductase (GRD) and Ascorbic Acid were done by standard methods.

Statistical Analysis:
The statistical analysis was carried out using Graph pad prism software version 5 and results were carried out by analysis of variance (ANOVA) followed by Dunnets test. P values <0.05 were considered as significant.

RESULTS

Preliminary Phytochemical Investigation:
The results of the preliminary phytochemical screening of the ethyl acetate extract of dried rhizomes of C. papaya L. were shown below. The ethyl acetate extract gave positive results for alkaloids, flavanoids, carbohydrates, tannins, glycosides, and absence of proteins, saponins, steroids, terpenes, phenols, gums and mucilage.

A pinch of the extract were dissolved in glacial acetic acid and few drops of ferric chloride solution was added followed by the addition of Conc. Sulphuric acid, formation of red ring at the junction of the two liquids indicates presence of glycosides.

Table-1. Phytochemical analysis of extract of C. papaya (EECP)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Phytochemical Tests</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Test for Alkaloids</td>
<td>+Ve</td>
</tr>
<tr>
<td>2</td>
<td>Test for Carbohydrates</td>
<td>+Ve</td>
</tr>
<tr>
<td>3</td>
<td>Test for Proteins</td>
<td>+Ve</td>
</tr>
<tr>
<td>4</td>
<td>Test for Steroids</td>
<td>+Ve</td>
</tr>
<tr>
<td>5</td>
<td>Test for Sterols</td>
<td>+Ve</td>
</tr>
<tr>
<td>6</td>
<td>Test for Phenols</td>
<td>-Ve</td>
</tr>
<tr>
<td>7</td>
<td>Test for Flavonoids</td>
<td>+Ve</td>
</tr>
<tr>
<td>8</td>
<td>Test for Gums and mucilage</td>
<td>+Ve</td>
</tr>
<tr>
<td>9</td>
<td>Test for Glycosides</td>
<td>+Ve</td>
</tr>
<tr>
<td>10</td>
<td>Test for Saponins</td>
<td>-Ve</td>
</tr>
<tr>
<td>11</td>
<td>Test for Tanins</td>
<td>+Ve</td>
</tr>
</tbody>
</table>

+Ve indicates the presence of compounds, -Ve indicates the absence of compounds

Acute Oral Toxicity Study:
The acute oral toxicity study was done according to OECD guidelines 423 (acute toxic class method). A single administration of starting dose of 200 mg/kg body weight/po of the EECP was administered to 3 female mice and observed for 3 days. There was no considerable change in body weight before and after treatment and no sign of toxicity were observed. When the experiment was repeated again with same dose level, 200mg/kg body weight/po of the EECP for 7 more days and observed for fourteen days no change was observed from first set of experiments. LD5 cut off dose per kilogram body weight was categorized as X (unclassified). The results are shown in table-2.

In-Vivo Pharmacological Activity: Behavioural studies:
EECP improves the cognitive defects in Scopolamine induced mice.

a. Effect of EECP on Water Maze:
There is an increase in escape latency in negative control group when compared with the control group (P<0.01) of the two groups of amnesia.
induced animals, both showed decreased time to escape on to the escape platform. The group treated with 20mg/kg EECP group 40mg/kg treated showed the significance of (P<0.01 and P<0.01) respectively (Figure-1).

b. Effect of EECP on Y-Maze:
The amnesia induced group (negative control) indicated decrease in the alternation of behaviour by the (P<0.01) in comparison with the control group I. The results presented by the treatment groups shows significance by (P<0.01 and P<0.01) increase in the alternation of behaviour in respect of 20mg/kg of EECP and 40mg/kg of EECP (Figure-2).

c. Elevated Plus Maze
The Inflexion ratio (IR) of the Group II animals were significantly (p< 0.001) decreased in comparison with the Group I (normal control) animals. EECP (2040mg/kg) dose dependently increased IR in Group III Group IV significantly (p< 0.001) in comparable with Group II. The results were presented in Figure-3.

Effect of EECP on AChE Activity:
Injection of Scopolamine significantly (P<0.01) increased the AChE activity when compared with control group. In the treated group there was a significance (P<0.05) reduction in enzyme levels on both 20mg/kg and 40mg/kg of EECP treated mice as shown in Figure-4.
Figure 3. Effect of EECP on Elevated Plus Maze

Figure 4. Effect of EECP on AChE Activity

Effect of EECP on Super Oxide Dismutase: SOD levels in the brain the significantly reduced (P<0.01) in A 25-35 induced group when compared to control group. Treatment with EECP at 200 mg/kg and 400 mg/kg dose level showed significant increase (P<0.01 and P<0.01) respectively when compared with negative control group as the results were shown in Figure 5.

Effect on EECP on Glutathione Peroxidase: The GPx in the dementia induced mice (group II) shown significant (P<0.01) reduction in the enzyme activity when compared with control group. The treatment with EECP at 20mg/kg and 40mg/kg shown the significance (P<0.05 and P<0.01) respectively when compared with negative control group as shown in Figure 6.

Effect of EECP on Glutathione Reductase: The glutathione reductase activity of A 25-35 induced group shown significant (P<0.01) decrease in the activity when compared with control group. The two groups with 200 mg/kg and 400 mg/kg doses of EECP treated animals showed significant increase (P<0.01) when compared with negative control group. The results are shown in Figure 7.

Figure 5. Effect of EECP on Super Oxide Dismutase

Figure 6. Effect of EECP on Glutathione Peroxidase

Figure 7. Effect of EECP on Glutathione Reductase
DISCUSSION

Natural products have played a significant role in the management of neuropsychiatric disorders. A number of Indian medicinal plants have been used for thousands of years in the traditional system of medicine (Ayurveda). Amongst these are plants used for the neurodegenerative diseases such as Alzheimers, loss of memory, degeneration of nerves and other neuronal disorders by the Ayurvedic practitioners.

Carica papaya L. is a medicinal plant with antioxidant properties. The investigation was carried out on cognitive impairment with relevance of the hypothesis, on Scopolamine induced AChE oxidative stress signaling and impaired behavioural performance.

The preliminary phytochemical screening of EECP shows the presence of various phytochemical constituents like alkaloids, carbohydrates, proteins, sterols, tannins and absence of gums and mucillages saponins etc.

The present study has revealed the neuroprotective effect of EECP on Scopolamine induced cognitive deficits in mice. Scopolamine induced impairment of memory assessed by Morris water maze test, Y maze and Elevated Plus maze. In the present study, we have found a significant decrease in the level of antioxidant enzymes and the elevated of AChE in mice brain after a single injection of Scopolamine.

The spatial learning in water maze task showed the significant memory retention indicated by the decrease in escape latency, improvement of percentage alternations in Y maze and inflexion ratio in elevated plus at both dose levels of EECP in 200 mg/kg and 400 mg/kg respectively.

EECP at 200 mg/ kg and 400 mg/kg had shown the significant reduction in the elevated enzyme level of acetylcholine esterase which indicates the potential to increase cognitive function through the decreased degradation of acetyl choline.

The oxidative stress involved by the administration of Scopolamine produced neurotoxicity indicated the decreased levels of super oxide dismutase, glutathione peroxidase, glutathione reductase. Treatment of EECP shows the protection of these antioxidant enzymes on both 200 mg/kg and 400 mg/kg dose level respectively due to the rejuvenating property of the extract.

In water maze test the impaired place learning by Scopolamine and the improvement of learning by EECP after injection shows the significant property of memory retention, this indicates the rejuvenation potential of the extract. The time required to escape on the platform is decreased on this task indicated the hippocampal learning ability of the extract.

The exploratory behavioral studies indicate that the Scopolamine induced neurotoxicity decrease the percentage alternations and decreases the habituation memory. The EECP showed improvement in the percentage alternations and the habituation memory indicated by the proper alternations.

The biochemical changes responsible for the cognitive impairment were assessed by the estimation of acetylcholine esterase, and antioxidant enzymes. The study against the Scopolamine induced amnesia by the treatment of EECP showed significant reduction in the activity of acetylcholine esterase.

The antioxidant value of EECP showed the regaining of the antioxidant enzymes SOD, GPx and GRD activity, it has been noted that the antioxidant properties of extract delayed the generation of free radical and also showed the reversal of the antioxidant enzyme after the memory impairment, the antioxidant property showed a significant improvement in the EECP treatment, further this showed the ability to reduce oxidative stress signaling of EECP.

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