Antiamnesic Potential of *Coccinia indica* (Linn.) Extract: A Mechanism Based Study

Atanu Bhattacharjee*, Biplab Kumar Dey

Faculty of Pharmaceutical Science, Assam down town University, Guwahati-781026, Assam, India

*Corresponding author: Dr. Atanu Bhattacharjee, Associate Professor
Faculty of Pharmaceutical Science, Assam Down Town University,
Guwahati – 781 026, Assam, India
Tel: +91-8073612051, E-mail: ATANU1983@GMAIL.COM

Keywords: *Coccinia Indica*, Amnesia, Acetylcholinesterase, Scopolamine, Donepezil

Abstract. *Coccinia indica* Linn., a well explored traditional edible medicinal plant of North East region of India, is routinely used as folkloric medicine to treat neurological disorders associated with cognitive dysfunction. The objective of the study was to evaluate the antiamnesic activity of *Coccinia indica* fruit extract. Antiamnesic activity of *Coccinia indica* was studied in Elevated plus maze and Y-maze against scopolamine induced amnesia. Moreover, we studied the influence of *Coccinia indica* extract on whole brain acetyl cholinesterase enzyme. Ethanolic extracts of *Coccinia indica* (100, 200 and 400 mg/kg body weight) were administered to healthy adult Wistar rats for successive seven days and acquisition, retention and retrieval of spatial recognition memory was determined against scopolamine (1 mg/kg, i.p.) induced amnesia in-vivo. Further, whole brain acetylcholinesterase was estimated as per Elman's method. Pre-treatment with *Coccinia indica* ethanolic extract significantly improved spatial learning and memory and decreased rat brain acetyl cholinesterase activity in a dose dependent manner and comparable with standard drug Donepezil. The results indicate that ethanolic extract of *Coccinia indica* might be a useful as natural source of antiamnesic drug to improve learning and memory deficit associated symptoms of dementia and Alzheimer's patients.

1. Introduction

Amnesia refers to the loss of cognitive abilities like memories, past information and experiences [1]. Cognition is the process to organize information inside the brain by an organism. The process involves acquiring (perception), selecting (attention), representing (understanding) and retaining (memory) information, and utilizing them to guide behavior (reasoning and coordination of motor outputs). Interventions to improve cognitive function may be directed at any one of these core faculties [2]. Memory function is vulnerable to a variety of pathological condition including many neuropsychiatric and neurodegenerative diseases like Alzheimer's disease (AD) [3]. AD is a progressive neurodegenerative disorder characterized by low level of neurotransmitter acetylcholine (ACh) in the brain. AD is an irreversible age-related form of dementia that slowly erodes the brain and robs the person memory and cognitive skills and causes changes in personality and behavior [4]. During last decade, significant growth in Alzheimer's prevalence has ignited the importance of more researches in the search of new medicine. One of the major clinical advances in the treatment of AD have been the use of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitors to alleviate ACh level in brain although cholinergic compounds with nicotinic and muscarinic agonist properties also have attracted some interest [5]. The cholinergic muscarinic antagonist like scopolamine is most widely used drug to induce amnesia in experimental animals [6]. Amnesic mild cognitive impairment (through scopolamine administration) represents a transitional state between cognitive changes of normal aging and the earliest clinical features of AD [7]. Nootropic drugs like Donepezil and cholinesterase inhibitors are clinically used to improve learning and memory abilities, mood and behavior in those neurodegenerative diseases [8]. But the resulting side-effects associated with these synthetic drugs have made their utility limited [9]. At present, there are very limited medications available to treat AD and most of the treatment is

available only to delay the progression of symptoms and symptomatic relief for a short period of time. Medicinal plants represent a great deal of untapped reservoir of natural medicines and potential source of natural AChE inhibitors [10]. The structural diversity of their phytoconstituents makes them a valuable source of novel lead compounds for the quest of drugs to treat AD. Therefore, systematic ethnopharmacological screening of these plants may provide useful leads in the discovery of new drugs for AD therapy.

Coccinia indica (*C. indica*) L. (Family: Cucurbitaceae) commonly known as Tundika, a well explored edible traditional Indian medicinal plant of North eastern region of India routinely used to treat various ailments in particular diabetes and neurological disorders [11-12]. The fruits are slimy in touch, pulpy and ovoid to ellipsoid shape. It is green in colour when its ripens, 5.0 cm long and 2.5 cm in diameter, glabrous, pulp red. The fruit processes numerous seeds which are oblong, 7.0 mm long, margin thickened [13]. It requires dry, hot climate and shady places to grow effectively [14]. With this back ground, the present study was designed to evaluate the anti-amnesic potentiality of *C. indica* through several in-vivo neuropharmacological assays and biochemical estimations.

2. Materials and Methods

2.1 Collection and authentication of plant material:

The whole plant of *C. indica* was collected from Narengi, Kamrup district of Assam in June, 2017. The plants yield maximum phytoconstituents during the flowering and fruiting season in May-July; hence, they had been collected during that period. The plant had been identified and authenticated by the Head, Department of Botany of Guwahati University, Guwahati (specimen no.: 18395 dated 10.02.2018). The herbarium of *C. indica* (voucher specimen no. AdtU/Hb-01/2017) was preserved in the institution.

2.2 Extraction and fractionation:

500 gm coarsely powdered raw material of *C. indica* fruits was extracted by continuous hot percolation with ethanol and concentrated through rotary flash evaporator at 40 °C under reduced pressure and stored in deep freezer at -20 °C (15). The yield was found to be 18.5 % w/w. A portion of concentrated ethanolic extract was fractionated with different solvents with increasing order of polarity (petroleum ether, chloroform, ethyl acetate, n-butanol and methanol). A flow chart of detailed method of extraction and fractionation is given in Figure 1

Figure 1: Schematic diagram of extraction and fractionation of *C. indica*

2.3 Phytochemical analysis:

The crude fractions were subjected to different qualitative phytochemical screening to identify the presence of various phytoconstituents as described by Harborne [16]. The phytochemicals analyzed were alkaloids, phenols, flavonoids, saponins, terpenoids, steroids, tannins, carbohydrates, proteins and coumarins.

2.4 Screening procedure:

2.4.1 Test for tannins and phenols:

5 ml of fraction was added to 2 ml of 5 % of alcoholic FeCl_3 solution. Blue-black precipitate indicated the presence of tannins and phenols.

2.4.2 Test for alkaloids:

2 ml of 2N HCl was added to 5 ml extract and heated over water bath for 10 min. The cooled solution was filtered and a few drops of Dragendorff's reagent were added. Reddish-brown precipitate indicated the presence of alkaloid.

2.4.3 Test for saponins:

About 1 g of dried powdered sample was boiled with 10 ml distilled water. Frothing persistence indicated the presence of saponins.

2.4.4 Test for terpenoids:

5 ml of fraction was mixed with 2 ml of chloroform and few drops concentrated H_2SO_4 was carefully added to form a layer. Red ring indicated the terpenoids are present.

2.4.5 Test for steroids:

5 ml of fraction was mixed with 10 ml CHCl_3 and 1 ml acetic anhydride and few drops of concentrated H_2SO_4 were added. Green ring indicated the presence of steroids.

2.4.6 Test for flavonoids:

To 5 ml of fraction few pieces of magnesium ribbon and few drops of concentrated HCl were carefully added. Red color indicated the presence of flavonoids.

2.4.7 Test for phlobatannins:

About 2 ml of fraction was boiled with 2 ml 1 % HCl. Deposition of a red color indicated the presence of phlobatannins.

2.4.8 Test for amino acids (Ninhydrin test):

5-6 drops of ninhydrin reagent were added in 5 ml of fraction and heated over boiling water bath for 5 min. Purple coloration indicated the presence of amino acid.

2.4.9 Test for proteins (Biuret test):

5-6 drops of 5 % NaOH and 5-7 drops of 1 % CuSO_4 were added in 2 ml fraction. Violet color indicated the presence of proteins.

2.5 Drugs and chemicals:

Scopolamine hydrobromide, donepezil, sodium carboxy methyl cellulose (Sodium-CMC), Acetylthiocholine iodide (ATC), 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) were collected from Sigma-Aldrich, Bangalore, India. All the toxic, standard and test drugs (suspended in 0.6 % w/v of sodium CMC solution) were administered in the morning session i.e. 9 AM- 10 AM on each day.

2.6 Acute toxicity study:

The acute toxicity of ethanolic extract of *C. indica* fruit extract was evaluated in female Wistar rats as per OECD guidelines 425 (Up and Down Procedure). A fixed dosage study was adopted for

acute toxicity study where the limit dose is 2000 mg/kg body weight of test animal. Clinical signs of toxicity, body weight changes, cage side parameters and mortality rate were observed every hour for the first 6 hours and every day for 7 days [17].

2.7 Experimental animals:

Healthy young Wistar rats of either sex aged between 8-12 week old and weighing between 150-200 g obtained from the institutional animal house were used for in-vivo studies. The animals were housed in a room under standard environmental condition (25 °C and 50-70 % relative humidity) of 12/12 h light/dark cycle and fed with standard rat pellet and water ad libitum. Animals were allowed to acclimatize for 7 days in laboratory conditions prior to experiment [18]. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) (Registration number and date of registration: 1574/PO/Re/11/CPCSEA dated 17/8/2015).

2.8 Exteroceptive behavioral model:

2.8.1 Elevated plus maze:

To access the effect of *C. indica* on spatial long term working memory of rats Elevated plus maze test was carried out [19]. A typical Elevated plus maze consists of two open (50x10 cm) and two close arms (50x10x40 cm) facing each other with a central square (10x10 cm). The entire maze has been elevated to a height of 50 cm from the floor. The animals were placed individually at the end of either of open arms facing away from center and the time taken by the animals to move from open arm to enclosed arm was noted on the first day (initial transfer latency, ITL). If the animal did not enter an enclosed arm within 90 seconds, it was pushed on the back into one enclosed arm and the transfer latency was given as 90 sec. Later, the animal was allowed to move freely to explore the apparatus for at least 20 sec. After the experiment, the animals were returned to their home cages and transfer latency was recorded again after 24 hours of the first exposure (retention transfer latency, RTL). The transfer latency measured on the first and second day trial served as an acquisition (learning) and retention (memory) respectively [20]. From these, inflexion ratio (IR) was calculated using the formula

$$IR = L_0 - L_1 / L_1$$

where,

IR = Inflexion ratio

L_0 = Initial transfer latency in seconds

L_1 = Retention transfer latency in seconds

A fall in transfer latency on subsequent maze exposures was taken as an index of successful retention [21].

2.8.2 Hebb's-William maze:

The Hebb's-William Maze is an incentive based exteroceptive behavioral model useful for measuring spatial and working memory of rats. A typical Hebb's-William maze (75x75x 25 cm) is a rectangular wooden box divided into three components viz. animal chamber (start box), which is attached to the middle chamber (exploratory area) and a reward chamber at the other end of the maze in which the reward (food) is kept.

The experiment was conducted under zero watt red colored bulb to minimize the nocturnal cycle disturbances. During study, the animals were exposed to food and water ad libitum only for 1 h after the maze exposure to ensure motivation towards reward chamber. Each rat was placed in animal chamber and the door of start box was closed immediately after the animal moved into the exploratory area to prevent its back entry. Time taken (in seconds) by the animals to reach reward chamber from start box (initial transfer latency, ITL) was noted for each animal. Each animal was allowed to explore the maze for additional 20 seconds.

After experiment, the animals were returned to their home cages and transfer latency was recorded again after 24 hours of the first exposure (retention transfer latency, RTL). A fall in transfer latency on subsequent maze exposures was taken as an index of successful retention [22].

2.9 Experimental protocol:

Thirty six healthy Wistar rats of either sex were selected randomly and divided into six groups (n=6) to evaluate their responses on exteroceptive behavior models. The groupings of animals were summarized below:

Group I (Normal control): Vehicle (0.6 % w/v sodium CMC) p.o.

Group II (Negative control): Scopolamine (1 mg/kg i.p.)

Group III (Positive control): Donepezil (50 mg/kg) p.o. + Scopolamine (1 mg/kg i.p.)

Group IV: *C. indica* ethanolic extract (100 mg/kg) p.o. + Scopolamine (1 mg/kg i.p.)

Group V: *C. indica* ethanolic extract (200 mg/kg) p.o. + Scopolamine (1 mg/kg i.p.)

Group VI: *C. indica* ethanolic extract (400 mg/kg) p.o. + Scopolamine (1 mg/kg i.p.)

The dosing for all groups were done for a period of 14 days; after 45 minutes of administration of the last dose on 14th day, amnesia was induced by administration of scopolamine (1 mg/kg i.p.) to Group II, III, IV, V and VI respectively. The negative control group (Group II) received just one dose of scopolamine (1 mg/kg i.p.) on 14th day and 45 minutes after the administration of scopolamine, trials were taken on Elevated plus maze and the retention was observed after 24 hours of the first exposure i.e. on day 15 [23].

The same experimental protocol was followed on the same experimental animals after one month of rehabilitation for assessment of learning and memory by Hebb's William maze model. During experiment, the animals were exposed to food and water ad libitum only for 1 hour after the maze exposure for the day to ensure motivation towards reward area [24].

3. Biochemical Estimation

3.1 Collection of brain sample:

Immediately after the experiment animals were sacrificed by cervical decapitation under light anesthesia and whole brain was carefully removed from the skull. The fresh whole brain was weighed, kept on ice bath, rinsed with ice-cold isotonic saline and homogenized (approximately 20 mg of tissue/ml of phosphate buffer (pH 8.0; 0.1M) in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 3000 rpm for 10 min and the resultant cloudy supernatant liquid was used for the estimation of acetyl cholinesterase (AChE) activity [25].

3.2 Estimation of brain acetyl cholinesterase (AChE) activity:

The AChE activity was assessed by Ellman's method [26]. The AChE activity was measured using an artificial substrate Acetyl thiocholine iodide (ATC). AChE acts on ATC and release thiocholine and acetate ion. Thiocholine further allowed to react with -SH reagent 5,5-dithio-bis-(2, nitro benzoic acid) (DTNB) which is reduced to thionitro benzoic acid, a yellow colored anion with $\lambda_{\max}$ 412 nm. The molar extinction coefficient of thionitro benzoic acid is 1.36×10^4 /molar/cm. The concentration of thionitro benzoic acid is determined using Specord 50-Plus Analytikjena UV-Vis spectrophotometer which is directly proportional to AChE activity. The rate of the reaction was calculated using following formula

$$R = 5.74 \times 10^{-4} \times \Delta A / C_0$$

where,

R = rate in moles of substrate hydrolyzed / minute / gm tissue

ΔA = change in absorbance / min

C_0 = original concentration of the tissue (mg/ml)

Statistical significance:

The results of in-vivo studies were expressed as mean $\pm$ SEM (standard error of mean). The difference between the control and treated means were analyzed using one way analysis of variance (ANOVA). P-values < 0.05 were taken to be statistically significant. Tukey's test was used for multiple comparisons between all columns. The statistical analysis was done using the software Graph-pad prism version no: 5.0.

4. Results

4.1 Phytochemical analysis:

Qualitative estimation revealed that *C. indica* fruit extract were enriched with alkaloids, phenolic compounds and tannins, phytosterols and triterpene, flavanoids, alkaloids, saponins, and cardiac glycosides. The detailed summary of phytochemical screening of *C. indica* fractions is shown below (Table 1).

Table 1: Preliminary phytochemical screening of *C. indica*

Compounds	Ethanollic extracts			
	Pet. Ether	Chloroform	Ethyl acetate	n-butanol
Phytosterols and Triterpene	+++	+	-	-
Alkaloids	-	+++	++	-
Saponins	-	-	-	-
Cardiac glycoside	-	-	-	-
Flavanoids	-	-	++	++
Tannins	-	-	++	++
Carbohydrates	-	-	+	++
Proteins	-	-	-	-

4.2 Acute toxicity study:

No mortality was observed following oral administration of *C. indica* ethanollic extract even with the highest dose (2000 mg/kg). Moreover, no significant changes in body weight and behavior were observed. Hence, *C. indica* could be safe up to the dose of 2000 mg/kg body weight of the animal.

4.3 Elevated plus maze:

Results suggest pre-treatment with ethanollic extract of *C. indica* (100, 200 and 400 mg/kg body weight) for 14 successive days did not exhibited much difference in ITL compared to normal control group but in presence of amnesia, highet dose of extract (400 mg/kg body weight) afforded a significant (### $p < 0.001$) decrease in TL (score: 33.83 ± 1.537) compared to other lower doses groups (score: 51.16 ± 1.759 and 42.20 ± 1.69 respectively) and closely approximated to standard drug Donepezil (score: 31.46 ± 1.558). However, all the three doses of extract showed improvement spatial learning and memory activity in dose dependant manner. Moreover, in Group IV, V and VI significant decrease in RTL on Day 15 compared to Day 14 were observed which elaboate the drugs responses to overcome the learning and memory deficits produced by scopolamine. Further, significant improvements in inflexion ratio in the treatment groups suggest its antiamnesic potential (Figure 2).

One way ANOVA followed by Tukey's test. Values are mean $\pm$ S.E.M., $n = 6$ in each group. *** $p < 0.001$ and * $p < 0.05$ when compared to control; ### $P < 0.001$, and # $p < 0.05$ when compared to negative control

Figure 2: Effect of *C. indica* extract on transfer latency (in seconds) on Elevated plus maze

4.4 Hebb's-William Maze:

Results suggest pre-treatment with ethanolic extract of *C. indica* (100, 200 and 400 mg/kg body weight) for 14 days did not exhibited much difference in ITL compared to normal control group but in presence of amnesia, highest dose of *C. indica* ethanolic extract (400 mg/kg body weight) afforded a significant (### $p < 0.001$) decrease in TL (score: 236.66 ± 2.15) compared to other lower doses groups (score: 271.13 ± 2.10 and 248.33 ± 1.87 respectively) and closely approximated to standard drug Donepezil (score: 224.36 ± 1.48). However, all the three doses of extract showed improvement spatial learning and memory activity in dose dependant manner. Moreover, in Group IV, V and VI significant decrease in RTL on Day 15 compared to Day 14 were observed which elaborate the drugs responses to overcome the learning and memory deficits produced by scopolamine (Figure 3).

One way ANOVA followed by Tukey's test. Values are mean $\pm$ S.E.M., $n = 6$ in each group. *** $p < 0.001$ and * $p < 0.05$ when compared to control; ### $P < 0.001$, and # $p < 0.05$ when compared to negative control

Figure 3: Effect of *C. indica* extract on transfer latency (in seconds) on Hebb's-William Maze

4.5 Estimation of whole brain acetylcholine esterase (AChE) activity:

The acetylcholinesterase activity of whole brain was significantly ($***p < 0.001$) elevated after scopolamine (1 mg/kg, i.p.) treatment. Pre-treatment with Donepezil (50 mg/kg, p.o.) and *C. indica* (100, 200 and 400 mg/kg, p.o.) significantly ($###p < 0.001$) lowered AChE activity (Figure 4).

One way ANOVA followed by Tukey's test. Values are mean $\pm$ S.E.M., $n = 6$ in each group. *** $p < 0.001$ and * $p < 0.05$ when compared to control; ### $P < 0.001$, and # $p < 0.05$ when compared to negative control.

Figure 4: Effect of *C. indica* extract on whole brain acetyl cholinesterase enzyme

5. Discussion

Learning and memory are two fundamental cognitive functions that confer knowledge from our experiences. Cognition is a process of storing sequence of information in a systematic manner by which one become aware of their surroundings, objects and thoughts [27]. Cognitive impairment is one of the major health problems in normal aged life as well as in some neurological disease conditions like Alzheimer's [28]. Herbal cognition enhancers can be used as an alternative to facilitate attention abilities and to attenuate the impairment of cognitive functions associated with age and age-related pathologies [29]. Since, no study had been conducted to evaluate the anti-amnesic potential of fruits of *C. indica*, the present study was conducted.

The phytochemical screening revealed fractions of *C. indica* fruits were enriched with different secondary metabolites in their respective fractions. Further, various bioactive phytoconstituents viz. β -sitosterol, stigmasterol, lupeol, Cephalandrin A and B, Cucurbitacin had been isolated from *C. indica* extract [30]. Anti-inflammatory and antioxidant activity of Cephalandrin and Cucurbitacin is well established which might be associated with the anti-amnesic activity of *C. indica* extract [31]. The findings from Elevated plus maze and Hebb's William maze test suggest *C. indica* ethanolic extract showed protective effect in transfer latency against scopolamine induced amnesia in dose dependent manner. Moreover, the decrease in transfer latency during retention period for both the test suggested the drug response to overcome learning and memory deficit produced by scopolamine. These provide an adequate scientific promise to validate the neuro-protective potency of *C. indica* extract.

Moreover, *C. indica* extract significantly inhibit whole brain AChE against scopolamine induced amnesia in dose dependent manner and thereby could increase the availability of acetylcholine level in cholinergic synapses. These observations postulated potent anticholinesterase activity of *C. indica* extract which might be one of the possible mechanisms to encounter with cognitive disorders.

Therefore, in the present study we observed that *C. indica* extract

- (i) showed improvement in memory of Wistar rats when tested on exteroceptive behavioral model viz. elevated plus maze and Hebb's-William maze
- (ii) inhibited whole brain acetylcholinesterase enzyme activity

6. Conclusion

Thus, a combination of anticholinesterase and neuroprotective effects exhibited by *C. indica* extract may be responsible for its anti-amnesic potentiality. However, molecular level investigations with isolated bioactive compounds may be required for scientific validation of the traditional use of *C. indica* as anti-amnesic drug.

Conflict of interest

We declare that we don't have any conflict of interest.

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