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Enhancement of cognitive performance in mice and *in vitro* acetyl-cholinesterase inhibitory activity of 3,3',4',5,7-pentahydroxyflavone isolated from *Cadaba indica*

Sundaram Dhivya, Subbiah Latha and Palanisamy Selvamani

Department of Pharmaceutical Technology, Centre for Excellence in Nanobio Translational Research Center, Anna University, Bharathidasan Institute of Technology Campus, Tiruchirappalli, Tamil Nadu, India.

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Abstract

Cadaba indica, a component of traditional siddha medicine, is used for the therapy of gonorrhea, inflammation, eczema etc. The present study shows that it exhibited acetylcholinesterase inhibitory activity (up to 81.5 ± 1.5%) at the concentration of 3 mg/mL, with the IC_{50} value of 1.8 mg/mL. Furthermore, the ethanol extract of *C. indica* therapy at a dose of 100 mg/kg p.o significantly ameliorated the cognitive impaired mice in Y-maze task. The active ingredient was identified as 3,3',4',5,7-pentahydroxyflavone. The above results suggest that *C. indica* would be a potential natural therapeutic remedy for cognitive disorders mediated by cholinergic dysfunction.

Introduction

Natural products from herbs have a long history in the use of treating neurological symptoms. Herbal therapy for cholinergic disorders such as Alzheimer's disease has been widely studied and reported during the past decade. The derived knowledge and identified bioactive molecules is promising and could be translated for clinical applications eg. Galantamine from *Galanthus nivalis L.* (Snowdrop) a potent acetylcholinesterase inhibitor is one of the most leading therapeutic options for Alzheimer's disease therapy (Orhan et al., 2011). However, such scientific studies and validation of neuroactive botanical ingredients for neurotherapies are still in its infancy. In this present study, *Cadaba indica* belongs to the Family: Capparaceae, which is a shrub common in scrubs jungles and wastelands was selected for pharmacological validation.

The Capparaceae family comprises 46 genera and 700 species distributed in the warm and tropical countries. The genus Cadaba consists of 30 species which were found distributed in tropical Africa, Arabia, India, Australia, Madagascar and the islands of Indian Ocean (Dyer, 1975). Its natural habitat is subtropical or dry shrub land. It is under greatest threat due to loss of habitat and reported as endemic on the Indian subcontinent: most widely in Bangladesh, India and Pakistan.

C. fruticosa (Tamil: Vizhuthi) is being used in siddha medicine practiced over years. The juice of the leaves is especially used to cure gonorrhea and the leaves were used to treat eczema, swelling and constipation (Selvamani and Latha, 2005). The roots of *C. fruticosa* has traditionally reported in the treatment of helminthiasis, syphilis, hemiplegia, dysentery, diarrhea, antidote against poisoning, stimulant, scorbutic and neurological disorders (Shashikanth et al., 2014).

The previous study on *C. indica* has reported for its potent anti-oxidant activity and elevated flavanoid and phenolic content was evaluated by *in vitro* methods. The leaves of the *C. indica* are taken as decoction with

other ingredients used in the treatment of dysmenorrheal, amenorrhea and uterine obstruction. The decoction of leaves was used in the treatment of antihelmintic activity. Methanol extract of *C. indica* was previously reported for its significant antimicrobial and anti-oxidant activity (Lavinya et al., 2014).

It was reported that the leaves and stem bark possesses L-stachydrine and L-3-hydroxyl stachydrine and also the presence of quercetin, isoorientin, hydroxyl benzoic acid, syringic acid, vanillic acid and 2-hydroxy,4-methoxy benzoic acid. The stem bark contains an alkaloid cadabicine and dry pods contain cadabalone (Khare, 2007).

This study was conducted to estimate the efficiency of crude ethanol extract of *C. indica* in cognitive enhancement and to identify the active botanical ingredient of *C. indica* which is responsible for the inhibition of acetylcholinesterase.

Materials and Methods

Chemicals

Acetylcholinesterase from electric eel (C3389-Sigma), eserine free base (E8375-250M-Sigma), α-napthyl acetate solution (RM1730-Himedia), fast blue salt (RM7003-Imedia), dimethyl sulfoxide (TC185–Himedia), scopolamine hydrobromide (S1875-Sigma) and sodium dodecyl sulfate (1610301-Biorad) were used. Ethanol and all other organic solvents of analytical grade were used in this study.

Plant material

The *C. indica* was collected in the month of July from Mathur, Tiruchirappalli, Tamil Nadu. It was authenticated by the Botanical Survey of India, South region, Coimbatore (BSI/SRC/5/23/2014-15/Tech/129). A voucher specimen of *C. fruticosa* or *C. indica* was maintained at Anna University, BIT campus, Tiruchirappalli.

Animals

Swiss albino mice (6 weeks old, 25-30 g) were selected for the study and well acclimatized for one week to the laboratory conditions before starting the experiment. Mice were housed in groups of five. Animals were maintained in the laboratory as per CPCSEA guidelines. Mice were fed with food and water *ad libitium* and kept under 12 hours light/dark cycle at room temperature.

Extraction

The leaves were shade dried and ground to a coarse powder. The plant materials were subjected to continuous hot extraction in 80% ethanol using soxhlet apparatus. The solvents were evaporated under reduced pressure and then lyophilized. Dried extracts were stored in -20°C until testing.

Thin layer chromatographic bio-autographic assay

Screening of possible cholinesterase inhibitors of the ethanol extract of *C. indica* was determined by rapid bio -autographic assay on TLC plates. This assay was carried out as described previously (Marston et al., 2002). The extract was dissolved in ethanol and then eluted with hexane : ethyl acetate (1:1; v/v) solvent system. After migration of the plant extract, the TLC plate was dried for complete removal of the solvent. Next, the plate was sprayed with acetylcholinesterase enzyme stock solution (concentration 6.7 U/mL) and pre-incubated for 20 min at 37°C in a humid atmosphere. α-Naphthyl acetate solution (10 mL) and fast blue B salt solution (40 mL) were mixed and sprayed onto the plate which will provide a purple coloration after 1–2 min due to the formation of purple colored diazonium dye. In presence of inhibitors, white spots were observed on the dye colored background on the TLC plates.

In vitro acetylcholinesterase inhibition activity by new micro well plate AChE inhibition assay (NA-FB)

The acetylcholinesterase inhibition activity was performed using a spectrophotometric test using 96-micro-well plates according to the method as described previously (Ali-Shtayeh et al., 2014). In each well, 10 µL plant extract, 50 µL (0.3 mg/mL) of α-naphthyl acetate dissolved in methanol, and 200 µL of acetylcholinesterase solution (3.3 U/mL) were added and the above mixture was incubated for 40 min at 4°C. Later, 10 µL (2.5 mg/mL) fast blue B dissolved in water were added to the mixture and the absorbance was measured at 600 nm. To overcome the error in absorbance reading as a result of the plant extract color, the absorbance readings before incubation were subtracted from the absorbance after the addition of the dye. The percentage of inhibition of each test solution was calculated using the following equation:

$$\text{Inhibition (\%)} = 1 - (A_{Sample} / A_{Control}) \times 100$$

Where A_{sample} is the absorbance of the sample extracts and $A_{control}$ is the absorbance of the blank

Isolation of the bioactive phytoconstituents

It is a challenging task to identify and isolate the bioactive compound from the complex mixture. Natural plant extract consists of mixtures of compound with varying polarities. Investigation of bioactive compounds from crude extracts was performed with bioactivity guided separation and isolation. To overcome this, screening test was performed first with a preliminary assay for all the isolated fractions from crude extract with a most sensitive bio-autographic assay on TLC for acetylcholinesterase inhibitory activity (as described above) which gave information on enzyme inhibition as well as selectivity.

Y-maze test

The Y-maze test used in the present study consists of three arms with angles of 120° between the arms, named A, B and C. The walls were fabricated using dark opaque polyvinyl plastic as previously described (Kim et al., 2006). Initially, the mice were placed within one arm, the number of arm entries and sequence of arm entries were recorded for 8 min. Percentage of triads in which alternative arm entries were recorded (i.e., ABC, CAB, or BCA but not BAB) to estimate the short-term memory (Sarter et al., 1988). An hour before the experiment mice were administered with ethanol extracts of *C. indica* (12.5, 25, 50, or 100 mg/kg, p.o.) or eserine (10 mg/kg, p.o.). Control group mice were treated with 0.9% saline solution rather than the test compounds or the standard drug. Half an hour before the test scopolamine (1 mg/kg, i.p.) was injected to induce cognitive impairment. Arms were regularly cleaned with water spray between each and every test to remove odors and residues. Percentage alternation score for each mouse was defined as the ratio of the actual number of alternations to the possible number. It is defined as the total number of arm entries minus two) multiplied by 100% Alternation = [(Number of alternations)/(Total arm entries -2)] × 100. The number of arm entries determines the locomotor activity of the mice (Park et al., 2010).

Isolation of phytoconstituents by column chromatography

The ethanol extract of *C. indica* was subjected to column chromatographic separation using normal phase silica gel column. The dark green solid of ethanol extract of *C. indica* was adsorbed on silica gel (20 g) and transferred to a column of silica gel (200 g equilibrated with benzene). Elution was performed with benzene (100%), benzene: chloroform (90:10), (70:30), (50:50), (30:70), and (100), chloroform: ethyl acetate (70:30), (50:50), (30:70), and (100), ethyl acetate: ethanol (80:20), (70:30) (50:50), (30:70) and (100).

Fractions of 100 mL were collected every time, distilled off the solvent and the homogeneity of the resulting residues was examined on TLC and similar fractions, identified by their TLC behavior, were mixed together. Prescreening of the isolated fractions was done by TLC bio-autographic assay to access their ability to inhibit acetylcholinesterase. The separation was carried out by using different solvent systems as eluent. Combining bioassay and fractionation had fastened the procedure to identify the active compound in the ethanol extract of *C. indica.* This bioactive fraction was further reconstituted with appropriate solvent after lyophilization and the active compound was characterized using IR, UV, NMR spectroscopy and mass chromatographic analytical procedures (Houghton, 2000).

Infrared spectral analysis

Isolated compound from the ethanol extract of *C. indica* was subjected to IR spectrum which was determined using a Nicolet 170SX. The fractions were grounded with KBr powder and then pressed into pellets. The spectral resolution for the FTIR measurement was 0.25 cm^{-1}, and the spectral data were stored in the database at intervals of 0.5 cm^{-1} at 4000-2000 cm^{-1} and of 0.3 cm-1 at 2000 cm^{-1}-400 cm^{-1}.

^{1}H NMR spectral analysis

An isolated fraction from the ethanol extracts of *C. indica* was subjected to ^{1}H NMR spectrum was recorded using JEOL AL-400 (399.6 MHz) equipment. The measuring conditions were set at flip angle of 22.5-30.0 degrees and pulse repetition time of 30 sec. The long pulse repetition time and flip angle were used to ensure precise relative intensities. The ^{1}H NMR chemical shifts were referred to TMS in organic solvents and TSP in D_2O.

^{13}CNMR spectral analysis

^{13}CNMR spectrum of the bioactive compound from the ethanol extracts of *C. indica* was recorded with a Bruker AC-200 (50.3 MHz). The measuring conditions were set a pulse flips angle of 22.4-45 degrees, a pulse repetition time of 4-7 sec and a resolution of 0.02-0.04 ppm. The spectra whose spectral codes started with "CDS" were reconstructed based on the peak positions, intensities and line widths. All resonance peaks were Lorenz lines. The chemical shift was referred to a TMS for all solvents.

Mass spectral analysis

Mass spectra of the compounds isolated from the ethanol extracts of *C. indica* was recorded by the electron impact method using JEOL JMS-700 where an electron is accelerating voltage 75eV and an ion accelerating voltage of 8-10 nV. The reservoir inlet system was used. The dynamic range for the peak intensities were 3 digits and the accuracy of the mass number was 0.5.

Results

Ethanol extract of *C. indica* furnished a white spot on purple background with the retention factor of 0.27. Extract of *C. indica* showed 81.5 ± 1.5 , 68.8 ± 1.0, 42.7 ± 1.2, 37.2 ± 1.0, 21.6 ± 1.6 and 7.7 ± 0.7% acetylcholinesterase inhibition at 3, 2.5, 2, 1.5, 1 and 0.5 mg/mL concentration respectively as compared to the standard drug eserine shown 85.6 ± 1.1 , 77.4 ± 0.7, 64.2± 0.8, 42.2 ± 1.0, 28.1 ± 0.6%and 18.5 ± 0.5% acetylcholinesterase inhibition at 3, 2.5, 2, 1.5, 1 and 0.5 mg/mL concentration respectively as shown in Figure 1. The IC_{50} values

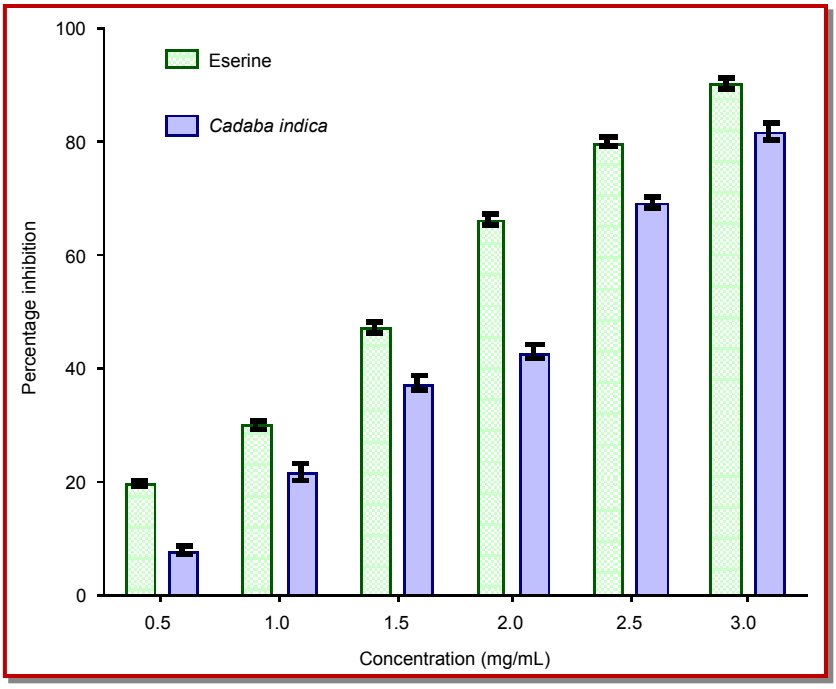

Figure 1: Graphical representation of percentage inhibition of acetylcholinesterase inhibitory by standard drug eserine and *C. indica*

of *C. indica* and eserine were found to be 1.8 mg/mL and 1.4 mg/mL respectively.

Figure 2 shows the effect of ethanol extract of *C. indica* in the presence or absence of scopolamine-induced memory deficit using the Y-maze task. A significant group effect was observed on spontaneous alteration behavior. In scopolamine treated group, percentage of spontaneous alteration behavior was found to be significantly lower when compared to the control group and this decreased spontaneous alteration was significantly ameliorated by ethanol extract of *C. indica* (100 mg/kg, p.o) and eserine (10 mg/kg, p.o) with a percentage alteration of 70.6 ± 2.9 and 82.4 ± 2.2% respectively. One-way ANOVA indicated that on administration of ethanol extract reversed the memory retention induced by scopolamine.

By combining TLC bio-autographic assay and fractionation had fastened the procedure to identify the active compound in the crude extract of *C. indica* to obtain bioactive fractions. About 120 fractions were eluted, of which fractions 58 to 86 eluted with ethyl acetate: ethanol 40: 60(v/v) showed a positive spot on thin layer bio-autographic assay representing the presence of acetylcholinesterase inhibitors which showed a Rf value of 0.3. These fractions were then combined and crystallized.

Infrared spectral data shows absorption at 3314 cm^{-1} indicates the presence of –OH group, whereas the strong band at 1666 cm^{-1} indicates the presence of carbonyl group. The absorption at 1609, 1561, 1552, 1456 and 1380 cm^{-1} reveals the presence of aromatic C=C stretching and bending vibrations. 939 cm^{-1} and 822 cm^{-1} low frequency peak assigns C-H bending vibrations of aromatic hydrocarbon. 1259 and 1204 cm^{-1} represents C-O stretching vibrations of aryl ether and phenols. 1165 cm^{-1} assigns C-CO-C stretching and bending vibrations of ketone. The presence of C-O-C ether linkage in the absorption at 1008 cm^{-1}. Infrared spectral data for the isolated bioactive compound was shown in Figure 3.

The ^{1}HNMR and ^{13}CNMR spectral data of the bioactive

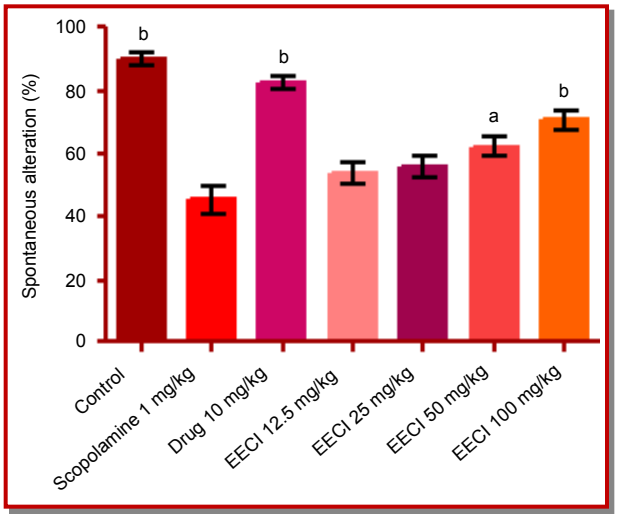

Figure 2: The effects of the ethanol extract of *C. indica* on scopolamine induced memory impairment in mice Y-Maze Task. One-way ANOVA test was analyzed using Dunnett's multiple comparison tests; each column represents the mean ± standard error of the mean of five mice. $^{a}p<0.01$, $^{b}p<0.001$ versus scopolamine treated group

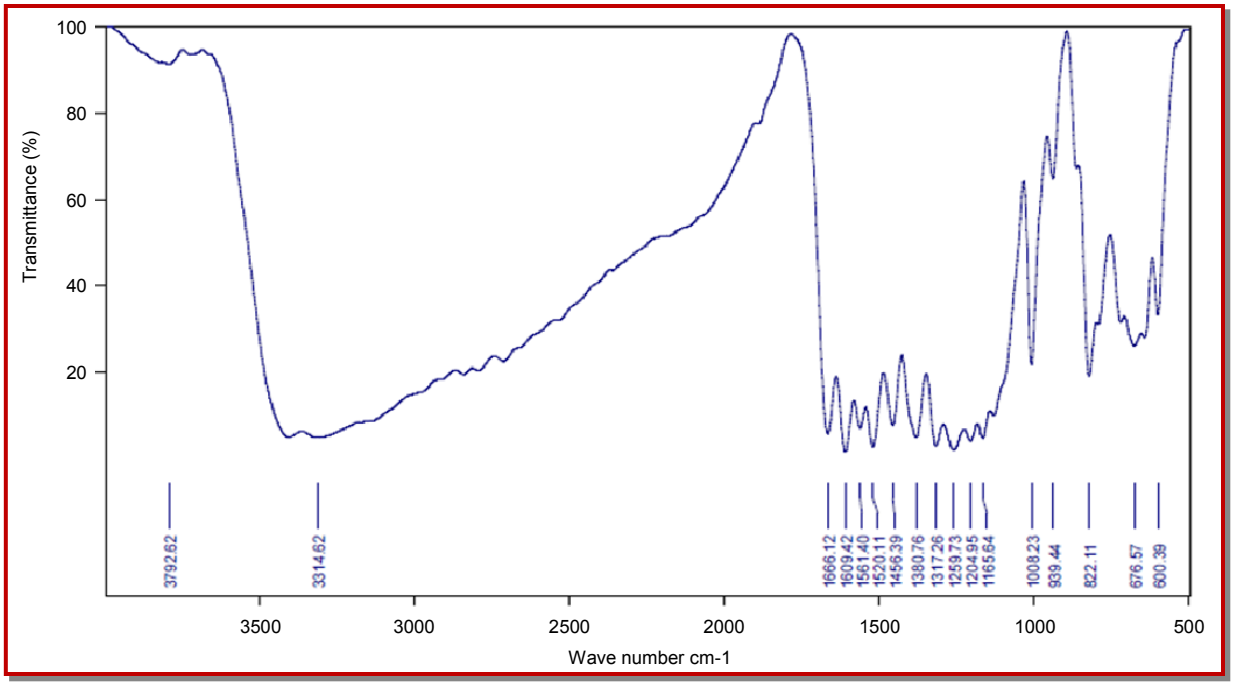

Figure 3: Infra red spectrum of the bioactive compound

compounds was analyzed. Based on the ^{1}HNMR (Table I) chemical shift values and ^{13}CNMR (Table II) chemical shift values of the isolated compound was found to be 3,3',4',5,7-pentahydroxyflavone.

The mass spectrum of the isolated compound is presented in Figure 4. The m/z value of the isolated compound of the molecular ion is found as 300 (M^{-2}) which includes the isotopes of corresponding atoms.

On the basis of spectral data (IR, ^{1}HNMR and ^{13}CNMR and mass spectra), the isolated bioactive compound was found to be 3,3',4',5,7-pentahydroxyflavone and the molecular formula was deduced as $C_{15}H_{10}O_7$.

Table I

^{1}HNMR spectral data of the bioactive compound

SL. No.	Chemical shift value	Functional group
1	δ 12.5	s, 1H, 3-OH
2	δ 10.8	hump , 1H, 5-H
3	δ 9.6	s, 1H, 4'-OH
4	δ 9.3	s, 1H, 3'-OH
5	δ 7.7	d, 1H, 7-OH
6	δ 7.56-7.5	d, 1H, 6-H
7	δ 7.5	d, 1H, 8-H
8	δ 6.90-6.9	d, 1H, 2'-H
9	δ 6.4	s, 1H, 5'-H
10	δ 6.2	s, 1H, 6'-H

Discussion

In the present study, ethanol extracts of *C. indica* was investigated for anticholinesterase activity and its ameliorative effect on scopolamine induced memory impaired mice model. Acetylcholinesterase inhibitory potential was examined by TLC bio-autography and fast blue salt assay. Enzyme inhibitory potential was detected by appearance of white spots on the dye-

Table II

^{13}CNMR spectrum of the bioactive compound

SL. No.	Chemical shift value (ppm)	Functional group
1	δ 175	C-4
2	δ 163	C-3
3	δ 160	C-5
4	δ 156	C-2
5	δ 147	C-7
6	δ 146	C-4'
7	δ 145	C-3'
8	δ 135	C-9
9	δ 121	C-2'
10	δ 119	C-6
11	δ 115.6	C-8
12	δ 115.1	C-10
13	δ 103	C-5'
14	δ 98	C-6'

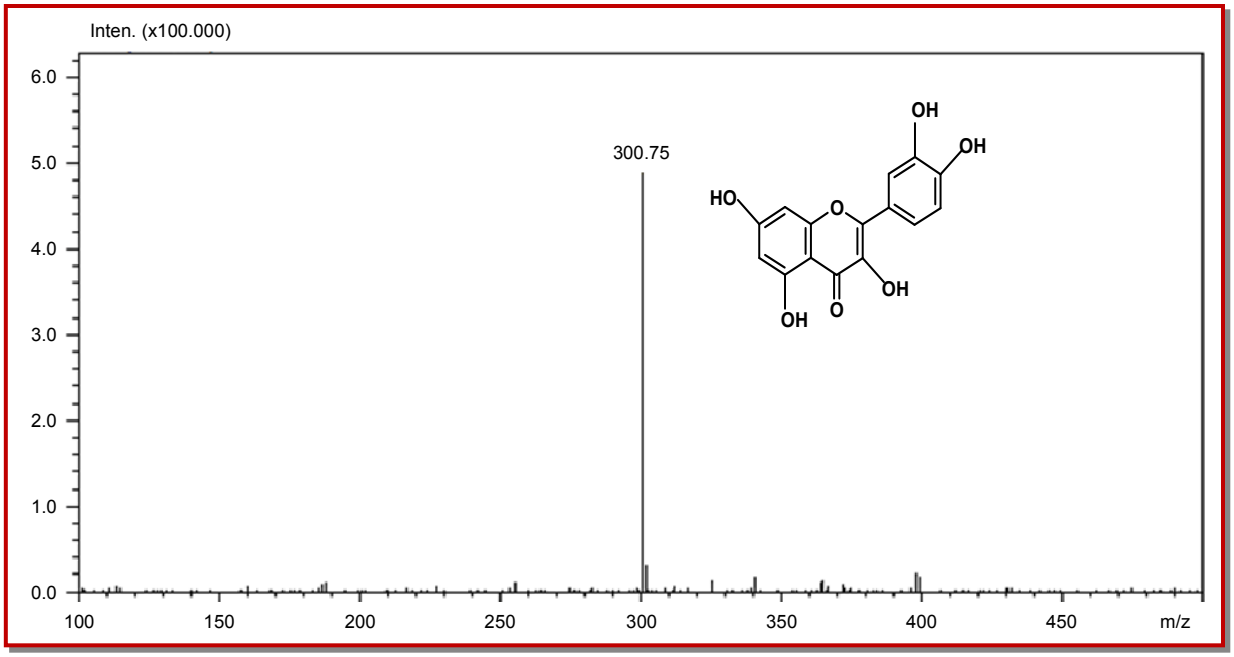

Figure 4: Mass spectrum data of the bioactive compound 7: 3,3',4',5,7-pentahydroxyflavone

colored background on the TLC plates; this confirms the presence of notable phytoactive compounds with acetylcholinesterase inhibiting activity. The results of fast blue salt assay showed potent acetylcholinesterase inhibition activity in a dose dependent manner and 81.5 ± 1.5% of inhibition was observed for the ethanol extracts of *C. indica*; whereas the standard drug eserine showed 89.8 ± 1.1% at 3 mg/mL concentration. The IC_{50} values of *C. indica* and eserine were found to be 1.8 and 1.4 mg/mL respectively.

Y-maze task is the most reliable and commonly used test to assess the spatial recognition memory. This task mainly relies on natural tendency of mice to explore a new environment (Yusuf et al., 2009). Short-term memory was determined by the spontaneous alteration behavior in the Y-maze task. Scopolamine, an anticholinergic drug was reported to interfere with cognitive function in humans; which functions in a very similar way to that of memory impairment observed in elderly subjects (Mintzer et al., 2010). In the current study a significant restoration of short-term memory loss was observed in the amnesic mice models when treated with ethanol extract of *C. indica* (100 mg/kg p.o). In the Y-maze task, the total number of arm (ABC) entries made by the mice subjects was found to be unaffected; this confirms the linearity in locomotor activity. As a concluding remark in the behavioral studies, the results suggest that the *C. indica* could possibly be developed as a drug for ameliorating cognitive dysfunction. This effect may be due to blockade of muscarnic cholinoreceptors. Furthermore, it has been reported that increasing of acetylcholine in the synaptic cleft by acetylcholinesterase inhibitors would activate presynaptic autoreceptors (Braida et al., 1996, Calabrese, 2008).

The bioactive compound containing acetylcholinesterase inhibitory activity was isolated and purified using column chromatography. The TLC bio-autography method for cholinesterase inhibitors was performed for bioguided selection of bioactive compound. Further from detailed spectral characterization studies the bioactive compound was found to be 3,3', 4',5,7-pentahydroxyflavone and its molecular formula was deduced as $C_{15}H_{10}O_7$ which was found to be a flavonoid quercetin.

It is noteworthy that quercetin was identified to be a major phytoconstituent with cholinesterase inhibiting potential which may enhance the cognitive effects although the presence of other active compounds cannot be excluded.

Furthermore, the effects of quercetin was previously reported by many researchers and has confirmed that quercetin on treatment has significant enhancement in the cognitive performance in Morris water and elevated plus maze tests (Dominik Szwajgier et., al 2013). Nasal administration of quercetin in AF64A rat model of Alzheimer's disease improved memory deficits in Morris water maze test by completely inhibiting the increase of malondialdehyde and lipid peroxidation followed by decrease in activities of superoxide dismutase and gluthatione peroxidase in hippocampal homogenates (Tong-Un et al., 2010). Quercetin has also been reported to inhibit the formation and extension of β-amyloid (Ono et al., 2003).

Bacopa monnieri and *Ginkgo biloba,* are well known cognitive enhancers used in Indian and Chinese

traditional medicine (Das et al., 2002). Investigation on use of *Ginkgo biloba* extracts EGb761 outperformed in enhancing the cognitive function (Hashiguchi et al., 2015). A randomized clinical trial for huperzine A, which is a sesquiterpene alkaloid separated from *Huperzia serrata* exhibited potent acetylcholinesterase inhibiting activity (Yang et al., 2013). Recently, acetylcholinesterase inhibiting activity was also reported in *Celosia argentea* var. *cristata* (Saqib et al., 2015), *Artemisia macrocephala* (Shoaib et al., 2015) and *Zygophyllum album* (Kchaou et al., 2016).

Conclusion

The ethanol extract of *C. indica* possesses a potent anticholinesterase activity and ameliorates short-term memory loss in the scopolamine–induced amnesic mice model.

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Conflict of Interest

All authors have completed the ICMJE uniform disclosure form and declare no support from any organization for the submitted work.

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Author Info
Palanisamy Selvamani (Principal contact)
e-mail: selva.autpharma@gmail.com