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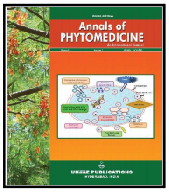

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Isolation, characterization and evaluation of endophytic fractions of *Centella asiatica* (L.) Urb. (Leaves) for the management of Alzheimer's disease

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Abstract

In the present investigation, we have carried out the isolation of fungal endophytes from *Centella asiatica* (L.) Urb. leaves followed by fermentation and extraction of fungal endophytes with non-polar solvents such as chloroform, ethyl acetate and n-butanol. Preliminary phytochemical investigation of endophytic crude fractions of plant was determined to detect the presence of primary/secondary metabolites. Based on the report of our earlier studies in *in vitro* antioxidant activity, endophytic crude fractions of plant ethyl acetate and n-butanol exhibited potential antioxidant activity. Therefore, potential endophytic fractions were further screened for nootropic activity, using various animal models such as Elevated plus maze, Morris water maze and Passive avoidance paradigm. To know the probable mode of action in these models, estimation of whole brain acetylcholinesterase and monoamines such as nor-adrenalin (NA), and dopamine (DA) was carried out. The pretreatment of ethylacetate fungal endophytic fractions (50 mg and 100 mg/kg, p.o) exhibited significant (p<0.01) improvement in learning and memory and also revealed significant (p<0.01) decrease in whole brain Ache activity as compared to n-butanol fraction. Results were further supported by histopathological studies of young mice brain. Further, investigations are required to isolate and characterize the potential metabolites from endophytic fungal fractions of *C. asiatica* leaves responsible for the anti-Alzheimer's activity.

1. Introduction

The word endophyte is derived from term (endon = within, phyton = plant). Endophytes are microbes that will reside in the internal tissue of the plant. Endophytes were first discovered in Germany by Freeman in 1904, who identified an endophytic fungus, in Persian darnel (annual grass). The first actinobacterial endophyte isolate was Frankia, a N-2 fixing actinobacterium that formed actinorhizae with 8 families of angiosperms (Arora and Ramawat, 2017). The presence of bacteria resident within the tissues of healthy plants was first reported as early as 1926. The microenvironment found inside the plant (between plant cells) that is colonized by microorganisms. Endophytes colonize the host tissue internally, sometimes in high numbers, without damaging the host or eliciting symptoms of plant disease. These endophytes are capable of producing novel secondary metabolites, which may or may not be present in the parent plant (Hallmann *et al*., 1997).

Alzheimer's disease (AD) is a neurodegenerative disorder, characterized by impairment of learning and memory, followed by more global cognitive deficits and behavioral disturbances (depression, agitation, and psychosis) which become progressively more severe. None of the pharmacological lines of interventions so far been able to stop the progression of AD. Nootropics are the drugs which are used for treatment for the mental and learning deficit in persons. Donepezil, tacrine, rivastigmine can be employed to increase the amount of acetylcholine in the body. Piracetam is considered as commonly used memory enhancing agent. The novel drugs which are used to cure loss of memory will damage the liver and causes other effects such as nausea, diarrhea, insomnia and fatigue. Compared to modern medicines, endophytes like bacteria/ fungi obtained from various parts of medicinal plants that were capable of producing novel metabolites will be considered as less toxic with the least side effects and can be an alternative approach in the management of Alzheimer's disease (Tripathi, 2003).

Centella asiatica (L.) Urb. (Apiaceae) is an ethnomedicinal plant, native to India, Madagascar, SriLanka, China, Indonesia, Australia and South Africa. It is a profusely branched prostrate herb, well known medicine to promote intelligence.The major chemical constituents found in the plant are triterpenoids, vallarine, asiaticoside, sitosterol, tannin, oxy-asiaticoside. Bioactive metabolites were isolated from an endophytic fungus, *Penicillium* sp. from *C. asiatica* (Ulrich-merzinch *et al*., 2010). A new fungal species, named *Echinosphaeria macrospora*, *teleomorph* of *Vermicu-lariopsiella endophytica*, were discovered from the stems of *C. asiatica*, *Chaetomium globosum,* a fungal species was isolated within healthy

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leaves of *C. asiatica*, collected from the Malnad region (Southern India) (Zheng and Qin, 2007). Inoculation of the growth-promoting endophytic fungus, *Piriformospora indica* to roots of *C. asiatica in vitro* cultures, resulted in the rapid enhancement of root and shoot biomass of the host plant and an increase in asiaticoside production in leaves (Devi and Prabhakaran, 2014), *Pantoea agglomerans* AR-PSBH2, an endophytic bacteria associated with *C. asiatica*, was shown high antioxidant potential (Gawas *et al*., 2006).

In vitro studies were conducted on the impact of endophytic bacteria, isolated from *C. asiatica* on the disease incidence, caused by the hemibiotrophic fungus, *Colletotrichum higginsianum* (Krishnamurthy *et al*., 2008). The total antioxidant power and free radical scavenging activity of the fungal extracts was estimated using the total phenolic content and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical assay along with the flavonoids and alkaloids content. The DPPH free radical assay showed that the ethanolic extract of endophytic fungi, *Aspergillus oryzae* CeR1 had higher radical scavenging activity than *Colletotrichum gloeosporioides* MKL1 (Sateeshan *et al*., 2012).

The antimicrobial potential of 6 endophytic fungi was isolated from leaves of *C. asiatica* plant. Maximum endophytes were isolated in PDA. The ethyl acetate extract of endophytic fungi showed growth inhibition on atleast one pathogenic bacteria, *Klebsiella* sp., *Staphylococcus* sp., *Pseudomonas* sp., *Salmonella* sp., *Proteus* sp., *Shigella* sp., *Serratia* sp. But, the activity against fungal pathogen was very low compare to bacteria (Rafat *et al*., 2012).

The effect of an aqueous *C. asiatica* extracts (100, 200 and 300 mg/kg for 21 days) was evaluated in intracerebroventricular (i.c.v.) streptozotocin (STZ)-induced cognitive impairment and oxidative stress in rats. The rats treated with *C. asiatica* showed a dose-dependent increase in cognitive behaviour in passive avoidance and elevated plus-maze paradigms. A significant decrease in MDA and an increase in glutathione and catalase levels were observed only in rats, treated with 200 and 300 mg/kg CA (Rakotoniriana *et al*., 2013).

The potential efficacy of *C. asiatica* (CA) extract was evaluated in rats in preventing the cognitive deficits, as well as the oxidative stress. Neuroprotection by *C. asiatica*, was further supported by the phosphorylation of cyclic AMP response element binding protein (CREB) by an increase in both a neuroblastoma cell line expressing amyloid beta 1-42 (A beta) and in rat embryonic cortical primary cell culture (Nath *et al*., 2014).

Oral treatment with 50 mg/kg/day of crude methanol extract of *C. asiatica* for 14 days significantly increased the antioxidant enzymes, like superoxide dismutase (SOD), catalase and glutathione peroxidase (GSHPx) in lymphoma-bearing mice. The antioxidants like glutathione (GSH) and ascorbic acid was decreased in the animals (Prabhakaran and Femina, 2012). Community structures of endophytic actinobacteria was assessed from a medicinal plant, *C. asiatica* based on a metagenomic approach using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) of 16S rRNA gene (Veerendra and Gupta, 2003).

Characterization of bacterial endophytes associated with *C. asiatica* was carried out and screened for their antifungal activity, using a well diffusion assay. Endophytic *Bacillus subtilis* B showed prominent antifungal activity, causing morphological distortion in the fungal morphology, offering scope for exploration an alternative antifungal agent (Xu *et al*., 2008).

The primary active constituents of *C. asiatica* are saponins (also called triterpenoids), which include asiaticosides, in which a trisaccharide moiety is linked to the aglycone asiatic acid, madecassoside and madasiatic acid. Other components isolated from CA, such as brahmoside and brahminoside, may be responsible for CNS and uterorelaxant actions, but are yet to be confirmed by clinical studies. Crude extract containing glycosides isothankuniside and thankuniside showed antifertility action in mice (Jayshree *et al*., 2003). However, there is no scientific proof, justifying the traditional use of *C. asiatica* leaves from the endophytic community in the management of Alzheimer's disease. Hence, the present work was undertaken to isolate, characterize and to evaluate its nootropic potential in different experimental models of Alzheimer's disease in albino mice.

2. Materials and Methods

2.1 Collection and authentication of plant

The plants were collected in and around Dharwad district in the month of June-2017 and were authentically identified as *Centella asiatica* (L.) Urb., belonging to family Apiaceae by Dr. Satyanarayan S. Hebbar, Assistant Professor, Department of Biology, Govt. P.U. College, Dharwad (SETCPD/Ref./35/2017).

2.2 Isolation of endophytic fungi from *C. asiatica*

The stem, root and leaves part of *C. asiatica* were washed thoroughly with distilled water and then they were surface sterilized with 90% ethanol, followed by treatment with 4% sodium hypochlorite solution for 30 sec to 1 min. For the preparation of culture media, petridishes were collected and washed properly, then they were rinsed with alcohol and left for drying. Two conical flasks of 250 ml were taken and 4 g of potato dextrose agar (PDA) and 25 mg of streptomycin were added to each flask and they were dissolved in 100 ml of distilled water in each flask. For proper solubility, the flasks were kept in a water bath for 5 min. The mouth of the flask was closed tightly. The petridishes and the conical flasks were collected and kept in autoclave for moist heat sterilization for 1 h. All the required materials were taken into the laminar airflow chamber. The PDA solutions were placed in petridishes and left for 10 min to solidify. After the broth media is properly solidified, then plant parts were separately embedded in the media in 3 petridishes. The petridishes were closed with cover plate and kept in an incubator for further growth of endophytic fungi at 25-26°C for 7 days. After 7 days, growth of endophytes was noticed and then they were subjected to subculture technique to get pure fungal strain (Ernawati *et al*., 2016; Nongklaw and Joshi., 2016; Heidari *et al*., 2007; Wiyakrutta *et al*., 2004; Silva *et al*., 2005).

2.3 Subculturing technique

Broth media was prepared using PDA (Potato dextrose agar). Petridishes and PDA solutions were sterilized by autoclaving for 120°C for 15-20 min. Small sections of grown endophytic fungi were transferred into media for proper growth. Transfer of endophytic fungi was done with flame sterilized inoculating loop by streaking and kept in incubator at 25-26°C for 7 days. After

incubation for 7 days, the fungi which were grown on PDA solidified media and were sent for identification by PCR sequential analysis to Bhat Biotech, Bengaluru (Tejaswi *et al*., 2008). Three samples were submitted for identification by colony morphology and compared with known organism from the literature.

2.4 Fermentation of isolated fungi

Fermentation of isolated fungi was carried out in 3 liters of PDB solution in Erlenmeyer flask and incubated at 25-26°C for 21 days under stationery condition. Fungal cultures were filtered through four layer of cheese cloth and homogenized at 4000 rpm to separate mycelia from broth.

2.4.1 Extraction of fungal broth with non-polar organic solvents

Extraction of fermented fungal broth was carried out using chloroform, ethyl acetate and n-butanol using separating funnel. The organic phase was separated to dryness under reduced pressure, using rotary flash evaporator and weighed to constitute crude fractions. All the crude fractions were weighed and calculated to know the % yield of each fractions. The % yield of chloroform fraction was found to be 4 g, ethylacetate fraction 5 g and n-butanol fraction 5.3 g, respectively. Phytochemical screening and TLC studies of above endophytic fractions were carried out which revealed the presence of alkaloids and steroids in chloroform fraction, tannins, flavonoids, volatileoils and coumarins in ethylacetate fraction, carbohydrates, tannins, flavonoids and volatileoils were present in n-butanol fraction, respectively (Shukla *et al*., 2012; Doty *et al*., 2005). Based on the results of our earlier studies on *in vitro* antioxidant activity, ethylacetate and n-butanol endophytic fungal fractions of *C. asiatica* were further screened for anti-Alzheimer's activity in various experimental models.

2.4.2 Phytochemical analysis

Qualitative chemical analysis and thin layer chromatographic studies were carried out to detect important phytoconstituents present in endophytic leaf fractions of *C. asiatica* (Staul, 1990; Wagner and Vladet, 1994).

2.4.3 Procurement of drugs

Piracetam (Sigma Aldrich, U.S.A), Phenytoin (Sigma Aldrich, U.S.A) and Scopolamine hydrobromide were procured from (Sigma Aldrich, U.S.A).

2.4.4 Acute toxicity studies

Acute toxicity studies were carried out using Swiss albino mice (25-30 g) by up and down/stair case method as per CPCSEA guidelines. Ethylacetate and n-butanol fractions were orally administered to different groups of young and aged mice at doses of 50 mg, 300 mg and 1000 mg/kg body weight, respectively. Animals were observed for 48 h to study the general behavior of animals, signs of discomfort and nervous manifestations. Endophytic leaf fraction of *C. asiatica* did not produced any toxicity up to dose level of 1000 mg/kg. Hence, 50 mg and 100 mg/kg were selected to screen for antiamnesic activity in mice (Reed and Munch, 1992; Sharma, 1986)

2.4.5 Animals

Swiss male mice weighing around 18 g (Young ones), and 25 g (Old ones) were used in the present study. Animals were procured from Adita Biosys Pvt. Ltd., Tumkur, Karnataka, India. They were acclimatized to the laboratory conditions for 5 days before behavioural studies. The animal had free access to food and water and were maintained under 12:12 h light and dark cycles. All the readings were taken at during the same time of the day (5-7 pm). Institutional Animal Ethical Committee (IAEC) had approved the experimental protocol and care of animals was taken as per CPCSEA guidelines, Animal welfare division, ministry of environment, Govt. of India.

2.4.6 Screening models for antiamnesic activity

2.4.6.1 Elevated plus maze

The elevated plus maze served as the exteroceptive behavioral model to evaluate learning and memory in mice. The apparatus consisted of two open arms (16 cm x 16 cm) and two covered arms (16 cm x 5 cm x 12 cm). The arms extended from a central platform (5 cm x 5 cm), and the maze was developed to a height of 25 cm from the floor. On the first day, each mouse was placed at the end of open arm, facing away from the central platform. Transfer latency (TL) was taken as the time taken by mouse to move in to one of the covered arm with all its four legs. TL was recorded on the first day. If the animal did not enter one of the covered arms within the 90 s, it was gently pushed in to one of the two covered arms and the TL was assigned as 90 s. The mouse was allowed to explore the maze for 10 s and then returned to its home cage. Memory retention was examined 24 h after the first day trial on the second day (Itoh *et al*., 1990; Parle, 2004; Rashmi and Dumka, 2019).

1: Photograph showing elevated plus maze.

The study comprises of nine different groups of young and old mice (n=6) for investigation as follows:

Young mice

Group 1: Normal control and saline (10 ml/kg, p.o) was administered orally for 8 days. TL were noted after 45 min of administration of last dose on the 8th day, after 24 h, on 9th day.

Group 2: Scopalamine (0.4 mg/kg, i.P) was injected and TL was noted after 45 min of administration on the 8th day and on the 9th day.

Group 3: Piracetam (200 mg/kg, p.o)+ Scopalamine (0.4 mg/kg, i.P) and TL was noted after 45 min of administration for 8th and 9th days.

Groups 4 and 5: Endophytic fractions of ethylacetate (50 mg and 100 mg/kg) were administered. After 45 min of last dose, TL was noted on 8th and 9th day.

Groups 6 and 7: Endophytic fractions of n-butanol (50 mg and 100 mg/kg) were administered. After 45 min of last dose, TL was noted on 8th and 9th day.

Group 8: Endophytic fractions of ethylacetate (100 mg/kg) were administered. After 45 min of last dose, scopolamine (0.4 mg/kg, i.p) was administered. TL was noted on 8th and 9th day.

Group 9: Endophytic fractions of n-butanol (100 mg/kg) were administered. After 45 min of last dose, scopolamine (0.4 mg/kg, i.p) was administered. TL was noted on 8th and 9th day.

Aged mice

Group 1: Normal control and saline (10 ml/kg, p.o) were administered orally for 8 days. TL was noted after 45 min of administration of last dose on the 8th day, after 24 h, on 9th day.

Group 2: Control (aged) (10 ml/kg, p.o) was administered orally for 8 days. TL was noted after 45 min of administration of last dose on the 8th day, after 24 h, on 9th day.

Group 3: Piracetam (200 mg/kg, p.o)+ Scopalamine (0.4 mg/kg, i.P) and TL were noted after 45 min of administration for 8th and 9th days.

Groups 4 and 5: Endophytic fractions of ethylacetate (50 and 100 mg/kg) were administered. After 45 min of last dose, scopolamine (0.4 mg/kg, i.p) was administered. TL was noted on 8th and 9th day.

Groups 6 and 7: Endophytic fractions of n-butanol (50 and 100 mg/kg) were administered. After 45 min of last dose, scopolamine (0.4 mg/kg, i.p) was administered. TL was noted on 8th and 9th day.

2.4.6.2 Morris water maze

Water maze is used to assess the long term memory in mice. It consists of a circular water tank (150 cm diameter, 45 cm height), filled with water and maintained at 25°C. The water was made opaque with a white colored non-toxic dye. The tank is divided in to four quadrants with the help of two threads, fixed at right angle to each other on the rim of the pool. A platform (10 cm^2) of 29 cm height was located in the center of one of these four quadrants. The position of platform was kept unaltered throughout the training sessions. In the present study, the target quadrant was Q4. Each animal was subjected to four consecutive trials on each day with a gap of 5 min for four consecutive days, during which they were allowed to escape on to the hidden platform and to remain there for 20 sec. In case, the animal was unable to locate the hidden platform within 120 sec, it was gently guided to the platform and allowed to remain on the platform for 20 sec. Escape latency time to locate the hidden platform in water maze was taken as an index of acquisition or learning. Starting position on each day to conduct four acquisition trials was changed as described below and Q4 was maintained as target quadrant in all the acquisition trials. The starting point for dropping the mice in to water maze on day one for four consecutive acquisition trials was the sequence Q1, Q2, Q3 Q4 and so on. Sequence change of starting point was as follows:

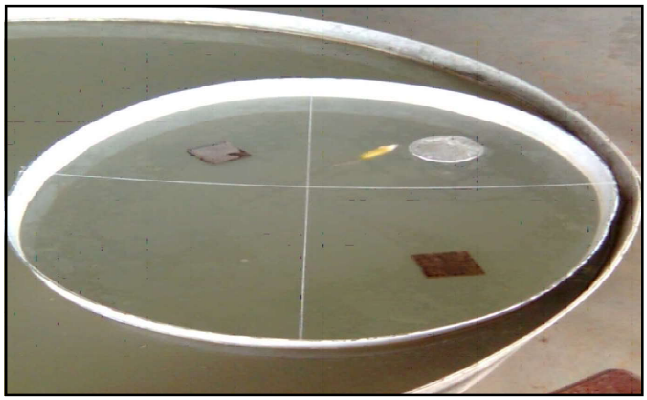
Day 1: Q1, Q2, Q3, Q4

Day 2: Q2, Q3, Q4, Q1

Day 3: Q3, Q4, Q1, Q2

Day 4: Q4, Q1, Q2, Q3

Mean escape latency time (ELT) was calculated for each day of the trial. On fifth day, the platform was removed, each mice was placed in water for 120 sec. The animal was subjected to four such trials and each trial had a different starting point, covering all the four quadrants. The mean time, spent by animals in all four quadrants was recorded. The time spent in the target quadrants Q4 as compared to time, spent in other quadrants in search of the missing platform was taken as an index of retrieval. Care was taken that relative location of water maze with respect to other objects in laboratory serving as visual clues was not disturbed during the total duration of the study (Parle Singh *et al*., 2007).



2: Photograph showing Morris water maze.

The study comprises of six different groups of young and aged mice (n=6) for investigation as follows:

Young groups

Group 1: Normal control and saline (10 ml/kg, p.o) were administered orally for 4 days. Acquisition trial were conducted before 45 min of administration of last dose on the day 1 to day 4. retrival trial on 5th day.

Group 2: Scopalamine (0.4 mg/kg, i.P) was injected 45 min before acquisition trial conducted on day 1-4 and saline(10 ml/kg, p.o) were administered 45 min before the retrival trial conducted on day 5.

Group 3: Piracetam (200 mg/kg, p.o)+ Scopalamine (0.4 mg/kg, i.P) was injected 45 min before acquisition trial conducted on day 1-4 and saline (10 ml/kg, p.o) was administered 45 min before the retrival trial conducted on day 5.

Group 4: Endophytic fractions of ethylacetate (100 mg/kg, p.o) were administered. It was administered 45 min before acquisition trial conducted on day 1-4 and scopolamine (0.4 mg/kg, i.p) was administered before 45 min of retrival trial on 5th day.

Group 5: Endophytic fractions of n-butanol (100 mg/kg) were administered. It was administered 45 min before acquisition trial conducted on day 1-4. scopolamine (0.4 mg/kg, i.p) was administered before 45 min of retrival trial on 5th day.

Group 6: Piracetam (200 mg/kg, p.o) was injected 45 min before acquisition trial, conducted on day 1-4 and saline (10 ml/kg, p.o) was administered 45 min before the retrival trial conducted on day 5.

Aged groups

Group 1: Normal control and saline (10 ml/kg, p.o) were administered orally for 4 days. Acquisition trial was conducted before 45 min of administration of last dose on the day 1-4 and retrival trial on 5th day.

Group 2: Piracetam (200 mg/kg, p.o) was injected 45 min before acquisition trial conducted on day 1-4 and saline (10 ml/kg, p.o) was administered 45 min before the retrival trial conducted on day 5.

Groups 3 and 4: Endophytic fractions of ethylacetate (50 and 100 mg/kg, p.o) were administered. It was administered 45 min before acquisition trial conducted on day 1-4 and Scopolamine (0.4 mg/kg, i.p) was administered before 45 min of retrival trial on 5th day.

Groups 5 and 6: Endophytic fractions of n-butanol (50 and 100 mg/kg) were administered. It was administered 45 min before acquisition trial conducted on day 1-4 and Scopolamine (0.4 mg/kg, i.p) was administered before 45 min of retrival trial on 5th day.

2.4.6.3 Estimation of brain acetyl cholinesterase activity

The whole brain acetyl cholinesterase (AChE) activity was measured using the Ellman method (Ellman *et al*., 1961) Animals were sacrificed on the 9th day by cervical dislocation and brain tissue was removed carefully to avoid any injuries. Tissue was homozenised in normal saline and centrifuged. The supernatant was used to estimate the AChE activity. The end point was the formation of yellow color due to the reaction of thiocholine from acetylcholine iodide in the presence of dithiobisnitrobenzoate ions. The rate of formation of thiocholine from acetylcholine iodide in the presence of tissue cholinesterase was measured using spectrophotometer. The sample was first treated with 5, 5'-dinitrobenzoic acid (DTNB) and the optical density (OD) of the yellow color compound formed during the reaction at 412 nm every minute was measured. Protein estimation was done using Folin's method. AChE activity was calculated using the following formula:

$$R = \frac{\S O.D.\ x\ volume\ of\ assay\ (5\ ml)}{E\ x\ mg\ of\ protein}$$

where R= rate of enzyme activity in 'n' mole of acetyl choline iodide hydrolysed/min/mg protein. § O.D. = Change in absorbance/ min, E= Extinction coefficient – 13,600/M/cm

Animals were grouped as follows:

Group 1: Control group treated with normal saline

Group 2: Treated with phenytoin (12 mg/kg, p.o)

Group 3: Treated with piracetam (200 mg/kg, p.o)

Groups 4 and 5: Treated with endophytic fraction of ethylacetate (50 and 100 mg/kg, p.o)

Groups 6 and 7: Treated with endophytic fraction of n-butanol (50 and 100 mg/kg, p.o)

2.4.6.4 Biochemical estimation of brain biogenic amines

The animals were sacrificed and whole brain was dissected out. Weighed quantity of tissue was homogenized in 10 ml hydrochloricacid–butanol, (0.85 ml of 37% hydrochloric acid in one liter n-butanol for spectroscopy) for 1 minute in a cool environment. The sample was then centrifuged at 3075 x g for 10 min. 0.08 ml of the supernatant phase was removed and added to an Eppendorf reagent tube containing 0.2 ml of n-heptane (for spectroscopy) and 0.025 ml 0.1M hydrochloric acid. After 10 min of vigorous shaking, the tube was centrifuged under the same conditions to separate two phases. The upper organic phase was discarded and to the aqueous phase of 0.02 ml, 0.05 ml 0.4 M and 0.01 ml EDTA/Sodium acetate buffer (pH 6.9) were added, followed by 0.01 ml iodine solution (0.1M in ethanol) for oxidation. The reaction was stored after 2 min by addition of 0.01 ml Na_2SO_3 in 5 M NaOH. Acetic acid was added 1.5 min later. The solution was then heated to100°C for 6 min. When the sample reached room temperature, excitation and emission spectra were read at 395-485 nm for Nor-adrenaline and 330-375 nm for Dopamine, respectively. The values were expressed as fluorescent excitation spectral height intensity correspondence to its concentration of biogenic amines present in the sample and percentage amine content in the brain was calculated (Schlumph *et al*., 1974).

2.4.6.5 Histopathology of hippocampus of the brain

After the treatment and behavioural studies, 24 h after administration of scopolamine, animals were sacrificed and Hippocampus was dissected out and kept in 10% formalin solution. The brain was stained with haematoxylin-eosin stain; hippocampus region was studied under microscope (Olympus India CH 20i). The samples were examined with the help of Dr. Ammanagi (Pathologist) Jawaharlal Nehru Medical College, Belgaum, Karnataka, India. Various parameters like cell damage, pyknotic black neurons, karryorhexis and number of cell death were observed (Chen *et al*., 1997)

2.4.6.6 Statistical analysis

The data were expressed as Mean ± SEM. The data were analysed using one-way ANOVA, followed by Tukey-kramer test. $p<0.01$ was considered significant (Dunnet, 1964).

3. Results

3.1 Isolation of endophytic fungi from leaf of the *C. asiatica*

3.1.1 Phenotypic identification

The colonies grown on the agar plate were studied by the colony characteristics and lactophenol cotton blue staining. The characteristics were compared with the known organisms from the literature.

3.1.2 Colony morphology

The colonies grown on the agar plate were studied by the lacto phenol cotton blue staining. The characteristics were compared with the known organisms from the literature.

3.1.3 Lactophenol cotton blue staining

Few drops of lacto phenol cotton blue stain (HiMedia) was placed in the center of the clean glass slide. With a sterile cooled loop, the mycelia was transferred to the slide and teased gently. A clean cover slip was placed on it without air bubble. Using a blotting paper, the excess stain was removed. The slide was visualized under low to high power objectives of the microscope.

3.1.4 Colony morphology of isolated fungi

Black surface, white reverse, smooth conidiophore, radiate, biseriate can be identified by its hyaline, septate hyphae. Sexual conidiophores can be identified by being long and globose at the tip, which appears to be a hymenial layer of structures, own spore.

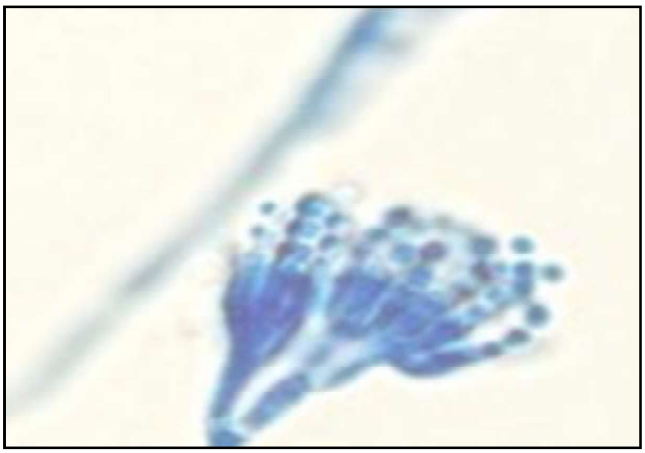


Figure 7: Lactophenol cotton blue-stain of the colonies observed on agar slant.

From the colony morphology and phenotypic identification, the above endophytic fungal sample (Figure 7) was identified as *Aspergillus niger*.

3.1.5 Phytochemical analysis

Preliminary phytochemical analysis and studies of chloroform, ethyl acetate and n-butanol fraction of leaf of *C. asiatica* revealed the presence of alkaloids and steroids in chloroform fraction, tannins, flavonoids and volatileoils in ethyl acetate and n-butanol fraction.

3.1.6 Effect of transfer latency (TL) an elevated plus maze

Aged mice showed higher transfer latency (TL) values on first day and second day as compared to young mice, indicating impairment in learning (ageing induced amnesia). Scopolamine (0.4 mg/kg, i.p.) increased the transfer latency significantly (p<0.01) in young mice on first and second day as compared to control, indicating impairment of memory. Treatment with Piracetam (200 mg/kg, i.p.) for 8 days, decreased TL as compared to control, indicating improvement in both learning and memory. pretreatment with endophytic *C. asiatica* leaf fraction (CALF) (50 and 100 mg/kg, p.o.) decreased the TL on 8th and 9th day in young and aged mice (p<0.01), when compared to control groups. The higher dose of endophytic ethylacetate fraction of C.A (EEAFCA) (100 mg/kg, p.o.) significantly enhanced antiamnesic property in aged animals rather than young mice as reflected by marked decrease in TL on 8th and 9th day when subjected to EPM tests. Endophytic n-butanol fraction of CA (ENBFCA) (100 mg/kg, p.o.) exerted moderate enhancement of memory in young mice and protected them from against scopolamine (p<0.01) and ageing induced amnesia. The results were summarized in Figures 1 and 2.

3.1.7 Effect on brain acetyl cholinesterase activity

EEAFCA and ENBFCA (50 and 100 mg/kg, p.o.) significantly produced a reduction in whole brain AChE activity of both young and aged mice as compared to respective control group. The brain AChE activity with phenytion (12 mg/kg, i.p.), exhibited significant elevation which was considered as a negative control. Piracetam (200 mg/kg, i.p.) profoundly reduced AChE activity as compared to control groups as shown (Figure 3).

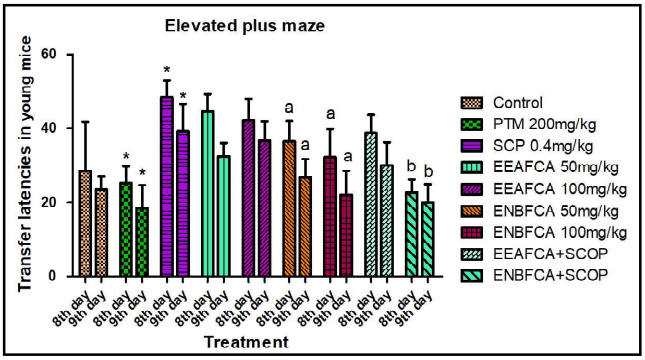


Figure 1: Effect of *C. asiatica* endophytic leaf extract on transfer latencies (TL) of young mice by elevated plus maze. *p<0.001 compared to control, ap<0.01 compared to control, bp<0.01 compared to negative control (scopolamine treated).

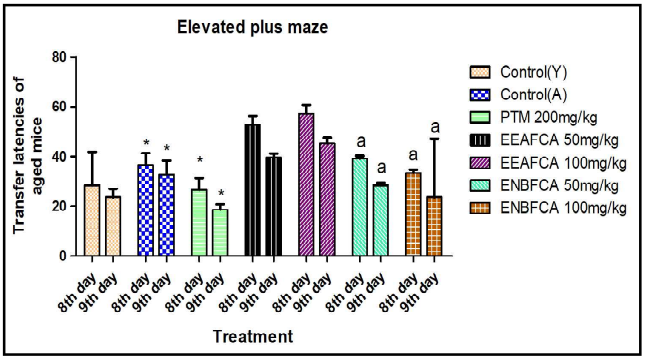


Figure 2: Effect of *C. asiatica* endophytic leaf extract on transfer latencies of aged mice by elevated plus maze. *p<0.001 compared to control, ap<0.01 compared to control.

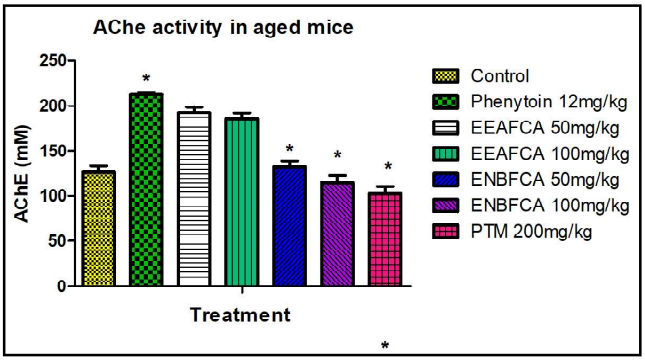


Figure 3: Effect of *C. asiatica* endophytic leaf extract and piracetam on AChE activity in aged mice. *p<0.01 compared to control.

3.1.8 Effect of endophytic leaf fractions of *C. asiatica* on scopolamine induced enhancement on escape latency time (ELT) in mice using water maze

A significant decrease (p<0.01) in the ELT was observed in control group mice in their 4 day trial. Scopolamine produced impairment of acquisition and increased the ELT during successive training trials. The action of scopolamine was reversed by pretreatment with EEAFCA (50 and 100 mg/kg, p.o.) as reflected by a significant decrease (p<0.01) in ELT of mice. The results were summarized in Figures 4 and 5.

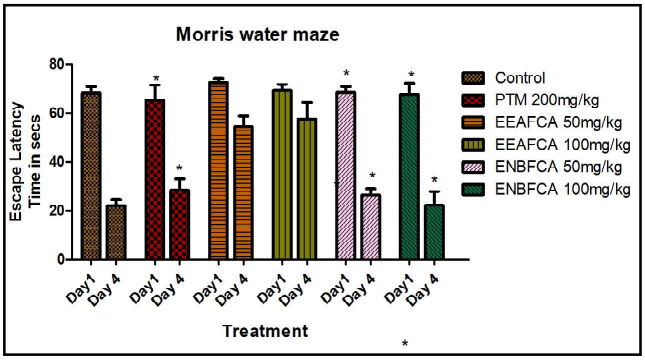


Figure 4: Effect of *C. asiatica* endophytic leaf extract on escape latency time (ELT) of young mice using Morris water maze. Each value represents ± SEM, * denotes $p<0.01$ as compared to control mice.

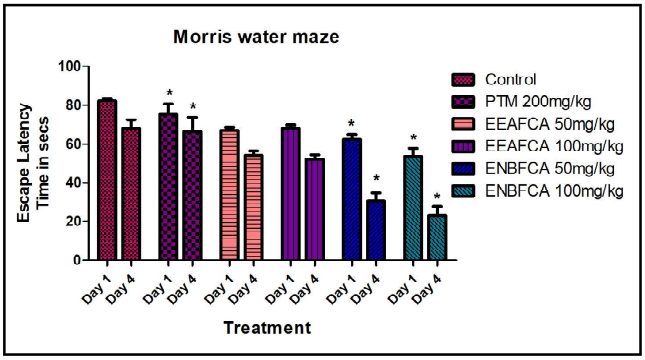


Figure 5: Effect of *C. asiatica* endophytic leaf extract on escape latency time (ELT) of aged mice using Morris water maze. Each value represents ± SEM, * denotes $p<0.01$as compared to control mice.

3.1.9 Effect of endophytic leaf fractions of *C. asiatica* on scopolamine and ageing induced alterations in the time spent target quadrant (TSQT) during retrieval trials on water maze

The time spent by young control mice in the target quadrant was more as compared to time spent on other quadrants during retrieval trial on 5th day. Further, scopolamine (0.4 mg/kg, i.p) administered before retrieval trial produced a significant decrease ($p<0.01$) in mean time spent in the target quadrant in search of the missing platform as compared to control (young). Aged mice also decreased TSQT significantly compared to control (young). The results were shown in Table 2. These observations indicate that scopolamine and natural ageing produced anterograde and retrograde amnesia. Mice treated with piracetam (200 mg/kg, i.p.) produced better effects only in aged mice by decreasing TSQT as compared to control (aged) mice. EEAFCA (50 and 100 mg/kg, p.o.) administered before training trial (from day 1 to day 4), significantly ($p<0.01$) attenuated scopolamine and ageing induced decrease in TSQT during retrieval test on 5th day (Tables 1 and 2).

Table 1: Effect of *C. asiatica* endophytic leaf extract on the mean time spent in the target quadrants (TSTQ) Q4 in young mice using Morris water maze

Group	Treatment	Dose (mg/kg, i.p./p.o.)	TSTQ (secs)
1	Control	10	67.7 ± 1.23
2	Piracetam	200	57.2 ± 2.13*
3	Scopolamine	0.4	39.2 ± 1.55
4	EEAFCA	50	34.2 ± 2.54
5	EEAFCA	100	38.4 ± 3.53
6	ENBFCA	50	60.4 ± 2.45[a]
7	ENBFCA	100	58.6 ± 7.23[a]
8	EEAFCA + Scopolamine	100	34.3 ± 1.24
9	ENBFCA + Scopolamine	100	58.9 ± 2.74[a]

Each value represents Mean ± SEM. * denotes $p<0.01$ as compared to control, 'a' denotes $p<0.01$ as compared to scopolamine treated mice.

Table 2: Effect of *C. asiatica* endophytic leaf extract on the mean time spent in the target quadrants (TSTQ) Q4 in aged mice using Morris water maze

Group	Treatment	Dose (mg/kg, i.p./p.o.)	TSTQ (secs)
1	Control	10	27.3 ± 2.44
2	Piracetam	200	58.4 ± 1.75*
3	EEAFCA	50	40.4 ± 3.44
4	EEAFCA	100	36.2 ± 4.34
5	ENBFCA	50	59.2 ± 4.43*
6	ENBFCA	100	55.9 ± 2.33*

Each value represents Mean ± SEM. * denotes $p<0.001$ as compared to control.

3.1.10 Brain biogenic amines

The administration of donepezil and endophytic ethylacetate fraction (EEAFCA) and n-butanol fraction of *C. asiatica* (ENBFCA) (100 mg/kg, p.o) to a normal group of animals indicated that there is decrease ($p>0.05$) in the height intensity of emitted spectra of dopamine and nor-adrenaline, comparative to young normal groups which were pre-treated with plain distilled water. Whereas, animals treated with scopolamine and natural aged animals showed a significantly ($p<0.05$) increase in the height intensity of emitted spectra of dopamine and an insignificant increase ($p>0.05$) in nor-adrenaline comparative to the young normal group. (Table 3).

Table 3: Effect of endophytic fractions of *C. asiatica* on brain biogenic amines in young mice

Sl. No.	Groups	Dose	Dopamine	Nor-adrenaline	Nor-adrenaline content (%)	Dopamine content (%)
1	Young (Normal)	10 ml/kg, p.o	873.06 ± 10.42	230.24 ± 11.96	100	100
2	Young + Donepezil	3 mg/kg, p.o	845.41 ± 21.44 a	204.74 ± 11.42 a	96.82	89.40
3	Young + EEAFCA	100 mg/kg	850.3 ± 19.3 a	213.90 ± 7.30 a	98.07	94.05
4	Young + ENBFCA	100 mg/kg	860.3 ± 19.3	218.93 ± 7.31b	95.06	92.06

Each group consists of 5 animals (n=5). The values obtained are fluorescent excitation height of biogenic amines. Values are in Mean ± SEM $p<0.05$ is considered as significant, [a]$p<0.05$ as compared to normal group.

The insignificant (p>0.05) reversal effect of increased height intensity of emitted spectra of dopamine and nor-adrenaline is seen with endophytic ethylacetate fraction (EEAFCA) and n-butanol fraction of *C. asiatica* (ENBFCA) (100 mg/kg, p.o), exhibited moderately significant (p>0.05) with donepezil compared to scopolamine and natural aged group of animals. Percentage of amine content in brain was calculated and depicted in (Table 4) of respective groups.

Table 4: Effect of endophytic fractions of *C. asiatica* on brain biogenic amines in aged mice

Sl. No.	Groups	Dose	Dopamine	Nor-adrenaline	Nor-adrenaline content (%)	Dopamine content (%)
1	Young (Normal)	10 ml/kg, p.o	875.05 ± 10.40	234.24 ± 11.94	100	100
2	Aged mice	10 ml/kg, p.o	956.13 ± 19.80 a	259.66 ± 12.87	107.16	112.44
3	Aged + Donepezil	3 mg/kg, p.o	905.56 ± 15.10	208.35 ± 8.86 b	96.54	76.58
4	Aged + EEAFCA	100 mg/kg	923.28 ± 22.22 a	217.5 ± 12.52 b	98.72	87.76
5	Aged + ENBFCA	100 mg/kg	935.28 ± 13.43	228.63 ± 13.20 b	99.12	96.42

Each group consists of 5 animals (n=5). The values obtained are fluorescent excitation height of biogenic amines. Values are Mean ± SEM p<0.05 is considered as significant, [a]p<0.05 as compared to normal group. [b]p<0.05 as compared to aged group.

4. Discussion

The acetylcholine is considered as the most important neurotransmitter involved in the regulation of cognitive functions. Alzheimer's disease is slow in onset, but causes personality changes, dementia and unusual behavior. Oxygen free radicals are implicated in the process of age related decline in cognitive performance and may be responsible for the development of Alzheimer's disease in elderly persons (Bickford *et al*., 2000). Oxygen free radicals and other by products of oxidative metabolism have been shown to be neurotoxic (Berr *et al*., 2002) and antioxidant rich diets improved cerebellar physiology and motor learning in older rats. Antioxidant constituents of endophytic fractions of *C. asiatica* may favourably contribute in enhancing the memory effect in mice (Butterfield and Landerback, 2002). Thus, the protective effect of *C. asiatica* may be attributed to its antioxidant property by virtue of which susceptible brain cells get exposed to less oxidative stress, resulting in reduced brain damage and improved neuronal function. The present study indicates that endophytic ethyl acetate fractions of *C. asiatica* is a potential antiamnesic agent as compared to n-butanol fraction. It also possesses nootropic activity in view of its facilitatory effect on retention and acquired learning. EEAFCA (50 mg and 100 mg/kg, p.o.) decreased transfer latencies in both young but more profoundly in aged mice in dose dependent manner as compared to respective controls (Figures 1 and 2). In our study, phenytoin (12 mg/kg, i.p.) significantly elevated brain AChE activity. Piracetam (200 mg/kg, i.p.) and EEAFCA (50 and 100 mg/kg, p.o.) on the other hand significantly (p<0.01) lowered this activity, indicating the counteracting action of the drugs on cholinergic system (Parle and Singh, 2004). Endophytic n-butanol fraction of *C. asiatica*, elicitated profound neuroprotective effect in scopolamine treated and older mice compared to control groups and piracetam treated mice. It significantly inhibited AChE activity in the whole brain homogenate in mice, indicating its potential in the attenuation of learning and memory deficits especially in aged mice (Figure 3).

In water maze model, a marked decrease in escape latency time (ELT), during subsequent trials as compared to the first exposure, denotes normal learning ability. The enhancement in the time spent by the animal in the target quadrant reflects successful retention of learned task (or memory).

The free radicals are implicated in the etiology of neuromuscular degenerative conditions such as AD and amnesia (Standert and Youung, 2006). Reactive oxygen free radicals and other byproducts of oxidative metabolism are shown to be neurotoxic (Joshi and Parle, 2006). Antioxidant may be beneficial in treatment of AD, amnesia and other neuromuscular degenerative conditions (Heo *et al*., 2009). In the present study, scopolamine (0.4 mg/kg, i.p.) showed anterograde amnesia as indicated by significant decrease in more time spent in target quadrant on 5th day in Morris water maze model (Tables 1 and 2). Our observations suggested that endophytic ethylacetate fraction of *C. asiatica* (50 and 100 mg/kg, p.o) significantly reversed the scopolamine and ageing induced amnesia as compared to endophytic n-butanol fraction of *C. asiatica*. (50 and 100 mg/kg, p.o). From the histopathological observations (Figure 6), it was obvious that hippocampus region of normal control mice and piracetam treated group showed normal neuronal cells with all its sub-cellular structures in intact as indicated in Figure (A) and Figure (C), while in scopolamine-induced mice, the neuronal cells have undergone significant damage and also the formation of plaque as shown in Figure (B) which is the important feature of Alzheimer's disease. Further, in Alzheimer's disease-induced mice, simultaneously treated with endophytic ethylacetate fraction of *C. asiatica* (EEAFCA) and endophytic n-butanol fraction of *C. asiatica* (ENBFCA), the number of plaques were reduced and the cellular damage caused by Alzheimer's disease induction was reversed to near normal condition, thus demonstrating the positive effect of EEAFCA and ENBFCA on the cytoarchitectural of the neurons as shown in the Figures (D-I).

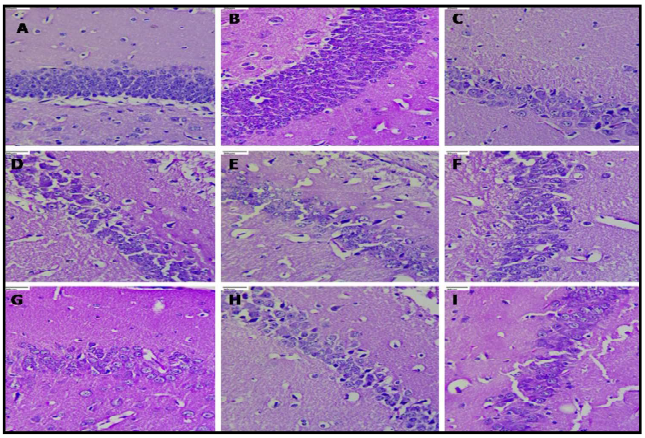


Figure 6: Histopathology of young mice brain.

The administration of donepezil and endophytic ethylacetate fraction (EEAFCA) and n-butanol fractions of *C. asiatica* (ENBFCA) (100 mg/kg, p.o) to a normal group of animals, indicated that there is decrease (p>0.05) in the height intensity of emitted spectra of dopamine and nor-adrenaline, comparative to young normal group which were pre-treated with plain distilled water. Whereas, animals treated with natural aged animals shows a significant (p<0.05) increase in the height intensity of emitted spectra of dopamine and an insignificant increase (p>0.05) with nor-adrenaline comparative to young normal groups (Tables 3 and 4).

5. Conclusion

In the present study, we observed that endophytic ethylacetate leaf fractions of *C. asiatica* inhibited acetylcholinesterase enzyme as compared to n-butanol fraction, thereby elevating acetylcholine concentration in brain homogenate and ultimately improved memory of both young and older mice. The brain content of biogenic amines such as dopamine and nor-adrenaline on treatment with donepezil and with endophytic fractions of *C. asiatica* showed significant decrease which is further supported by histopathological studies of hippocampus of mice brain. From the study, we can conclude that endophytic fractions of *C. asiatica* can be effectively employed in the management of Alzheimer's disease and their associated neurological problems.

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Conflict of interest

The authors declare that there are no conflicts of interest in the course of conducting the research. All the authors had final decision regarding the manuscript and decision to submit the findings for publication.

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