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# Isolation, Characterization and Screening of Endophytic Fractions of *Centella asiatica Linn* (Leaves) for Antiamnesic Activity

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## ABSTRACT

**Background:** In the present investigation, we have carried out the isolation of fungal endophytes from *Centella asiatica Linn* leaves followed by fermentation and extraction of fungal endophytes with non-polar solvents such as chloroform, ethylacetate and n-butanol. Prelimnary screening of endophytic crude fractions of plant were carriedout to detect the phytoconstituents. Based on the report of our earlier studies, endophytic crude fractions of plant ethylacetate and n-butanol exhibited potential antioxidant activity. Therefore, potential endophytic fractions were further screened for antiamnesic activity.

**Methods**: Animal models such as the elevated plus maze were used to assess the exploratory behaviour in mice to evaluate memory. 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. 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.

**Result:** The pretreatment of ethyl acetate endophytic fractions (50 mg and 100 mg/kg, p.o.) exhibited significant ($p<0.01$) improvement in learning and memory as compared to the n-butanol fraction. Scopalamine-induced groups showed more time to move from the open arm to the closed arm, indicating memory impairment, whereas standard drug groups showed significant improvement in memory.

**Conclusion:** The study indicates that endophytic fractions of *C. asiatica* can play significant role in enhancing the memory of both aterograde and retrograde amnesia.

**Key-words:** *Centella asiatica*, endophyte, elevated plus maze, scopolamine, Anti-amnesic

## INTRODUCTION

The term "endophytic fungi" refers to fungi that live in plant tissues throughout the entire or partial life cycle by establishing a mutually beneficial symbiotic relationship with its host plant without causing any adverse effect or disease. They are natural components of the plant micro-ecosystem that positively affect the physiological activities of the host plant in several ways.

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These include producing hormones such as indole acetic acid and biosynthesizing and acquiring nutrients essential for plant growth and development. They also secrete stress-adaptive metabolites that protect the host plant against herbivores and pathogens. Furthermore, they improve the host plant's adaptability to abiotic stressors [1,2].

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 mental and learning deficit in persons. Donepezil, tacrine, rivastigmine can be employed to increase the amount of acetylcholine in the

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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, which are capable of producing novel metabolites, will be considered less toxic, with fewer side effects, and can be an alternative approach in the management of Alzheimer's disease [3,4].

*C. asiatica* (L.) Urb (Apiaceae) is an ethno-medicinal plant, native to India, Madagascar, SriLanka, China, Indonesia, Australia and South Africa. It is a profusely branched prostrate herb, a well-known medicine to promote intelligence. The major chemical constituents found in the plant are triterpenoids [5], Vallarine, Asiaticoside, Sitosterol, Tannin, Oxy–asiaticoside. Bioactive metabolites were isolated from endophytic fungus *Penicillium* sp. from *C. asiatica* [6], A new fungal species, named *Echinosphaeria macrospora*, teleomorph of Vermicu-lariopsiella endophytica, was discovered from the stems of *C. asiatica* [7], *Chaetomium globosum* a fungal species was isolated from healthy leaves of *C. asiatica* collected from the Malnad region (Southern India) [8]. 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 [9], *Pantoea agglomerans* AR-PSBH$_2$, an endophytic bacteria associated with *C. asiatica*, was shown high antioxidant potential [10].

*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* [11], 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 the endophytic fungus Aspergillus oryzae CeR1 had higher radical-scavenging activity than that of *Colletotrichum gloeosporioides* MKL1 [12].

The potential efficacy of *C. asiatica* (CA) extracts was evaluated in rats in preventing the cognitive deficits, as well as the oxidative stress. Neuroprotection by CA 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 [13].

However there is no scientific proof justifying the traditional use of *C. asiatica* leaves from endophytic community in the management of alzheimers disease. Hence, the present work was undertaken to isolate, characterize and to evaluate its nootropic potential in different experimental model of alzheimer's disease in albino mice.

## MATERIALS AND METHODS

**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 *C. asiatica Linn* belonging to family Apiaceae by Dr. Satyanarayan. S. Hebbar, Lecturer, Department of biology, Govt P.U. College, Dharwad Karnataka.

**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, Petri dishes were collected and washed properly; then they were rinsed with alcohol and left to dry. Two conical flask of 250 ml were taken and to it 4gm 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 water bath for 5 m. The mouth of flask was closed tightly. The Petri dish and the conical flask were collected and kept in autoclave for moist heat sterilization for 1 hr. All the required materials were taken into laminar airflow chamber. The PDA solution were placed in Petri dish and left for 10 m to solidify. After the broth medium was properly solidified, plant parts were separately embedded in the medium in 3 Petri dishes. The Petri dish were closed with cover plate and kept in incubator for further growth of endophytic fungi at 25-26$^0$C for 7 days. After 7 days, growth of endophytes was noticed then they were subjected to subculture technique to get pure fungal strain [14-18].

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**Sub-culturing technique**- Broth media was prepared using PDA (Potato dextrose agar). Petri dish and PDA solution were sterilized by autoclaving for 120$^0$C for 15-20 m. Small sections of grown endophytic fungi were transferred into the medium for proper growth. Transfer of endophytic fungi were done with flame sterilized inoculating loop by streaking and kept in incubator at 25-26$^0$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. Three samples were submitted for identification by colony morphology and compared with known organism from the literature.

**Fermentation of Isolated fungi-** Fermentation of isolated fungi was carried out in 3 liters of PDB solution in an Erlenmeyer flask and incubated at 25-26$^0$C for 21 days under stationary conditions. Fungal culture was filtered through four layers of cheesecloth and homogenized at 4000 rpm to separate mycelia from broth.

**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 a separating funnel. The organic phase were separated to dryness under reduced pressure using rotary flash evaporator and weighed to constitute crude extract. All the crude fractions were weighed and calculated to know the % yield of each fractions. % yield of the chloroform fraction was found to be 4 gm, the ethyl acetate fraction 5 gm, and the n-butanol fraction 5.3 gm, respectively. Phytochemical screening and TLC studies of the above endophytic fractions were carried out, which revealed the presence of alkaloids and steroids in the chloroform fraction; tannins, flavonoids, volatile oils, and coumarins in the ethyl acetate fraction; and carbohydrates, tannins, flavonoids, and volatile oils in the n-butanol fraction [20-22]. Based on the phytochemical analysis and TLC results, chloroform, ethylacetate and n-butanol endophytic fungal fraction of *C. asiatica* were further screened for anti-alzheimer's activity [18].

**Phytochemical analysis-** Qualitative chemical analysis and thin-layer chromatographic studies were carried out to detect important phyto-constituents present in endophytic leaf fractions of *C. asiatica.*

**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).

**Acute toxicity studies-** Acute toxicity studies were carried out using Swiss albino mice (25-30 gm) by up and down /stair case method as per CPCSEA guidelines. Ethyl acetate 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. The endophytic leaf fraction of *C. asiatica* showed no toxicity up to 1000mg/kg. Hence, 50 mg and 100 mg/kg were selected to screen for antiamnesic activity in mice [19].

**Animals:** Male Swiss 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 animals had free access to food and water and were maintained under a 12:12 h light and dark cycle. All the readings were taken during the same time of the day (5-7 PM). Institutional Animal Ethics Committee (IAEC) approved the experimental protocol, and animal care was carried out as per CPCSEA guidelines, Animal Welfare Division, Ministry of Environment, Govt. of India.

**Screening models for anti-amnesic activity**

**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 maze was developed to a height of 25 cm from the floor. On the first day, each mouse was placed at the end of an 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

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90 s. The mouse was allowed to explore the maze for 10 s and then returned to its home cage. We examined memory retention 24 h after the first-day trial on the second day [20,21].

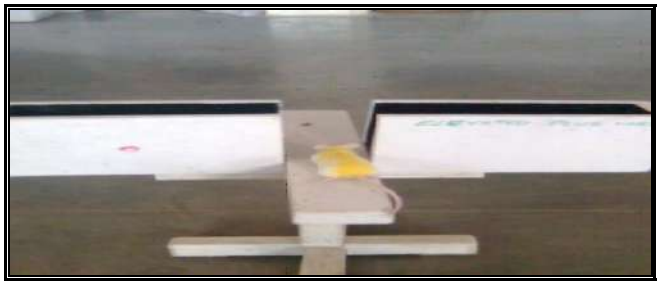


**Fig. 1:** Photograph showing elevated plus maze

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

Young groups:

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

Group 4 and 5: Endophytic fractions of ethyl acetate (50 mg and100 mg/kg) was administered. After 45 min of last dose, TL was noted on 8th and 9th day.

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

Group 8: Endophytic fractions of ethyl acetate (100 mg/kg) was 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) was 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 groups:

Group 1: Normal control, saline (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 24h, 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 24h, on 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.

Group 4 and 5: Endophytic fractions of ethyl acetate (50 and 100 mg/kg) was 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 6 and 7: Endophytic fractions of n-butanol (50 and 100 mg/kg) was administered. After 45 min of last dose, scopolamine (0.4 mg/kg, i.p) was administered. TL was noted on 8th and 9th day [22,23].

**Statistical analysis-** The data were expressed as mean ± SEM. The data were analysed using one way ANOVA followed by Tukey-kramer test. The p-value< 0.01 was considered significant [24].

**RESULTS**

Phenotypic identification was performed using colony morphology, lactophenol cotton blue staining, and sequential PCR analysis. The characteristics were compared with the known organisms from the literature and the isolated fungi was identified as *Aspergillus niger.* It has a bluish surface, with smooth conidiophores, is radiate and biseriate and can be identified by its hyaline, septate hyphae. Asexual conidiophores can be identified by being long and globose at the tip, which appears to be a hymenial layer of structures and with its own spore.

Preliminary phytochemical analysis and t.l.c studies of chloroform, ethyl acetate, and n-butanol fractions of the leaf of *C. asiatica* revealed the presence of alkaloids and steroids in the chloroform fraction, and tannins, flavonoids and volatile oils in the ethyl acetate and n-butanol fractions.



**Fig. 2:** Colony morphology of *Aspergillus niger*

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Aged mice showed higher 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 group, 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 8$^{th}$ and 9$^{th}$ day in young and aged mice ($p<0.01$) when compared to control groups. 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 8$^{th}$ and 9$^{th}$ 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 are summarized in Table 1 and 2.

**Table 1:** Effect of *C. asiatica* endophytic leaf extract on transfer latencies (TL) of young mice by elevated plus maze

| Group | Treatment | Dose(mg/kg) | TLT(8$^{th}$ day) | TLT(9$^{th}$ day) |
|---|---|---|---|---|
| 1 | Control | 10 | 28.4±13.45 | 23.5±3.57 |
| 2 | Piracetam | 200 | 25.2±4.57$^{*}$ | 18.4±6.24$^{*}$ |
| 3 | Scopolamine | 0.4 | 48.4±4.56$^{*}$ | 39.2±7.38$^{*}$ |
| 4 | EEAFCA | 50 | 44.6±4.56 | 32.4±3.67 |
| 5 | EEAFCA | 100 | 42.2±5.78 | 36.6±5.34 |
| 6 | ENBFCA | 50 | 36.4±5.67$^{a}$ | 26.8±4.89$^{a}$ |
| 7 | ENBFCA | 100 | 32.2±7.64$^{a}$ | 22.0±6.45$^{a}$ |
| 8 | EEAFCA+<br>Scopolamine | 100<br>0.4 | 38.8±4.79 | 30.0±6.25 |
| 9 | ENBFCA+<br>Scopolamine | 100<br>0.4 | 22.6±3.65$^{b}$ | 20.1±4.93$^{b}$ |

*Each group consists of 5 animals except group I (n=6). Values are mean ±SEM.$^{*}$p< 0.001 compared to control, $^{a}$p< 0.01 compared to control, $^{b}$p< 0.01 compared to control (scopolamine treated)*

**Table 2:** Effect of *C. asiatica* endophytic leaf extract on transfer latencies of aged mice by elevated plus maze

| Group | Treatment | Dose(mg/kg) | TLT(8$^{th}$ day) | TLT(9$^{th}$ day) |
|---|---|---|---|---|
| 1 | Control(Y) | 10 | 28.4±13.45 | 23.5±3.57 |
| 2 | Control(A) | 10 | 36.6±4.67$^{*}$ | 32.7±5.65$^{*}$ |
| 3 | Piracetam | 200 | 26.7±4.68$^{*}$ | 18.6±2.13$^{*}$ |
| 4 | EEAFCA | 50 | 52.6±3.60 | 39.6±1.56 |
| 5 | EEAFCA | 100 | 57.2±3.54 | 45.2±1.21 |
| 6 | ENBFCA | 50 | 39.2±1.02$^{a}$ | 28.4±1.08$^{a}$ |
| 7 | ENBFCA | 100 | 33.4±1.32$^{a}$ | 23.5±2.21$^{a}$ |

*Each group consists of 5 animals except group I and XV (n=6). Values are mean ±SEM.$^{*}$p<0.01 compared to control, $^{a}$p<0.01 compared to control (young), $^{a}$p<0.01 compared to control (aged)*

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## DISCUSSION

Acetylcholine is considered as one of the most important neurotransmitter which is involved in the maintenance of cognitive functions. There is extensive evidence of link between central cholinergic systems to memory [25]. It was observed that reduced cholinergic transmission leads to cognitive dysfunction. Loss of cholinergic neurons and decreased cholin acetyl transferase activity was found to be a characteristic feature of senile dementia of the Alzehemer's type [26]. Oxygen free radicals are involved in the process of age related decrease in cognitive function and may be cause for the development of Alzheimer's disease in elderly. Oxygen free radicals and other oxidative metabolites have been shown to be toxic to neuron and antioxidant rich food improved cerebellar physiological function and increase motor learning in older rats Antioxidant compounds of endophytic fractions of CA may favorably contribute to enhancing the memory in mice. Thus, neuroprotective effect of CA may be attributed to its antioxidant property of which susceptible neurons in the brain get exposed to less oxidative stress resulting which less damage tobrain and improved neuronal physiology. The present research indicates that the endophytic ethylacetate fractions of *C. asiatica* a potential Antiamnesic and neuroprotective agent as compared to n-butanol fraction. It also possesses nootropic activity in view of its facilitator effect on retention and acquired learning. EEAFCA (50 mg and 100 mg/kg, p.o.) decreased transfer latencies in young but more profoundly in aged mice in dose dependent manner as compared to respective control group animals. Cholinergic system plays a vital role in memory and learning [27-29]. Piracetam (200 mg/kg, i.p.) and EEAFCA (50 mg and 100 mg/kg, p.o.) on the other hand significantly ($p < 0.01$) lowered this effect indicating the counteracting action of the drugs on cholinergic system. Endophytic n-butanol fraction of CA showed profound neuroprotective activity in scopolamine treated and older mice as compared to control group.

## CONCLUSIONS

In the present study, we observed that endophytic ethylacetate leaf fractions of C. asiatica improved memory of both young and older mice as compared to n-butanol fraction, whereas scopolamine treated group lowered the memory level in mice from the study, we can conclude that endophytic fractions of C. asiatica can be effectively employed in the management of Alzheimer disease. Further work should be explored to isolate and characterize the important secondary metabolites from the endophytic fungal community of C. asiatica leaves and to know the mechanism of action responsible for anti-amnesic activity.

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## CONTRIBUTION OF AUTHORS

**Research concept –** Dr RA Shastri
**Research design –** Dr PV Habbu
**Supervision –** Dr PV Habbu
**Materials –** Dr RA Shastri
**Data collection –** Dr RA Shastri
**Data analysis and interpretation –** Dr CB Savant
**Literature search –** Dr Smita DM
**Writing article –** Dr RA Shastri
**Critical review –** Dr PV Habbu
**Article editing –** Dr VH Kulkarni
**Final approval –** Dr VH Kulkarni

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