International Journal of Pharmacy
Journal Homepage: http://www.pharmascholars.com

Original Article **CODEN: IJPNL6**

ACETYLCHOLINESTERSAE INHIBITORY POTENTIAL OF ENDOPHYTIC FUNGI INHABITING THREE INDIAN MEDICINAL PLANTS

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Received on: 04-11-2015; Revised on: 15-12-2015; Accepted on: 08-01-2016

ABSTRACT

Fungal endophytes isolated from medicinal plants viz. *Withania somnifera, Tinospora cordifolia* and *Ficus religiosa* were screened for their ability to produce acetylcholinesterase inhibitors, which are important in the treatment of various diseases and disorders related to decline in acetylcholine levels. Nine cultures were found to exhibit significant inhibitory activity (>50%). Molecular characterization of the producer isolates was carried out using ITSI-5.8S-ITSII rDNA sequencing. The generated dendrogram showed that the isolates were distributed within Ascomycota phylum and belonged to three classes *Eurotiomycetes, Dothideomycetes* and *Sordariomycetes.* Maximum inhibition (95.8%) was evinced by *Cladosporium uredinicola* isolated from *Tinospora cordifolia* after 10days of incubation on malt extract medium when diethyl ether was used as an extraction solvent. Thin layer chromatography based bioassay revealed the presence of at least two bioactive molecules. Acetycholinesterase inhibitory activity is being reported for the first time from endophytic *Cladosporium uredinicola*, *Nigrospora* sp. and *Colletotrichum* sp.

Key words: *Endophytic fungi, Acetylcholinesterase inhibitors, Cladosporium uredinicola, Tinospora cordifolia*

INTRODUCTION

Alzheimer's disease (AD) is a progressive, degenerative disease characterized by memory loss, language deterioration, poor judgment, impaired visuo-spatial skills etc. It is the most common cause of dementia among elderly. According to the cholinergic hypothesis, the memory impairment in the patients with senile dementia of Alzheimer's type results from a deficiency in cholinergic function in the brain leading to decreased levels of acetylcholine (ACh) [1].The enzyme responsible for the degradation of acetylcholine is acetylcholinesterase (AChE). Reversible inhibition of this enzyme leads to an increase in neurotransmitter concentration within the synaptic cleft, which positively affects AD disease patients [2]. This makes enzyme AChE an attractive target for rational drug design and the discovery of mechanism based inhibitors for the treatment of AD. The aim of administering acetylcholinesterase inhibitors (AChEIs) is to boost the endogenous levels of ACh in the brain of AD patients by inhibiting the activity of AChE, thereby increasing cholinergic transmission. AChEIs are the most effective approach to treat the cognitive symptoms of AD [3] and other possible therapeutic applications in the treatment of Parkinson's disease, senile dementia and ataxia etc [4]. AChEIs such as eserine, tacrine, donepezil, rivastigmine and galantamine are the drugs currently approved for the treatment of Alzheimer's disease; however, these drugs are known to have limitations for clinical use due to their low bioavailability, short half-lives and unfavorable side-effects like hepatotoxicity [5,6]. Therefore the search for novel AChEIs with better properties is necessary. AChEIs are also important as they possess insecticidal

properties and can be used as pest control agents [7]. AChEIs have been reported to be produced by some fungi, but traditionally most fungi have been isolated from soil samples. The fungi, *Aspergillus terreus* [8,9] and *Penicillium* sp. [10-12], *Chrysosporium* sp. [13] and *Xylaria* sp. [14] have been reported to produce AChEIs. Although soil fungi have provided a broad spectrum of secondary metabolites with diverse chemical structures, the most recent exciting discoveries have come from exploration of fungi living in unusual ecological niches, such as endophytic fungi which reside between and among the tissues of living plant cells. The relationship that they establish with the plant varies from symbiotic to pathogenic [15]. Some of these endophytic fungi produce various useful bioactive molecules, which has encouraged a worldwide scientific effort to isolate and study them [16-18]. Endophytic fungi, in spite of their immense potential as sources of novel and important bioactive molecules, however, have not been screened and characterized for AChEIs. There are few reports of screening of endophytic fungi for AChEIs [19,20]. Thus, keeping in view the vast potential of endophytic fungi to produce bioactive molecules, this study aimed at isolating and screening the endophytes for the production of AChEIs from three medicinal plants *Withania somnifera*, *Tinospora cordifolia* and *Ficus religiosa*.

MATERIALS AND METHODS

Isolation of endophytic fungi: For isolation of endophytic fungal cultures, plants *viz*. *W. somnifera*, *T. cordifolia* and *F. religiosa*, exhibiting the potential to produce AChEIs were used. Different samples (stems, leaves) from healthy plants were collected from Guru Nanak Dev University campus of Amritsar (India). The plant parts were thoroughly washed with distilled water, followed by treatment with 70% ethanol for 2 min and 5% sodium hypochlorite for 3 min to accomplish surface sterilization. It was again rinsed in sterile distilled water prior to plating. The samples were cut into 5-6 pieces (2-6 mm size) and were placed on potato dextrose agar (Himedia Laboratories, Mumbai) plates supplemented with chloramphenicol (200µg/ml) and incubated at 30˚C for 3-4 days to few weeks till the growth initiated. Similar procedure, but without surface sterilization, was used as a control to check for surface contaminating fungi or epiphytes. To further ensure elimination of epiphytes, the water obtained after last rinsing was also plated. The hyphal tips, that emerged from the plant parts were picked, purified and maintained on potato dextrose agar plates for further studies.

Molecular characterization of endophytic fungi:

Isolation of DNA: For DNA extraction, the fungi were grown on potato dextrose broth for 24h at 30ºC under shaking conditions (120 rpm) and the resultant mycelium was harvested by vacuum filtration and stored at -70˚C. The chilled mycelia (200 mg) was ground in 550 µl of extraction buffer (50 mmol l^{-1} TrisHCl, pH 8.0; 700 mmol l^{-1} NaCl; 10 mmol l^{-1} EDTA, 1% (v/v) β- mercaptoethanol and 10% (w/v) SDS and then 300 µl of equilibrated phenol was added. The contents were homogenized and incubated for 15 min at 65°C. The DNA in the aqueous phase was purified with repeated extractions using equal volumes of saturated phenol, chloroform, iso-amyl alcohol (PCI) mixture (25:24:1). The DNA was precipitated with 9 parts of ice cold isopropyl alcohol and 1 part of sodium acetate (3 mol l^{-1}; pH 8.0) and kept at -20°C for 2 h, followed by centrifugation for 15 min at 8000 *g*. The DNA pellet was rinsed with 70% (v/v) ethanol, air dried, suspended in 50µl of sterilized double distilled water and stored at 4°C [21].

PCR Amplification: DNA coding for internal transcribed spacers (ITSI & ITS II) and the intervening 5.8S rDNA region was amplified using universal primers, ITS1 (5' TCCGTAGGTGAACCTGCGG3') and ITS4 (5' TCCTCCGCTTATTGATATGC3'). The PCR amplification was carried out in 0.2 ml PCR tubes, using Master cycler personal (Eppendorf). The PCR reaction mixture (50 µl) contained 25 µl of PCR master mix (Genei, Bangalore, India), 2.5 µl of DMSO, 1 pmol l^{-1} of each primer and 100 ng of template DNA. Thermal cycling conditions were as follows: initial denaturation (4 min at 95°C), followed by 30 cycles of denaturation (94°C for 50 sec), annealing (51°C for 1 min), and primer extension (72°C for 1 min), followed by final extension step for 10 min at 72°C. Amplification products were electrophoretically resolved on 1.4% (w/v) agarose gel containing ethidium bromide, using 1X TAE buffer at 70 V.

Internal transcribed spacer sequence analysis: The purified amplified internal transcribed spacer (ITS) region was sequenced by single primer anaysis (SPA) services (Genei, Bangalore, India). The ITS sequences of different fungi were aligned with each other as well as the sequences retrieved from NCBI databases, using multiple sequence alignment software (CLUSTAL X). Dendrogram was generated using neighbour joining (NJ) plot and the boot strapping was carried out using 100 replications. The ITS sequences were deposited with NCBI gene bank (Table 1).

Production of cholinesterase inhibitor: Erlenmeyer flask (250 ml) containing 50 ml of liquid production medium (malt extract 20g/l, dextrose 20g/l, peptone 1g/l) was inoculated with three agar plugs (8mm diameter) taken from periphery of actively growing endophytic isolates on PDA plates. The flasks were incubated at 250 rpm on a rotary shaker at 30°C for 10 days. Thereafter, the culture broth along with the fungal biomass was extracted with 50 ml of ethyl acetate under shaking conditions at 150 rpm and 45ºC for 2 h. The upper organic phase thus obtained was separated and concentrated on rotary evaporator (BUCHI). The concentrated samples were then re-suspended in HPLC grade water for further assay. All the experiments were repeated in triplicate.

In Vitro Acetylcholinesterase Inhibition Assay: AChE inhibition assay was carried out by modified Ellman's method [22] as followed by Devkota *et al.* [23]. Acetylthiocholine iodide (ATCI) (Himedia Laboratories, Mumbai) was used as substrate to assay AChE activitiy. The reaction mixture contained 140 µl of sodium phosphate buffer (100mM, pH 8.0), 10 µl of 5, 5'- dithiobis [2- nitrobenzoic acid] (Himedia Laboratories, Mumbai) (DTNB 3mM), 20 µl of test compound solution and 20 µl of AChE from electric eel (Sigma, USA) solution, which were mixed and incubated for 10 min at 25ºC. The reaction was then initiated with the addition of 10µl ATCI (15mM). After the incubation hydrolysis of ATCI was monitored at a wavelength of 412 nm by the formation of a yellow colored 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine. All the reactions were performed in duplicate in 96-well micro-plate. The percent inhibition was calculated as: control absorbance-sample absorbance/ control absorbance X 100. Galanthamine, a known AChEI was used as positive control.

Estimation of the IC_{50} value: The test extract was serially diluted in double distilled water. The concentration of the extract at which 50% (IC_{50}) AChE inhibition occurred was evaluated.

TLC bioautography for Acetylcholine esterase inhibitor: AChE inhibitory activity was also determined using TLC assay which involves staining with Ellman's reagent (DTNB). Ten µl of each sample was spotted on the silica gel TLC plates and developed with the solvent system $CHCl_3$:MeOH:H_2O (7:3:1); 10µl of galantamine solution in HPLC grade water was also spotted as a reference. After developing the TLC plate, enzyme inhibitory activities of the developed spots were detected by spraying it with the substrate, dye and enzyme. The presence of AChE inhibitor molecule was determined by the formation of well-defined white spots made visible by spraying with DTNB, which gives a yellow background [24].

Optimization of inhibitor production: The culture (TC-9II) showing maximum AChE inhibitory activity was selected for further optimization studies. Production was carried out as previously described. The effect of media types was studied by growing the culture in 50 ml of different media broth: malt extract, Czapekdox, Sabouraud dextrose, potato dextrose and malt yeast extract glucose peptone. The flasks were incubated for 14 days to study the effect of incubation time on the production of the metabolite. The flasks were withdrawn at regular intervals of 24 h and the inhibitory activity of the crude extract was measured. The crude solution was extracted with different solvents (ethyl acetate, diethyl ether, butanol, dichloromethane and chloroform) to select the best solvent showing high extraction efficiency thus providing maximum AChE inhibitory activity. Stability of the inhibitor was checked by analyzing the inhibitory activity of the stored extract (at 4°C) at regular intervals. All the experiments were repeated in triplicate.

RESULTS AND DISCUSSION

Methanolic plant extracts of *W. somnifera, T. cordifolia* and *F. religiosa* have been found to possess AChE inhibitory activity. It is quite possible that the endophytes which are isolated from these plants may also produce the AChEIs. This has been exemplified in the case of paclitaxel which is a highly functionalized diterpenoid and famed anticancer agent that is found in Yew trees (*Taxus* spp). In 1993, a novel fungus *Taxomyces andreanae*, from the Yew *Taxus brevifolia* was isolated, capable of producing taxol. HuperzineA, an AChEI produced by a moss *Huperzia serrata* has also been found to be produced by endophytic fungi *Cladosporium cladosporides* and *Shiaria* sp. residing in it [25, 26]. This production could be due to the genetic recombination of the endophytes with host that occurs in evolutionary time [17]. Thus, if endophytes can produce the same and important bioactive compounds as their host plants, this would not only reduce the need to harvest slow growing and possibly rare plants but also preserve the world's ever diminishing biodiversity. Furthermore, a microbial source of a valued product may be easier and more economical to produce, effectively reducing its market price. So keeping this in view, in the present study endophytic fungi were isolated from

three medicinal plants (*W. Somnifera, T. cordifolia and F. religiosa)* which are reported to possess AChE inhibitory activity. A total of 75 endophytic cultures were screened for AChE inhibitors production. Nine cultures exhibited more than 50% AChE inhibition whereas 11 endophytes gave moderate inhibition (more than 30%) and 47 cultures showed negligible activity.

From *W. somnifera* 16 endophytes were screened for AChEIs, out of which only one culture (Wi-25) showed significant AChE inhibition (above 50%). 27 cultures were screened from *F. religiosa* with only 2 exhibiting 50% AChE inhibition. From *T. cordifolia* 32 cultures were screened with 6 of them possessing more than 50% AChE inhibitory activity. Maximum inhibitory activity of 95.8% was observed in a fungal strain TC-9II isolated from *T. cordifolia.* The potent cultures exhibiting inhibitory activity were identified morphologically (Figure 1) using standard taxonomic key [27] and on molecular basis using ITSI-5.8S-ITSII rDNA sequencing (Table 1). The amplified ITSI-5.8S-ITSII rDNA region of the selected isolates was sequenced and compared with the ITS sequences of organism represented in the NCBI database gene bank using BLAST search to generate a phylogenetic tree (Figure 2). The sequences that showed E=0.0 and highest % similarity with the amplified sequences were included for alignment and bootstrapping using CLUSTAL X. The generated dendrogram showed that the isolates are distributed within Ascomycota phylum and belonged to three classes *Eurotiomycetes, Dothideomycetes* and *Sordariomycetes.* The dendrogram showed all the endophytes isolated from different plants to be phylogenetically distinct as they were clubbed into different branches. The producer strains namely TC-3, TC-9II, TC-31, identified as *Cladosporium sp*. clustered together but the branches were supported by low boot strap values (>50) showing large extent of genetic evolution among these endophytes. The best producer culture TC-9II showed maximum similarity with *Cladosporium uredinicola*. Other endophytic isolates were identified as *Cladosporium cladosporioides, Cladosporium oxysporum, Nigrospora oryzae, Hemigera* sp. and *Colletotrichum gleosporoides, Aspergillus fumigatus* and *Penicillium* sp.. Some *Penicillium* sp. isolated from soil and insects have been found to exhibit AChE inhibitory activity [10, 11, 19]. Terreulactones produced by *A. terreus* have been reported to exhibit AChE inhibition [8]. But there is no report of inhibitory activity in other species of *Aspergillus*. Endophytic fungus *C. uredinicola* (TC-9II) showing maximum AChE inhibition was selected for further studies.

Optimization of medium components is of primary importance in any fermentation process to enhance the productivity. To achieve enhanced production of the AChE inhibitor, optimization of various parameters was carried out using one factor at a time (OFAT) approach. The production of AChE inhibitor was studied on five different media types: MEB (malt extract broth), CDB (Czapek dox broth), SDB (Sabouraud dextrose broth), PDB (potato dextrose broth) and MYGP (malt yeast extract glucose peptone broth). Maximum AChE inhibitory activity was obtained on malt extract broth followed by Czapek Dox broth. Other media types showed negligible inhibitory activity (Figure 3 i). Effect of incubation time on the production of metabolite was studied for 14 days. It was observed that maximal AChE inhibitory activity was exhibited on 10th day of incubation, after which it declined sharply (Figure 3ii). An attempt was made to establish the relationship of production with growth profile of the organism. Maximum biomass was obtained after six days of incubation after which it remained constant. This indicates that the inhibitory compound is a secondary metabolite produced in the stationary phase of growth and loss in activity could be due to the degradation of the compound. The effect of different solvents on the extraction of the active compound was analyzed. AChE inhibitory metabolite was obtained maximally when extraction was carried out with ethyl acetate or diethyl ether (Figure 4 i). But the stability of active compound was more in diethyl ether than ethyl acetate (Figure 4 ii). The production of the metabolite could be enhanced to give 99% inhibition by optimizing the process parameters. Zhu *et al* [25] have revealed the production of huperzine in an endophytic *Cladosporium cladosporides* isolated from *Huperzia serrata* but there is no report of the presence of AChE inhibitory activity in *C. uredinicola*. Differences were observed among isolates of endophytic fungi within the same fungus species with respect to their ability to produce metabolites with cholinesterase inhibitory activities. Similar differences in the activity of isolates of same fungal species but from different plants and plant parts have also been reported by Rodrigues *et al* [19]. Endophytic *Alternaria* spp isolated from different plants have been shown to differ in their AChE inhibitory potential [20, 28]. TLC based bioassay for the inhibitor was carried out using the method described by Rhee et al[24]. Two closely spaced white spots were observed against yellow background indicating the presence of at least two bioactive compounds (Figure 5). IC_{50} of the ethyl acetate extract was determined to be 542.2 µg/ml. AChEIs are also agriculturally important as insecticides. In another study conducted by our group, the compounds produced by Tc-9II and have also demonstrated to

possess good insecticidal potential against *Spodoptera litura*, which is a polyphagous pest of commercial crops [29]. It is known that the one of the benefits conferred by the endophytes on the host plant is providing resistance against insect pests. From this study, it can be hypothesized that AChEIs produced by endophytic fungi could be having a role in imparting resistance against insect pests.

CONCLUSION

In the present study, endophytic culture of *C. uredinicola* isolated from T. *cordifolia*, may be a potential source of novel compounds exhibiting AChE inhibitory property. The yield of these compounds can be significantly enhanced after optimization of process parameters. Endophytic fungi *C.uredinicola*, *Nigrospora* sp. and *Colletotrichum* sp. are being reported for the first time for production of AChEIs.

ACKNOWLEDGMENT

The financial support from Council of Scientific and Industrial Research (CSIR), New Delhi, for carrying out this work is duly acknowledged.

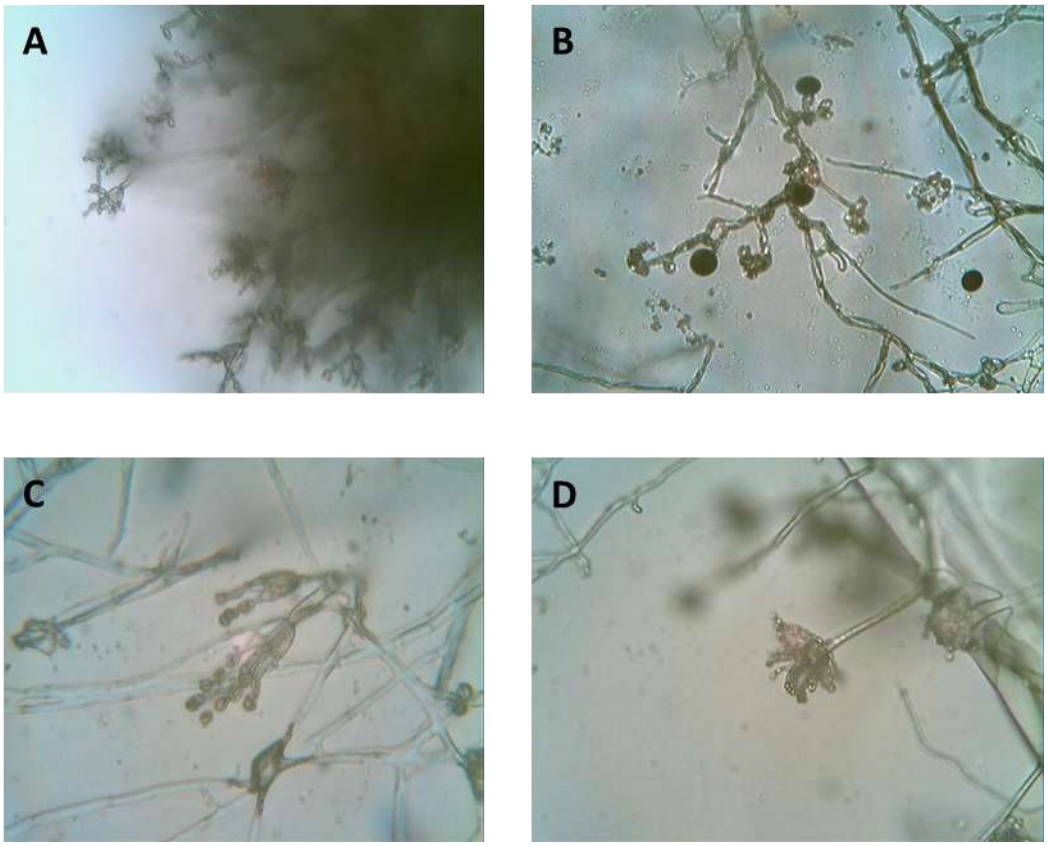

Figure 1. Colony morphology of producer isolates (A) TC-9II (*Cladosporium uredinicola)*, (B) TC-42 (*Nigrospora oryzae)*, (C) Fi-7 (*Penicillium sp*.), (D) Wi-25 (*Aspergillus fumigatus*)

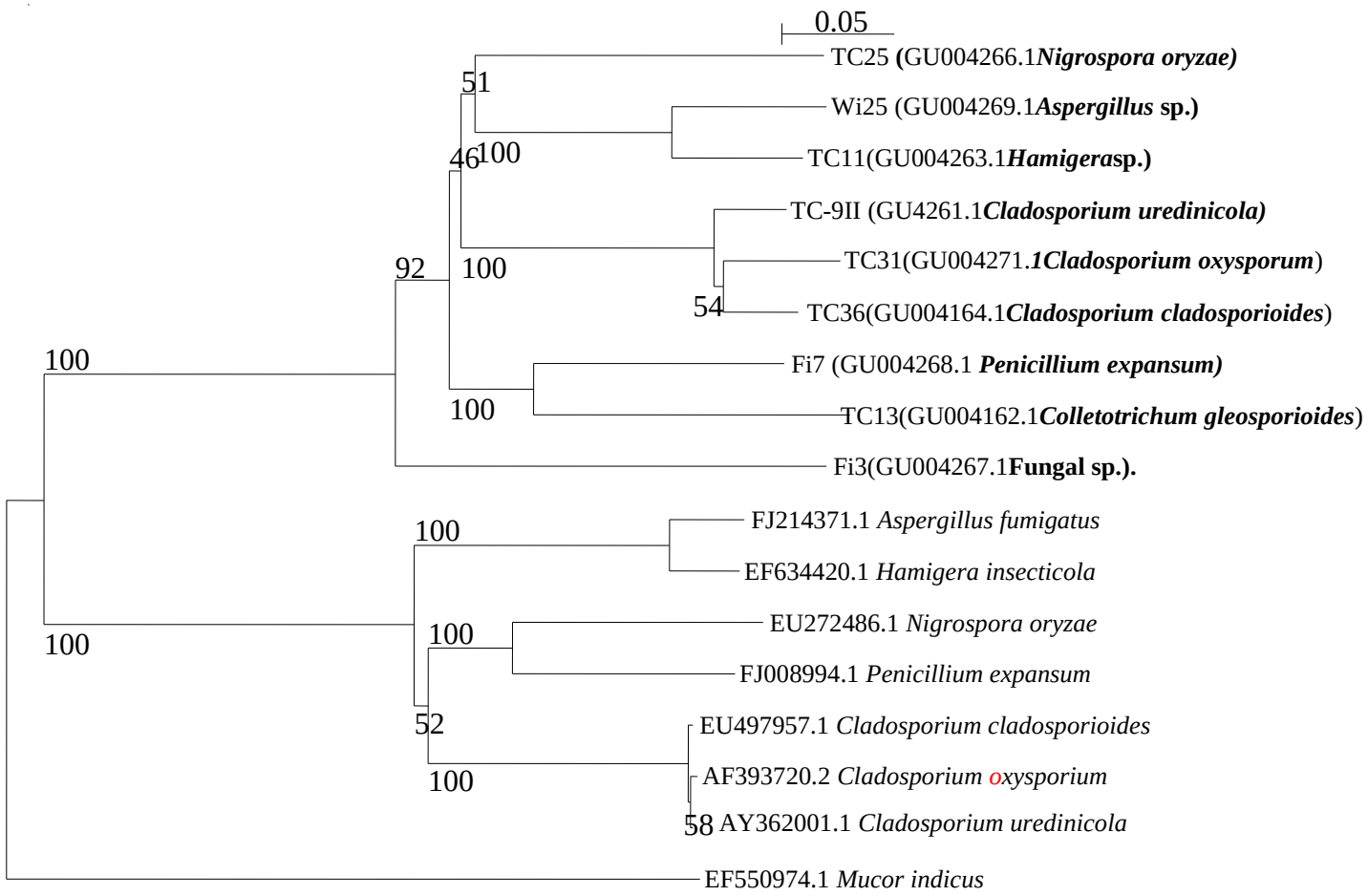

Figure 2. ITSI-5.8S-ITSII rDNA sequence based phylogenetic tree of endophytic isolates exhibiting AChE inhibitory activity. A consensus neighbour joining dendogram with bootstrap values was based on multiple aligned sequences using CLUSTAL X program.

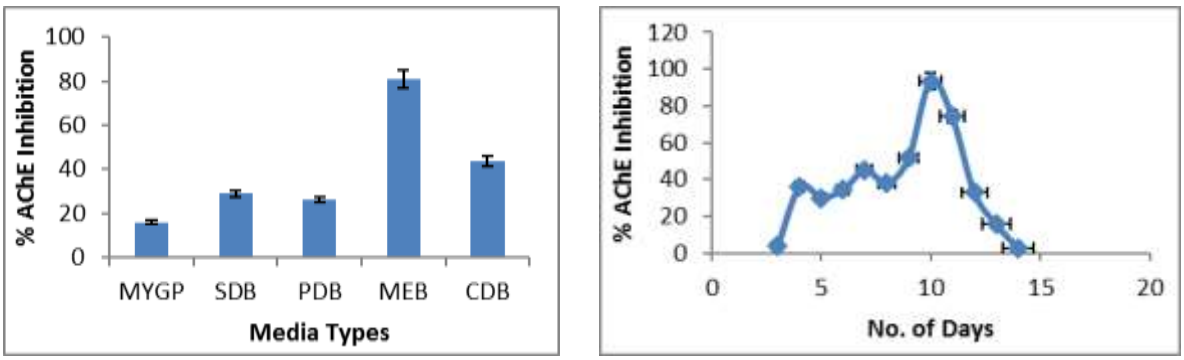

Figure 3. (i) Effect of (i) different media types **(ii)** incubation time on inhibitor production by TC-9II. Error bars indicate the standard error of mean.

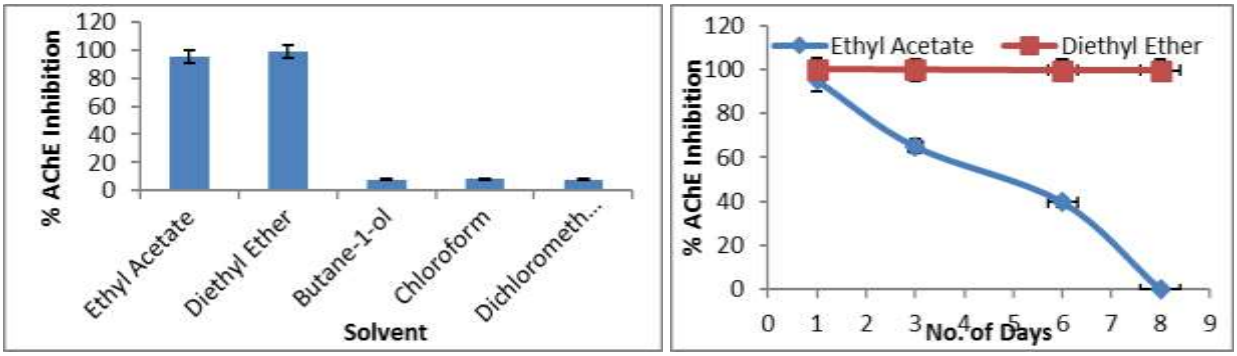

Figure 4 (i) Effect of solvents on inhibitor extraction **(ii)** stability of diethyl ether and ethyl acetate extracts of TC-9II. Error bars indicate the standard error of mean.

Lane 1 Lane 2

Figure 5 Bioautograph showing the presence of AChE inhibitor (Lane 1: extract of TC-9II, Lane 2: galanthamine (positive control).

Table 1. AChE inhibitory activity and molecular characterization of selected endophytic isolates

Plant name	Isolated culture	% AChE inhibition	Database match	Accession number
Tinospora cordifolia	TC-3	50.3	*Cladosporium cladosporioides*	GU004264.1
Tinospora cordifolia	TC-9II	95.8	*Cladosporium uredinicola*	GU004261.1
Tinospora cordifolia	TC-11	54.91	*Hamigera* sp.	GU004263.1
Tinospora cordifolia	TC-13	51.89	*Colletotrichum gloeosporioide*	GU004262.1

Tinospora cordifolia	TC-25	54.45	*Nigrospora oryzae*	GU004266.1
Tinospora cordifolia	TC-31	49.80	*Cladosporium oxysporum*	GU004271.1
Ficus Religosa	Fi-3	50.57	*Fungal sp. YX-3*	GU004267.1
Ficus religosa	Fi-7	50.54	*Penicillium expansum*	GU004268.1
Withania somnifera	Wi-25	56.06	*Aspergillus fumigatus*	GU004269.1

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