Isolation and identification of endophytic fungi with acetylcholinesterase inhibitory potential from *Bacopa monnieri*

A thesis submitted in partial fulfillment of the requirement for

the award of the degree of

MASTERS OF SCIENCE

IN

BIOTECHNOLOGY

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CERTIFICATE

I hereby declare that the work that has been presented in this thesis **"Isolation and identification of endophytic fungi with acetylcholinesterase inhibitory potential from *Bacopa monnieri*"** submitted by **Ananaya Bharat** in partial fulfillment of the requirement for the award of the degree of Masters of Science in Biotechnology, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab, is a record of student's own work carried out under my supervision. This report has not been submitted for the award of any other degree or certificate in this university or any other university.

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DECLARATION

I **Ananaya Bharat**, hereby declare that the work that has been done and presented in this thesis **"Isolation and identification of endophytic fungi with acetylcholinesterase inhibitory potential from *Bacopa monnieri*"** submitted in partial fulfilment of the requirement for the award of the degree of Masters of Science in Biotechnology, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab, is a record of my own original research work done under the supervision of **Dr. M. Vasundhara** during the period of January 2023 to July 2023. This report has not been submitted for the award of any other degree or certificate in this university or any other university.

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LIST OF ABBREVIATIONS

Abbreviation	Name
%	Percentage
L	Litre
Ml	Millilitre
Mg	Milligram
µg	Microgram
µl	Microlitre
°C	Degree Celsius
Cm	Centimeter
A	Absorbance
AA	Ascorbic Acid
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
Ach	Acetylcholine
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase inhibition
DPZ	Donepezil
pH	Potential of Hydrogen ion
sp.	Species
Rf	Retention factor
TLC	Thin Layer Chromatography
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
HRMS	High resolution mass spectrometry
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
FTIR	Fourier-transform infrared spectroscopy

Abstract

Endophytic fungi are the source of potent bioactive compounds resides in medicinal plants with various bioactivities like anti-acetylcholinesterase, antifungal, antibacterial, and antioxidant etc. In the current study, the medicinal plant *Bacopa monnieri* was selected for the isolation of the endophytic fungi. A total of seven endophytic fungi isolated from *B. monnieri*. The crude extracts from these fungal isolates have been evaluated for preliminary acetylcholinesterase inhibitory and antioxidant activities. Among the seven crude extracts the maximum acetylcholinesterase inhibitory activity of 64.10% was shown by the fungal extract BM-16 at 250 µg/ml followed by BM-6, with inhibitory activity of 60% at 125 µg/ml, in comparison to DPZ, which was used as a reference compound. The fungal crude extracts were also tested for antioxidant activity. Among these 7 selected fungal isolates, BM-6 showed maximum antioxidant activity of 75.51% and BM-16 58.29 % at 250 µg/ml. The fungal extract BM-6 revealed the presence of condensed tannins, alkaloids, flavonoids, steroids, glycosides, and triterpenoids, and the fungal extract BM-16 showed the presence of alkaloids, steroids, glycosides, saponins, and triterpenoids. BM-6 and BM-16 were selected for further analyses. TLC was done by selecting an appropriate solvent system. Column chromatography was also performed for the selected fungal isolates. After purification via column chromatography, total of 11 fractions obtained, 4 from BM-6 and 7 from BM-16. Out of these fractions, fraction F-4 from fungal extract BM-6 and fractions F-6 and F-7 from fungal extract BM-16 showed maximum acetylcholinesterase inhibitory activity of 61.75%, 71.70%, and 73.17% at 160 µg/ml of concentration, respectively. Out of these 3 selected fractions, fraction F7 of BM-16 showed the maximum acetylcholinesterase inhibitory activity of 73.17%. The same fraction was analyzed using UV-spectroscopic, HRMS and FTIR analyses. Endophytic fungus BM-16 was identified through microscopic and molecular techniques and it was observed that BM-16 showed morphology similar to *Alternaria tenuissima*. Molecular and phylogenetic analysis confirmed that BM-16 was *Alternaria tenuissima*.

Introduction

Brain is a complex organ, and diseases that affect its functioning are known as neurological or neurodegenerative disorders. Neurodegenerative illnesses can be difficult to diagnose precisely, and typically the doctor will base their decision on symptoms that have been present throughout life (Holzgrabe *et al.,* 2007). Currently, neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, Down syndrome, myasthenia gravis, etc. are serious concerns for human health, especially in the elderly people (Zaki *et al.,* 2020) all around the world. Beyond their distinct pathogenic characteristics, the cholinergic system's degeneration emerged as a common pathological feature in neurodegenerative illnesses, and AChE gained significant interest as a probable common therapeutic target (Holzgrabe *et al.,* 2007). Studies have shown an increase of 117% in cases of dementia from 1990 to 2016. In 1990, the number of people suffering from dementia was 20.3 million, whereas in 2016, it had increased to 43.8 million. By 2050, it is estimated that around 152 million people will suffer from Alzheimer's disease and other brain-related disorders (Li *et al.,* 2022). Alzheimer's disease (AD) is the most common neurodegenerative disorder of the central nervous system and can be seen more prominently in elderly people around 60–65 years of age. But in a few cases, it can also have an impact on people younger than 60 (generally in their 40s or 50s) and is referred to as early-onset Alzheimer's disease. Early-onset Alzheimer's disease is further categorized into two categories: genetic Alzheimer's disease and common Alzheimer's disease. Genetic Alzheimer's disease is a very rare ailment in which people have some genes that are directly responsible for the disease and generally show its symptoms in the early 30s to 50s. On the other hand, common early-onset Alzheimer's disease is the same as what can be seen in elderly people over 65 (Johns Hopkins Medicine). According to the World Health Organization (WHO), AD alone accounts for about 65–70% of overall cases of dementia. Smoking, extensive alcohol intake, physical inactivity, hypertension, and air pollution are some major risk factors for dementia (Livingston *et al.,* 2020). The first indication is the contemporary memory ailment, which is followed by persistent cognitive impairment, loss of reasoning abilities, dysfunction in movement, etc. Cholinergic nerve injury is considered the most accepted assumption for this disease; although the pathological process is not very clear (Wang *et al.,* 2015).

The slow loss of learning ability and memory functions due to the decreased production of the neurotransmitter acetylcholine is considered one of the known or accepted mechanisms of Alzheimer's disease (AD) (Lima *et al.,* 2018). The enzyme acetylcholinesterase (AChE) is involved in this process, and the inhibition of this enzyme fulfills the approach for the treatment of Alzheimer's disease (Mukherjee *et al.,* 2007). Acetylcholine (ACh) is an important neurotransmitter that plays a key role in the peripheral nervous system as well as in the central nervous system. In the peripheral nervous system, nerve cells release the neurotransmitter acetylcholine, which binds to receptors and eventually leads to muscle contraction. In the central nervous system, acetylcholine is associated with memory-related functions (Santillo and Liu 2015). Acetylcholinesterase (AChE) is a secretory carboxyl esterase enzyme particularly present at postsynaptic neuromuscular junctions, mainly in nerves and muscles (Su *et al.,* 2017).

The AChE inhibition might guard against the detrimental consequences of beta-amyloid aggregation and its neurotoxicity. In accordance with the amyloid-beta hypothesis, which states that neuropathology related to AD incorporates reduced analytical or cognitive functioning, mitochondrial ailment, intemperate (uncontrolled) energy metabolism, and neuronal cell death, this corresponds with the extracellular accumulation of amyloid-beta (Aβ) and its corresponding neurotoxicity (Althobaiti *et al.,* 2022). A report from Johns Hopkins Medicine says that there are two proteins that are responsible for AD. The first one is beta-amyloid, whose fragments are known as plaques. The other one is tau protein with twisted fibers known by the name tangles. These proteins lead to impairment of the nerve cells, which ultimately causes the death of the nerve cells.

Researchers are also concentrating on herbal or traditional remedies due to the natural origin of the compounds obtained from the medicinal plants, their diversity of applications, and lack of adverse effects compared to contemporary synthetic medication. Different herbal treatments are recognized in Ayurveda as successful treatments for numerous brain-related illnesses. The detailed acetylcholine release pathway and cholinergic postulation of AD with different stages is shown in the Fig 1. (1) Nerve impulses that cause Ca2+ invasion and synaptic vesicle membrane docking; (2) Gradual depolarization in post synaptic cells is caused by the neurotransmitter acetylcholine binding to receptors; (3) Acetylcholine is hydrolyzed into choline and acetate molecules by the enzyme acetylcholinesterase and then reabsorbed by the presynaptic neurons (Stanciu *et al.,* 2020).

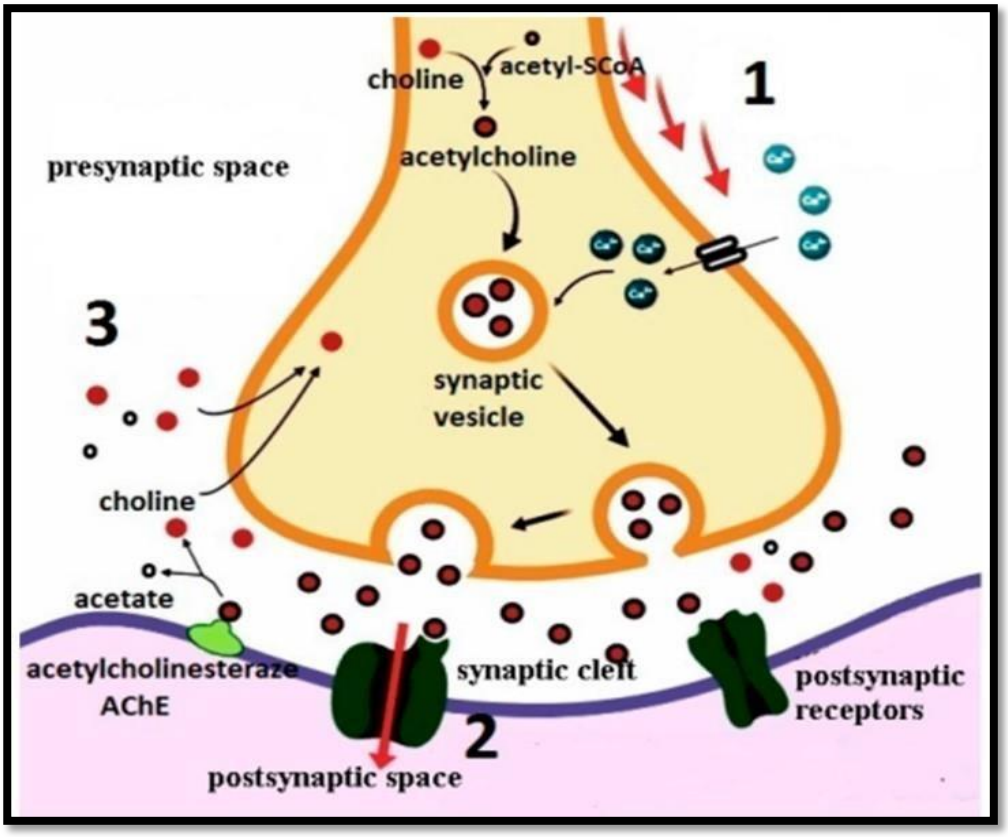


Fig 1: Acetylcholine release pathway and cholinergic postulation of AD with different stages (Stanciu *et al.,* 2020)

The conventional medicinal system has remarkably contributed to the contemporary pharmaceutical industry (Xu *et al.,* 2020). But the adverse effects of the same are already known. In the present studies the importance of natural products obtained from a wide range of medicinal plants are considered as one of the incredible source of coverted compounds with therapeutic potential that plays main role in contemporary drug research and development processes (Xiao *et al.,* 2022). The literature survey has illustrated that there are few synthetic drugs in the market for the management of mild dementia authorized by the FDA, like galantamine, donepezil (DPZ), memantine, and rivastigmine, which are being used as acetylcholinesterase inhibitors. Earlier, tacrine was being used as an acetylcholinesterase inhibitor for the treatment of AD, but due to its neurotoxicity, it has been no longer in use since 2013 (Marucci *et al.*, 2020).

So, there is a need to look over the new drugs extracted from natural products from different medicinal plants that can act as acetylcholinesterase inhibitors in order to improve their

efficiency and reduce their side effects, as we are well aware that these synthetic drugs are less efficient, less reliable, and might have a wide range of side effects too (Vig *et al.,* 2018).

Numerous medicinal plants generate a diverse array of secondary metabolites, some of which inhibit the activity of the neurotransmitter acetylcholinesterase and maintain its level within the brain. These natural acetylcholinesterase inhibitors are effective therapeutic agents for treating a variety of neurodegenerative diseases. Many theories contend that endophytes, which have long coevolved with host plants, ingest plant DNA into their own genomes. According to Lu *et al.,* (2002), plant endophytes are microorganisms (fungi, bacteria, or actinomycetes) which reside within the host plant and establish a mutually beneficial partnership with it over time or their entire life span. As a result of this adaptation and genetic variation, endophytes may have acquired the capacity to synthesize phytochemicals that had been linked to the host plant. The ability of the endophyte to synthesize related plant compounds results from horizontal gene transfer. The ability of endophytes to synthesize depends on the interaction between the host and the endophyte. Under stressful conditions, endophytes affect the host plant's capacity to create chemicals that defend it from invasive pathogens. The capacity of endophytes to synthesize variety of novel substances also rises in this hostile environment. Environment thus influences the host plant, which in turn influences the endophyte to alter its metabolic profile and increase the physiological activity of its secondary metabolites overall (Selim *et al.,* 2012; Kaul *et al.,* 2017). Endophytic fungi provide multiple benefits to the host. These protect their hosts from transmissible agents, promote growth, hold out against adverse conditions, and strengthen their resilience to biotic and abiotic stresses by producing active or secondary metabolites (Sivaramakrishnan *et al.,* 2015). In contrast to mycorrhizal fungi, which establish a colony inside plant and tend to spread into the rhizosphere, endophytic fungi only exist within host tissues and become apparent after host senescence (Kaul *et al.,* 2017). According to Vasundhara et al. (2016), a number of secondary metabolites produced by endophytic fungi have the potential to be antioxidant, antibacterial, anticancer, cytotoxic, and AChE potent chemical constituents.

Ayurvedic physicians have valued *B. monnieri* as a reviving herb. It is categorized as a medhyarasayana, a medhya (drug) used to enhance cognition and memory. Since the sixth century AD, the herb has been mentioned in numerous ancient literature of Ayurveda, such as the "Charaka Samhita," where it is advised in formulations for the management of a variety of mental conditions, including anxiety, poor cognition, and lack of concentration, as well as a diuretic and an energizer for the nervous system and the heart. The management of

Asthma, insanity, and epilepsy are some specific applications. The plant has been widely used to enhance learning, memory, and lung function, as well as a digestive aid. The plant is a little creeper from the Scrophulariaceae family with multiple branches, tiny oblong leaves, and tiny white or light purple flowers (Gohil and Patel 2010). Also, it is known to amplify long-term potentiation (LTP) and can have a definite impact on neurological disorders (Kulkarni *et al.*, 2021). In the present study, endophytic fungi has been isolated and screened for the production of AChE inhibiting compounds from medicinal plant *B. monnieri*. The potent compound has been analyzed via different techniques such as UV, HRMS and FTIR.

OBJECTIVES

1. **Isolation and screening of endophytic fungi for the production of bioactive compounds for AChE inhibition from *Bacopa monnieri***
2. **Extraction, purification and characterization of crude extract from selected endophytic fungi**
3. **Identification of the selected endophytic fungi**

REVIEW OF LITERATURE

2.1 Neurodegeneration

Neurodegeneration refers to the neural cell death caused by the slow and progressive loss of neural structure and functioning, which gives rise to different types of neurodegenerative disorders that result in motor or cognitive deterioration. Mild motor and cognitive changes seen in the elderly are similar to those seen in neurodegeneration. This finding gave rise to the widely held hypothesis that ageing may be a "benign" form of neuronal degeneration. This theory was backed up by the previously commonly held belief that normal ageing, like neurodegeneration, is invariably accompanied by the death of neurons (Serge *et al.*, 2003). Neurodegenerative disorders are age-dependent disorders that are more prevalent in elderly people of age above 65 and represent a major concern for human health (Gitler *et al.*, 2017). Inflammation, cell death, and elevated oxidative stress prevail during depressive disorders and neurological conditions (Justyna *et al.*, 2021). Some of the commonly seen neurodegenerative disorders are shown in Fig 2.

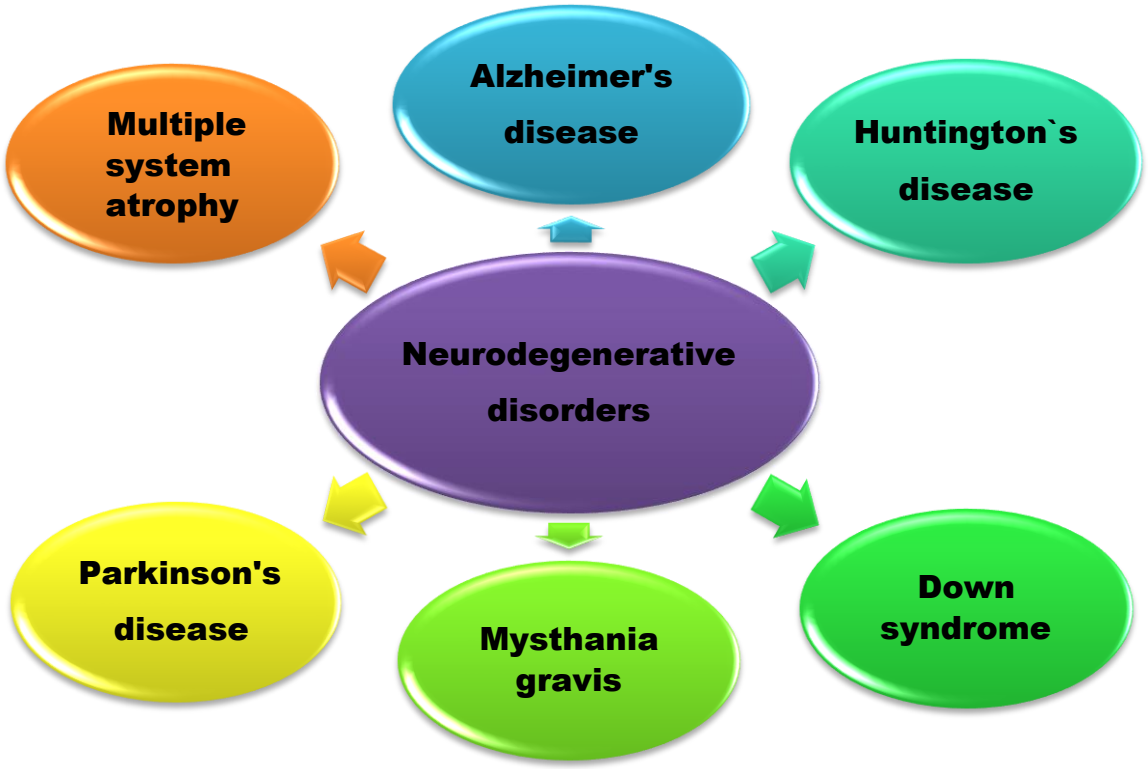


Fig 2: Outline of neurodegenerative disorders

These neurodegenerative diseases are linked to insufficient production of neurotransmitters like acetylcholine due to an increase in the amount of enzymes like acetylcholinesterase, followed by inflammation and oxidative stress in the central nervous system. Some of the frequently seen symptoms of neurodegenerative disorders are poor memory, anxiety, apathy, forgetfulness, agitation, mood swings, etc. Different studies have shown that altered RNA processing has also become apparent in recent times by including model systems as a crucial factor in a number of neurodegenerative diseases (Gitler *et al., 2017*).

2.1.1 Acetylcholinesterase inhibition

Acetylcholinesterase inhibitors (AChEI) are chemical compounds that are considered among the most preferred therapeutic tools for many neurodegenerative disorders like Alzheimer's disease (AD), Huntington's disease, Parkinson's disease, myasthenia gravis, Down syndrome, etc. (Amira G. Zaki 2020). The rise in the proportion of the enzyme acetylcholinesterase has an impact on the production level of acetylcholine and is the main reason behind the shorter half-life of the neurotransmitter acetylcholine. Acetylcholine is a neurotransmitter of the central nervous system and has a principal effect on the motor neurons associated with memory formation and functioning (Vig *et al.,* 2021).

In the central nervous system and peripheral nervous system, the enzyme acetylcholinesterase (AChE) is responsible for the hydrolysis of the neurotransmitter acetylcholine at cholinergic synapses into inactive compounds like choline and acetic acid (Bhagat *et al.,* 2016; Chapla *et al.,* 2020). For intensification of memory and analytical functions in patients suffering from neurodegenerative disorders, AChE inhibitors are regarded as well-regulated therapeutic agents (Amira G. Zaki 2019).

Certain fungus found in soil can create cholinesterase inhibitors. In particular, acetylcholinesterase and butyrylcholinesterase inhibition can be seen in extracts made by soil isolates of *Penicillium citrinum*, *Penicillium sp*., and *Aspergillus terreus* (Kuno *et al.,* 1996; Kim *et al.,* 2001, 2003; Rodrigues *et al.,* 2005). *Huperzia serrata,* a traditional Chinese medicine plant, naturally contains a substance called hupA, a lycopodium alkaloid. After being found to be a potent cholinesterase inhibitor, HupA became the focus of many researchers and has received a lot of attention (Ishiuchi *et al.,* 2013). Table 1 shows the list of some natural AChE inhibitors that have been reported from different medicinal plants. HupA

is a more effective reversible inhibitor when compared to the popular and frequently used AChE inhibitors (Zaki *et al.,* 2020).

Table 1: List of some natural Acetylcholinesterase inhibitors

S.No.	Compound name	Source	Reference
1.	Bacoside-A3	*B. monnieri*	Logesh and Sathasivampillai 2023
2.	Protopine	*Corydalis ternate*	Kim *et al.,* 1999
3.	Harmol	*Peganum nigellastrum*	Zheng *et al.,* 2009
4.	Palmatine	*Coptis chinensis*	Mak et *al.,* 2014
5.	Berberine	*Coptis chinensis*	Mak *et al.,* 2014
6.	Quinizarin	*Rubia cordifolia*	Zengin *et al.,* 2016
7.	Purpurin	*Rubia cordifolia*	Zengin *et al.,* 2016
8.	Galantamine	*Galanthus caucasicus*	Santos *et al.,* 2018
9.	Huperzine-A	*H. elmeri*	Santos *et al.,* 2018
10.	Quercetin	*Tinospora cordifolia*	Vig *et al.,* 2021

2.2 Endophytes

The term endophytes were given by De Bary in 1866 (Al-Qaralleh *et al.,* 2021). Endophytes are a common term used for both bacteria and fungi. Endophytic fungi are the one that resides inside the host plant by establishing a symbiotic alliance with the host plant without causing deleterious consequences to the plant. These endophytic fungi are known to produce different categories of secondary metabolites or bioactive compounds which provide plant the defense mechanism against various biotic and abiotic stresses and also help the host plant to adapt to the changing environmental conditions (Khan & Ali 2015).

The microbes are transmitted via seeds are typically part of a symbiotic relationship, but those that transmitted by vertical modes live in conflict with their hosts (Aly *et al.,* 2011).Various studies have shown the presence of different types of bioactive compounds in

plants produced by a variety of endophytic fungi, such as alkaloids, carbohydrates, lipids, glycosides, fats, volatile oils, terpenoids, terpenes, etc.

Endophytes hold very much importance in today's world, as it is a well-known fact that since ages, fungi have been considered a rich source of different pharmaceutical compounds with therapeutic applications including immunosuppressive, antioxidant, antimicrobial, acetylcholinesterase inhibitors (AChEI), antitumor agents, etc. (Rodrigues *et al.,* 2005). According to a study, out of 1.5 million fungal species, the number of familiar species is still around 1 lakh (Kaul *et al.,* 2017).

2.2.1 Endophytic fungi isolated from medicinal plants

Medicinal plants have been used to cure a wide range of diseases for ages. Researchers around the world have been inspired to investigate medicinal plants for endophyte isolation. This is due to the significance of secondary metabolites in pharmaceuticals derived from medicinal plants and their effect on the biosynthetic capability of endophytes. Endophytes are known to imitate the bioactive compounds produced by the host plant. Therefore, it is essential to bioprospect endophytes from medicinal plants, which have been a source of important bioactive compounds for centuries. Endophytes isolated from medicinal plants have the potential to result in the boundless and cost-effective extraction of desired compounds, thereby conserving biodiversity. Endophytes from medicinal plants are thought to be the source of compounds with diverse biological actions. Endophytic fungi isolated from medicinal plants live in an area of interest where they interact with several microbial groups, and diverse interactions occur between these microbial groups (Kaul *et al.,* 2017). A list of endophytic fungi isolated from different medicinal plants has been shown in table 2. These phytochemicals are widely used as raw materials for the pharmaceutical, cosmetic, and perfumery industries (Soni *et al.,* 2021). Different studies are going on to explore more new medicinal plants for the isolation of novel compounds and their residing endophytes. This will further help to treat those diseases whose treatment is still not known (Harman *et al.,* 2019).

Small molecules that are not necessary for typical growth or development are referred to as secondary metabolites. Despite the fact that endophytes are unable to replicate an enormous number of compounds in vitro, it is intriguing to note that under regulated fermentation ailments, the endophytes can be streamlined for the synthesis of additional naturally

occurring compounds by changing the observable culture and proceeding factors (media composition, air circulation, pH, time of incubation, shaking conditions, inoculum size, etc.). Endophytes isolated from medicinal plants may result in the cost-effective production of preferred compounds and therefore help to preserve biodiversity (Kaul *et al.*, 2017). Some major bioactivities shown by endophytes are given below in Fig 3**:**

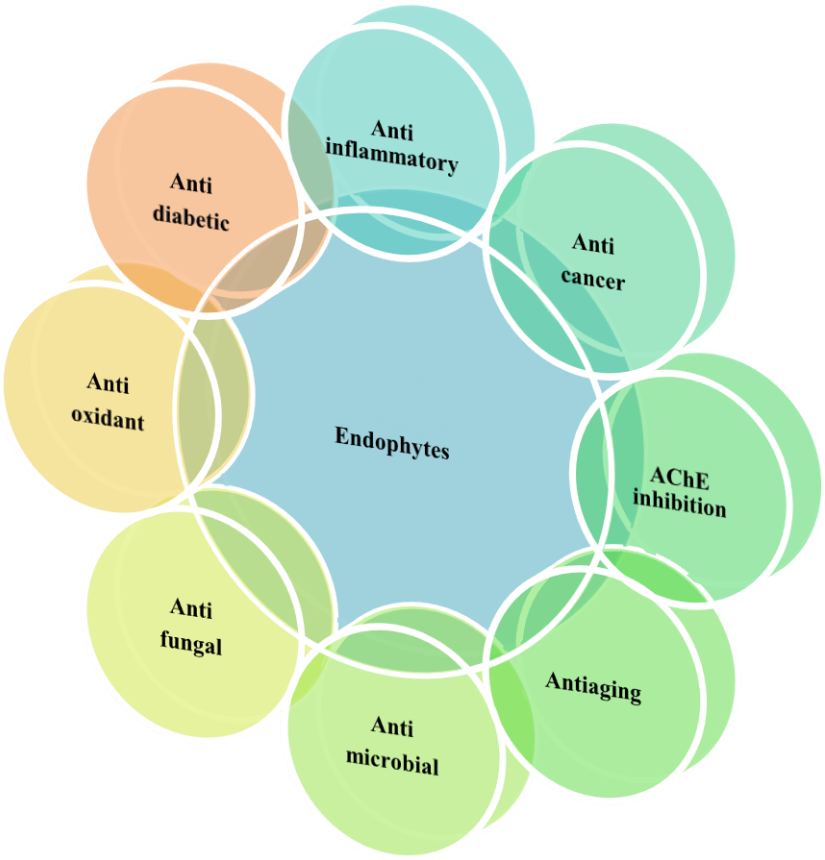


Fig 3: The major reported bioactivities of the endophytic fungal extracts

Table 2: Endophytic fungi isolated from different medicinal plants

S.No.	Endophytic fungi	Medicinal plant	Activity	Reference
1	*Nigrospora oryzae*	*Tinospora cordifolia*	AChE inhibition Antioxidant	(Vi g *et al.*, 2021)
2	*Alternaria alternate*	*Catharanthus roseus*	AChE inhibition Antioxidant Insecticidal	(Bhagat *et al.*, 2016)

3	*Fusarium sp.*	*Euphorbia sp*.	Neuroprotective Antioxidant Antimicrobial	(Al-Qaralleh *et al.* , 2021)
4	*Trichoderma sp.*	*B. monnieri*	Cytotoxic Antimicrobial	(Katoch *et al.*, 2014)
5	*Phaeosphaeria sp.*	*Huperzia serrata*	AChE inhibition	(Xiao *et al.*, 2022)
6	*Curvularia sp.*	*Rauwolfia macrophylla*	AChE inhibition Antioxidant Antimicrobial	(Kaaniche *et al.*, 2019)
7	*Saccharicola sp.*	*Eugenia jambolana*	AChE inhibition Antifungal	(Chapla, *et al.*, 2020)
8	*Paecilomyces sp.*	*Nicotiana tabacum L.*	AChE inhibition	(Xu, *et al.*, 2020)
9	*Alternaria brassicae*	*Huperzia serrata*	AChE inhibition	(Zaki et al., 2021)
10	*Aspergillus sp.*	*Limonia acidissimaa*	Cytotoxic	(Siriwardne *et al.*, 2015)
11	Not reported	*B. monnieri*	Antidiabetic Antioxidant	(Ghosh *et al.*, 2006)
12	Not reported	*Polygonum multiflorum*	Antioxidant Antiaging Antiarteriosclerosis	(Li *et al.*, 2017)
13	*Aspergillus terreus*	*Corchorus olitorius*	Extracellular enzymes	(Ahmed *et al.*, 2016)
14	*Penicillium oxalicum*	*Cupressus torulosa*	Antimicrobial Antidiabetic	(Bisht *et al.*, 2016)
15	*Aspergillus fumigates,Fusarium sp.*	*Gracinia spp.*	Anti-inflamatory Antimicrobial Antioxidant	(Ruma *et al.*, 2013)
16	*Curvularia lunata*	*Cymbopogon caesius*	Antimicrobial potential	(Avinash *et al.*, 2015)

17	*Penicillium sp.*	*Ginkgo biloba*	Antioxidant	(Yuan *et al.*, 2013)
18	*Claviceps purpurea*	*Mimosops elengi*	Anti-inflammatory Anticancer	(Deshmukh *et al.*, 2009)
19	*Lasiodiplodia theobromae*	*Morinda citrifolia*	Anticancer	(Pandi *et al.*, 2011)
20	*Arisaema erubescens*	*Phoma sp.*	Antitumor Antimicrobial	(Wang *et al.*, 2012)

2.3 Medicinal plant

Brahmi (***B. monnieri*****):** *B. monnieri* (L.) is an important medicinal herb (creeper) shown commonly known as Brahmi (Fig 4) (Mehta et al., 2022) and an adherent of the family Scrophulariaceae used in indigenous medicinal preparations for the treatment of neurodegenerative disorders and to strengthen memory and boost brain functions (Jasim et al., 2017). It is widely used in ayurveda (the Indian system of traditional medicine) for the treatment of many diseases like anxiety, poor memory, neurosis, psychosis, epilepsy, and the renaissance of sensory organs (Soni *et al.*, 2021). Bacoside A and A3 are the bioactive compounds present in *B. monnieri* that show inhibitory potential against the enzyme acetylcholinesterase. AChE is the enzyme responsible for the catabolism of Ach. The reduction in the level of beta-amyloid by *B. monnieri* in the brain gives the idea of mechanisms of action applicable to Alzheimer's disease. The reason behind the selection of this plant in this study is because it is the source of many important phytoconstituents and because of its importance in the treatment of brain-related diseases (Garg *et al.*, 2015). The phytoconstituents obtained from *B. monnieri* have shown different bioactivities like acetylcholinesterase inhibition (AChE) activity, β- amyloid reduction, antioxidant neuroprotection, antibacterial, antifungal, neurotransmitter modulation, cardio-tonic properties, increased cerebral blood flow, etc. (Jeyasri *et al.*, 2020). Bacosides are the main components of *B. monnieri* and are characteristic triterpenoid saponins. Bacosides have a wide range of functions, as they can be used as acetylcholinesterase inhibitors, memory enhancers, anti-inflammatory, anticancer, antiulcer, antiepileptic, antidiarrheal, and also in the treatment of anxiety and depression (Banerjee *et al.*, 2021). Classification of *B. monnieri* is given below in table 3.

Fig 4: *B. monnieri*

Table 3: Classification of *B. monnieri*

S.No.	**Classification**	***B.monnieri***
1	Kingdom	Plantae
2	Division	Magnoliophyta
3	Class	Magnoliopsida
4	Order	Lamiales
5	Family	Scrophulariaceae
6	Genus	*Bacopa*
7	Species	*monnieri*

2.3.1 Chemical constituents: Drug manufacturers spend a lot of money trying to find phytoconstituents, which are potentially helpful substances that can be used to treat a variety of illnesses. This is the rationale behind why a number of plants have been chosen based on their historical applications. One of these plants, *B. monnieri* (Brahmi; family: Scrophulariaceae), has long been used to treat mental health conditions. In India's marshy regions, Brahmi is frequently grown (Garg *et al.,* 2015). *B. monnieri* is known to produce wide range of phytoconstituents with pharmaceutical importance (Table 4).

Table 4: List of some phytoconstituents obtained from *B. monnieri*

S. No	Compounds
1	Nicotine
2	D-Mannitol
3	Bacoside A
4	Bacopasaponin A
5	Bacopasaponin B
6	Bacopasaponin C
7	Bacopasaponin D
8	Bacopasaponin E
9	Bacopasaponin F
10	Bacopasaponin G
11	Bacopaside I
12	Bacopaside II
13	Bacopaside III
14	Bacopaside IV
15	Bacopaside V
16	Bacopaside VIII
17	Bacopaside XII
18	Plantainoside B
19	Betulinic acid
20	Cucurbitacin A

21	Cucurbitacin B
22	Cucurbitacin C
23	Cucurbitacin D
24	Cucurbitacin E
25	Stearic acid
26	Rosavin
27	3,4Dimethoxycinnamic acid
28	Ascorbic acid
29	Asiatic acid
30	Brahmic acid
31	Wogonin
32	Oroxindin
33	Loliolide
34	Stigmasterol
35	β-sitosterol
36	Ebelin lactone
37	Stigmastanol
38	Bacosterol
39	Bacosine
40	Heptacosane
41	Octacosane
42	Nonacosane

43	Triacontane
44	Hentriacontane
45	Dotriacontane
46	Apigenin
47	Quercetin
48	Ursolic acid
49	Luteolin
50	Asiaticoside
51	Bacopaside VI
52	Bacopaside VII

2.3.2 Endophytic fungi isolated from *B. monnieri:* Researchers have been working for years to explore endophytic fungi from the *B. monnieri* plant, and a number of fungal isolates have been isolated from *B. monnieri* (Table 4).

Table 5: List of Endophytic fungi obtained from *B. monnieri*

S. No.	Endophytic Fungi	Medicinal Plant	References
1	*Nigrospora oryzae, Alternaria alternate, Aspergillus terreus*	*B. monnieri*	(Soni *et al.,* 2021)
2	*Aspergillus fumigatus*	*B. monnieri*	(Thakur *et al.,* 2015)
3	*Klebsiella aerogenes*	*B. monnieri*	(Shukla *et al.,* 2022)
4	*Trichoderma sp.*	*B. monnieri*	(Katoch *et al.,* 2014)

Material and Methods

3.1 Collection of samples

Stems and leaves of *B. monnieri* were collected from TIFAC-CORE, Thapar Institute of Engineering & Technology, Thapar Technology Campus, Patiala, India.

3.2 Surface disinfection of the samples

The collected samples (stem and leaves) from *B. monnieri* were washed thoroughly by giving them an initial wash under running tap water for 10 minutes to remove the dust and debris from the surface. After that, the samples were surface disinfected by immersing in 70% ethanol for 1 minute and then in 1% sodium hypochlorite solution for 1 minute, followed by washing with 3 sets of sterile distilled water and then being blot dried (Gill *et al.,* 2019). Water from the final washing was spread on the water agar plate, which was taken as a control to check whether the surface disinfection was done properly or not.

3.3 Isolation of the endophytic fungi from the samples

After surface disinfection, the stem and leaves were divided into pieces that were each 1 cm long. The sterilized samples were put on a water agar-coated plate (2% agar) for 7–14 days at 25 ± 2°C and observed after every 2-3 days for the growth of the endophytic fungi. After 7–14 days, mixed populations of endophytic fungi were transferred onto potato dextrose agar (PDA) media by the hyphal tip method (Gill *et al.,* 2019).

3.4 Purification of fungal endophytes

The fungal endophytes were purified from a mixed population of fungal endophytes with the help of a sterile blade. From the mother plate, the inoculum was taken and placed onto the PDA media. Incubation of the plates was done for 7–14 days at 25 ± 2°C. The mother plates were stored at 4°C for further use.

3.5 Labeling of fungal endophytes

Labeling was done for the isolated and purified endophytic fungi from *B. monnieri* accordingly. For example, isolated and purified endophytes from the stem and leaves of the medicinal plant *B. monnieri* were labeled as BM followed by numbers (for example: BM-

1,2,3 etc.), S-Stem or Leaves (BM for *B. monnieri*, S for sample) respectively (for example: BM-1 S-Stem or Leaves).

3.6 Sub-culturing of the endophytic fungi

For sub-culturing of the endophytic fungi, about 1 cm^2-shaped discs of the mycelia were taken from the mother plates with the help of sterile blades. These discs were further transferred onto fresh PDA plates, one on each plate, and incubated for 7–10 days at 25 ± 2°C they were observed every 2-3 days for the growth of the endophytic fungi.

3.7 Production of secondary metabolites

After about 7 days of incubation of sub-cultured mycelia, 4-5 mycelial plugs of 5.0 mm diameter were taken from the edges of actively growing cultures from the PDA plates and were then inoculated aseptically into 250 ml of potato dextrose broth (PDB) in 500 ml Erlenmeyer flasks. The flasks were then incubated at 25 ± 2°C under stationary conditions for 21 days. After 21 days of incubation, filtration of the culture was done using sterile muslin cloth to separate mycelium from the broth (Prabavathy & Nachiyar, 2011). The mycelium was dried in the oven and weighed.

3.8 Solvent extraction

To obtain the fungal metabolites, the culture filtrate was first reduced up to 50% by using a rotary evaporator, and then the filtrate was extracted with an equal volume of ethyl acetate by solvent extraction. A separate funnel was used, in which equal volumes of ethyl acetate and filtrate were taken and shaken for 20 minutes. Then the two phases, i.e., the organic and inorganic phases, were allowed to settle in order to attain complete separation without causing any disturbance. After separation, the organic phase was collected in a separate flask, and the inorganic phase was also collected in a different flask. Sodium sulphate anhydrous was used to imbibe the water molecules present in the organic phase. The extract obtained was put in the sterile vial and further dried completely at room temperature. Then the dry weight of the vial was taken. The crude extract was then stored at 4°C for further studies.

3.9 Preliminary bioactive screening of the fungal extracts

3.9.1 Antioxidant assay of crude extracts

DPPH assay: DPPH (*2,2-diphenyl-1-picryl-hydrazyl-hydrate)* assay was used to determine the free radical scavenging effect of the isolated fungal crude extracts. Five different concentrations of fungal extracts (62.5, 125, 250, 500, and 1000 µg ml^{-1}) were used to evaluate the antioxidant activity. In this assay, ascorbic acid (150 µg ml^{-1}) was used as a positive control. Different concentrations of the isolated fungal crude extracts were mixed with 150 µl of DPPH (100 µmol l^{-1}) and methanol in a 96-well microtiter plate. The total volume taken in each well was 200 µl. The entire test was performed in triplicate. The well plate was wrapped in aluminium foil and incubated for 30 minutes in the dark. The absorbance was recorded at 517 nm using an ELISA plate reader (Vig *et al.*, 2021).

The formula used for the calculation of free radical scavenging activity is given below:

$$\text{Free radical scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

3.9.2 Acetylcholinesterase Inhibition Assay

AChE inhibitory activity with slight modifications was estimated in accordance with Ellman *et al.,* 1961. Donepezil (DPZ) was being used as a standard AChE inhibitor. Five different concentrations of 50, 100, 200, 400, and 800 µg/ml of individual fungal extracts (BM-1, BM-7, BM- 3, BM- 31, BM- 6, BM- 5, and BM-16) were used to estimate percentage inhibition of AChE. 10 µl of AChE (1.00 U mL−1) and 20 µl of above concentration of fungal extract were incubated in a 96-well plate for 30 min at room temperature. 20 µl (1.5 mM) of DTNB was added to the above solution. Acetylthiocholine iodide (ATCI) (15 mM, 20 µl) was added to it to commence the reaction, and absorbance was recorded immediately at 415 nm using an ELISA plate reader. The same steps were followed for reference compound DPZ.

$$\text{Percentage inhibition of AChE} = \frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{Absorbance of blank}} \times 100$$

3.10 Preliminary qualitative analysis of crude extracts BM-6 & 16

Phytochemical screening of crude fungal extracts BM 6 & 16 was done to confirm the presence of diverse classes of natural compounds in the extracts (Hadacek *et al.*, 2000).

3.10.1 Test for tannins and phenols (Ferric chloride test):

50 µl of crude extract was treated with 50 µl of 5 % alcoholic ferric chloride solution, and the appearance of a greenish color indicated the presence of condensed tannins.

3.10.2 Test for alkaloids (Wagner's reagent test):

50 µl of crude extracts were taken, and a few drops of Wagner's reagent were added. Reddish-brown precipitates indicated the presence of alkaloids.

3.10.3 Test for triterpenoids:

100 µl of chloroform, 50 µl of acetic anhydride, and 50 µl of concentrated sulfuric acid were added to 50 µl of crude extracts, and the formation of a reddish-violet color indicated the presence of triterpenoids.

3.10.4 Test for flavonoids:

100 µl of 2% NaOH was added to 100 µl of crude extracts, concentrated yellow color was produced, which became colorless after the addition of a few drops of diluted acid. These results indicated the presence of flavonoids.

3.10.5 Test for glycosides (Keller-Killiani test for deoxysugars):

50 µl of glacial acetic acid, one drop of 5% alcoholic ferric chloride solution, and a few drops of concentrated sulfuric acid were added to 100 µl of crude extracts. A reddish brown color appeared at the junction of two liquid layers, which confirmed the presence of deoxy sugars.

3.10.6 Test for saponins (Saponification test):

50 µl of crude extracts were taken, and a few drops of 0.5 N alcoholic KOH were added along with a drop of phenolphthalein. The test tubes were then placed in a pre-heated water bath for some time. The formation of soapy bubbles indicated the presence of saponins.

3.11 Purification of crude extracts

3.11.1 Thin-layer chromatography (TLC)

Finding out how many compounds are present in a crude extract is simple and quick with a little TLC. The endophytic fungus BM-6 and BM-16 methanolic extracts were spotted 1 cm from the bottom of TLC plates (Silica Gel F254-LOBA). For running samples on TLC plates, an optimized mobile phase was utilized. The elements were detached by the mobile phase into different Rf value ranges. UV light at 254 and 366 nm was used to view the produced chromatogram.

3.11.2 Mobile phase optimization

By adjusting the solvent strength in accordance with the polarity index, the mobile phase in TLC optimized to have an Rf value that is between 0.15 and 0.85. Pure forms of solvents were employed. Finally, to run the extract on TLC plates, gradients of chloroform, methanol, and ethylacetate were employed.

3.12 Purification of different fractions of BM-6 and BM-16 through column chromatography

3.12.1 Preparation of the Sample

Ethyl acetate extract obtained from the fermentation of endophytic fungal cultures was evaporated, dried, and then weighed. Different fractions were separated using dried extract.

3.12.2 Preparation of Column

The adsorption of the extract, charging, and distribution of the column were all included in the preparation of the column. On the stationary phase, the extract that was selected for fractionation was adsorbed. Based on the size of the sample to be separated, a glass column with an appropriate dimension was selected. In order to remove contaminants, the selected column was washed with solvent. A cotton cushion was strategically placed at the base to block the stationary-phase current. Wet packing was used to charge the column in the stationary phase. To avoid the formation of air bubbles, the slurry was smoothly discharged at the top of the column and packed with gently pushed air. Following a hexane rinse, the

column was set up by passing a sufficient amount of the initial solvent mixture across it. A hexane suspension of the crude extract was used to load the column. 100% hexane was used for the elution of the column; gradients of hexane and chloroform, chloroform and methanol, hexane:chloroform (9:1), hexane:chloroform (7:3), hexane:chloroform (5:5), hexane:chloroform (3:7), hexane:chloroform (1:9), chloroform:methanol (9:1), chloroform:methanol (7:3), chloroform:methanol (5:5), chloroform:methanol (3:7), chloroform:methanol (1:9) and then for the final cleaning, the column was eluted out with 100% methanol (Andriana *et al.,* 2019). All the fractions eluted were collected in test tubes for further use. Each fraction was then spotted on the pre-coated TLC plates. After using different solvent systems based on the review of the literature, the plates were then visualized under long and short UV at 366 and 254 nm, respectively. Similar fractions were pooled together based on the TLC profile. To create dried fractions, these pooled fractions were evaporated.

3.13 Screening of bioactive fractions

The fractions obtained after purification through column chromatography were further tested for acetylcholinesterase inhibitory activity. The fractions showed maximum acetylcholinesterase inhibitory potential and were characterized for the identification of the bioactive compounds present in the fractions.

3.14 Characterization of the selected fractions showing maximum bioactivity

3.14.1 UV-visible spectroscopic analysis

Ultraviolet absorption spectroscopy quantifies the decrease in intensity of a light ray as it passes through a sample. Dimensions of absorption can be at a specific wavelength or above an extensive spectral range. The UV-Vis spectrometer-1900 (Shimadzu), was activated, and the lamps were allowed to preheat up for an adequate amount of time. Cuvette was filled with 100 percent methanol to serve as a control. Sets the reading for the blank to zero. The sample was placed in the cuvette. Both the sample and the standard were dissolved in 100 percent methanol. For the accuracy of the results, the cuvette was cleansed twice with the sample and then filled three-fourths of the way with it. Cuvette was accurately positioned within the spectrophotometer. The wavelengths 190-800 nm were measured (Catauro *et al.,* 2015).

3.14.2 HRMS

The LC-HRMS analysis of bioactive fraction was performed using a Waters, SYNAPT-XS HDMS, UK Agilent 6520 (DBA064), UPLC ACQUITY H CLASS Series System equipped with a G1311A quaternary pump at Sophisticated Analytical Instrument Facility (SAIF), Punjab University, Chandigarh. The scan parameter settings include the dissolution temperature: 550^0C, dissolution gas flow: 850Lts/Hr, capillary voltage: 3.22keV, cone gas flow:50Lts/Hr, cone voltage: 50V, Collision energy: 4eV, and the source offset was 80v. The two mobile phases were used: solvent A: 0.1% Formic acid: LC-MS grade water and solvent B: 0.1% Formic acid: LC-MS grade acetonitrile at flow rate of 0.3 mL/min. During the whole process, column temperature was maintained at 30°C. After passing through the flow cell of the diode array detector, the column elute was directed to SYNAPT-XS HDMS fitted with an electrospray interface. The mass spectrum analysis was carried out using positive electron spray ionization (ESI-positive mode) within the mass range of 100-2000 Dalton's at a scan rateof 2 min with error of 2ppm.

3.14.3 Analysis of fraction 7 of BM-16 through fourier-transform infrared spectroscopy

The range of infrared spectrum was from 500 to 4000 cm-1. Analysis was done through IRT racer-100 (SHIMADZU). The peaks of sample were recorded. FTIR spectrum of bioactive fraction was recorded as percentage of light transmitted vs wavenumber (Catauro *et al.*, 2015).

3.15 Identification of endophytic fungi

3.15.1 Morphological identification

To investigate the hyphae structure, spore morphology, texture, and colony morphology of endophytic fungi, lacto phenol cotton blue staining was used. A drop of dye was applied to a glass slide inside a laminar hood, and then endophytic fungal hyphae from a PDA plate were added. After piercing the fungal tissue, a cover slip was placed on it, and the hyphal structure and spores were carefully examined under a phase contrast microscope (Ranganathan *et al.*, 2019).

3.15.2 DNA extraction, PCR amplification, and molecular identification

Endophytic fungus mycelium plugs were cut and placed in a 500-ml flask with PDB. The flask was incubated for 7 days at 25 °C. On the seventh day, the mycelia was separated using

muslin cloth, dried in an oven to eliminate moisture, and then crushed with liquid nitrogen before being kept at -80°C to isolate the genomic DNA (Kaur *et al.,* 2022).

3.15.3 DNA extraction using the CTAB method

In a 2 ml centrifuge tube, 100 mg of powdered fungal tissue was placed. 600 µl of preheated extraction buffer was added to the tube, which was then incubated for 60 minutes at 65 °C. To remove detritus, centrifugation at 13,500 g for 20 minutes at 4°C was performed. The supernatant was transferred into a new microfuge tube. 800 µl of 25:24:1 Phenol:Chloroform:Isoamyl Alcohol was added to the tube containing the supernatant. To obtain aqueous layer, centrifugation at 13,500 g for 20 minutes at 4°C was carried out. After obtaining the aqueous layer in a new tube, 800 µl of isopropanol was added to precipitate the DNA. After 20 minutes of centrifugation at 13,500 g and 4°C, pellet was collected. 70% ethanol was added to the above centrifuge container, which was then centrifuged at 13,500 g for 20 minutes at 4°C. The pellet was dissolved in 25 µl of sterile MQ water and the supernatant was disposed (Latiffah et al., 2018). The obtained DNA was examined by passing it through a 0.8% agarose gel. After heating, 0.32 g of agarose material was weighed and combined with 40 ml of 0.5X TBE buffer.

2 µl of ethidium bromide was added to it. The mixture was then taken into a casting tray containing a comb. Gel was permitted to harden at ambient temperature. After removing the comb, the tray was inserted in the electrophoresis chamber.

3.15.4 Polymerase chain reaction (PCR)

The amplification of ITS regions was done using forward primer ITS1 and reverse primer ITS4. A 25 µl volume of reaction mixture was used to perform PCR. PCR consists of three steps: a preliminary denaturation at 98°C for 5 minutes, 32 cycles of 1 minute at 98°C, annealing at 55°C for 1 minute, and extension at 72 °C for 7 minutes (Kaur *et al.,* 2022). A gel electrophoresis unit was supplied with ladder and PCR samples for evaluation. The PCR product was quantified with the help of Nano drop. Nano drop was calibrated using Millipore water. After loading 1µl of sample, the concentration of the PCR product and the A260/A280 ratio was determined.

3.15.5. Sequence analysis of ITS region

PCR product was then sequenced by employing Sanger sequencing. Sequences obtained were then searched for sequence similarity with the non-redundant database maintained by NCBI. Homology in the sequence was searched by comparing sequence of interest with already available data using BLAST tool. Similar sequences were aligned using ClustalW. Neighbor joining method was used to construct the phylogenetic tree in Mega 11 software.

3.16 Statistical analysis

All results were shown as mean ± standard errors of mean (S.E.M.). Statistical analysis of data was carried out by using One-way ANOVA followed by Tukey's multiple comparison post hoc test.

Results

In the present study, the medicinal plant *B. monnieri* has been selected for the isolation and identification of endophytic fungi. The plant *B. monnieri* is selected because this plant has held great importance for ages and because not much work has been done on the isolation of endophytic fungi and characterization of bioactive compounds from this plant. This indigenous medicinal plant is very potent, holds therapeutic value, and could be explored for endophytic fungi having the proficiency to produce a wide range of secondary metabolites.

4.1 Collection of samples

Stems and leaves of *B. monnieri* were collected from TIFAC-CORE, Thapar Institute of Engineering & Technology campus, Patiala, India (Fig 5). Samples were then moved to sterilized bottles in the TIFAC-CORE lab for further studies.

Fig 5: Collection of samples from *B. monnieri* (Leaves of *B. monnieri*)

4.2 Surface disinfection and isolation of endophytic fungi

The collected samples were first washed under running tap water to remove dirt (Fig 6). After initial washing with running tap water, the samples were cut into small pieces and then surface disinfected. After that, the small leaf samples were put on Petri dishes containing agar and then incubated in the incubator under static conditions for 10–14 days at 25 ± 2°C to

encourage the growth of endophytic fungi (Fig 7). For the growth of endophytic fungi, the plates were routinely checked. After 7-8 days the endophytic fungi emerged from the plates.

Fig 6: Washing of samples under running tap water

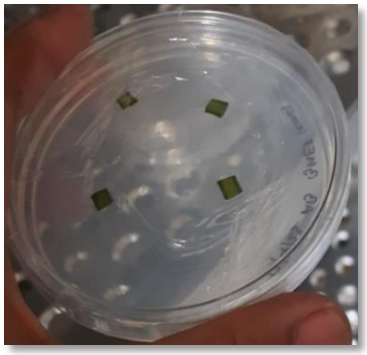


Fig 7: Leaf samples on water agar plate

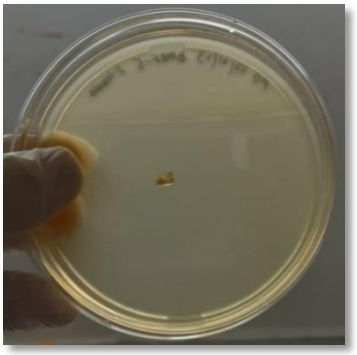
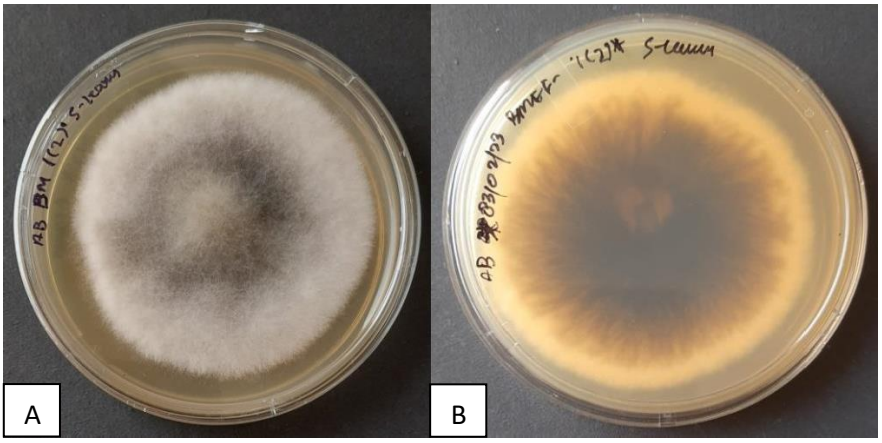


Fig 8: Inoculum transferred onto PDA plate

After this, from a mixed population of endophytic fungi, purification was done in order to get the pure strains. The process was carried out using a sterile blade. With the help of a blade, the inoculum was taken from the agar plate and moved on to the fresh PDA plates (Fig 8). Incubation of the plates was done for 7–14 days at 25 ± 2°C. The mother plates were stored at 4°C for further use. The leaves and stems of *B. monnieri* yielded a total of 32 endophytic fungi out of which 7 different kinds of endophytic fungi were selected, which were designated as BM-1, BM-3, BM-5, BM-6, BM-7, BM-16, and BM-31. Fig 9 shows the front and back side of different endophytic fungi.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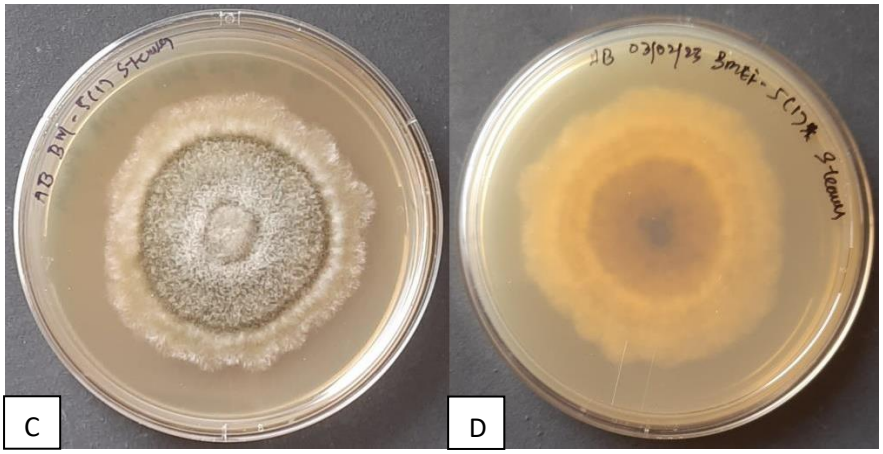
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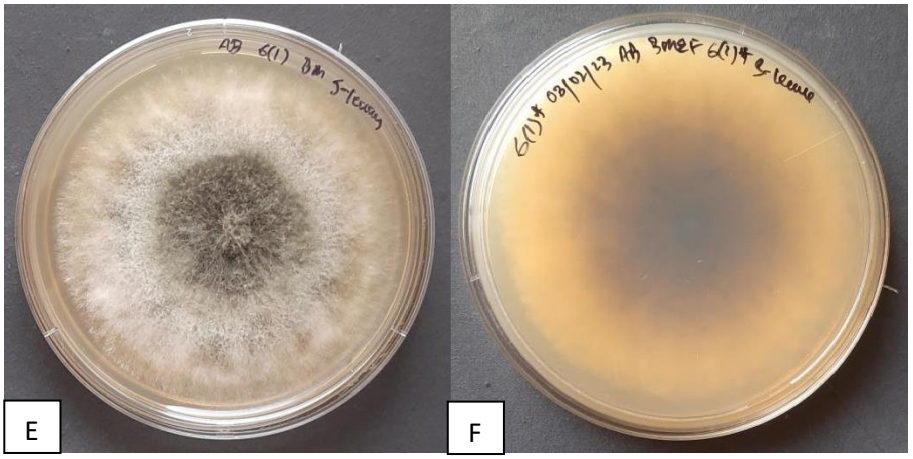
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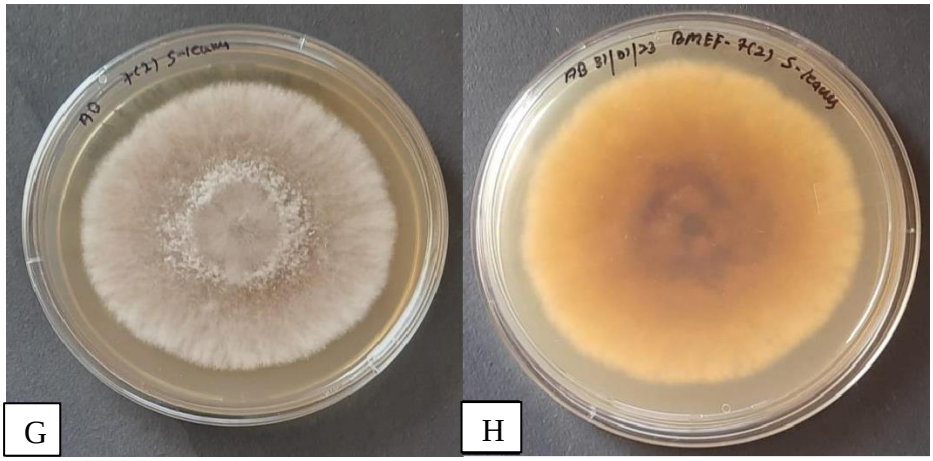
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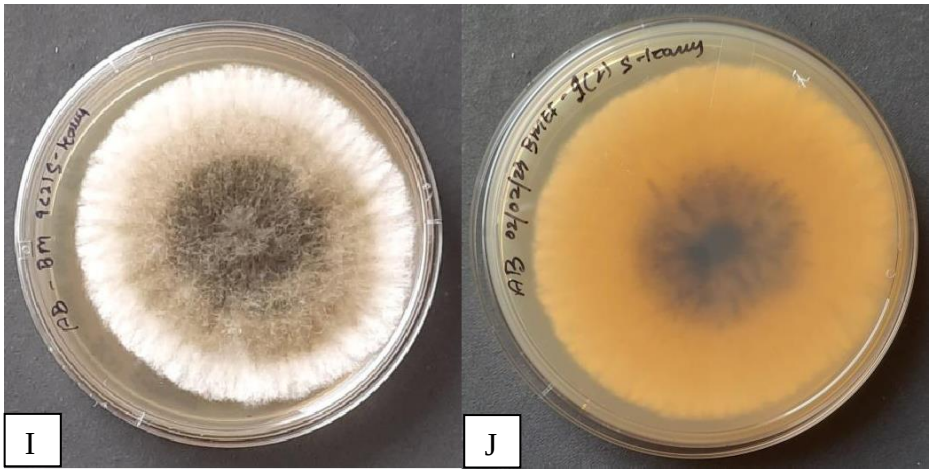
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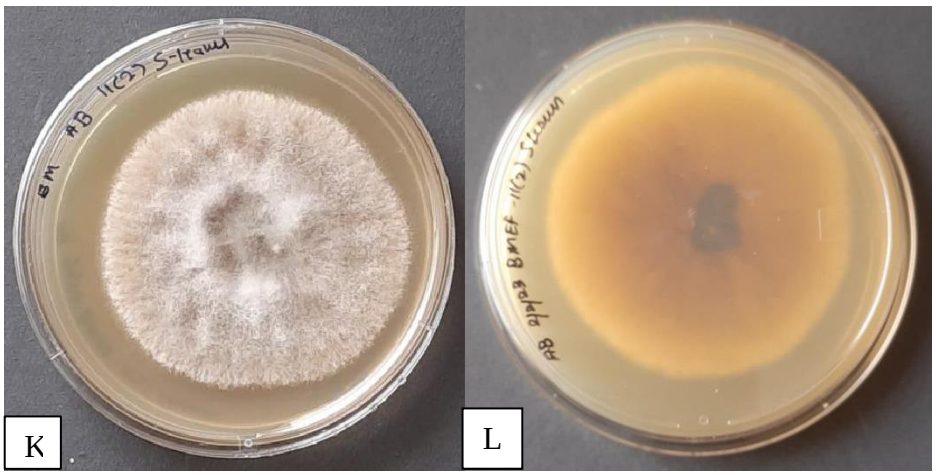
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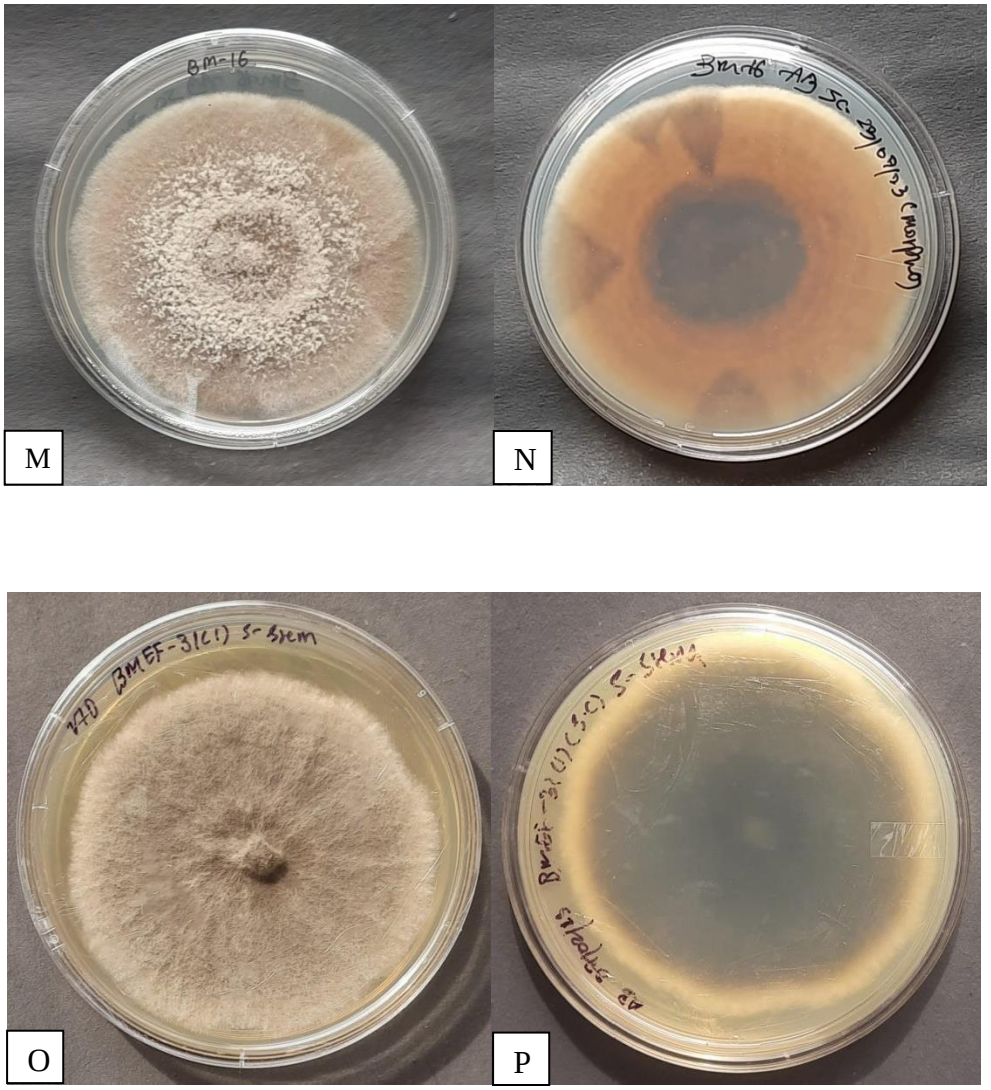


Fig 9: (A) BM-1(front); (B) BM-1(back); (C) BM-5(front); (D) BM-5(back); (E) BM-6 (front); (F) BM-6(back); (G) BM-7(front); (H) BM-7(back); (I) BM-9(front); (J) BM-9 (back); (K) BM-11(front); (L) BM-11(back); (M) BM-16(front); (N) BM-16(back); (O) BM-31 (front); (P) BM-31(back)

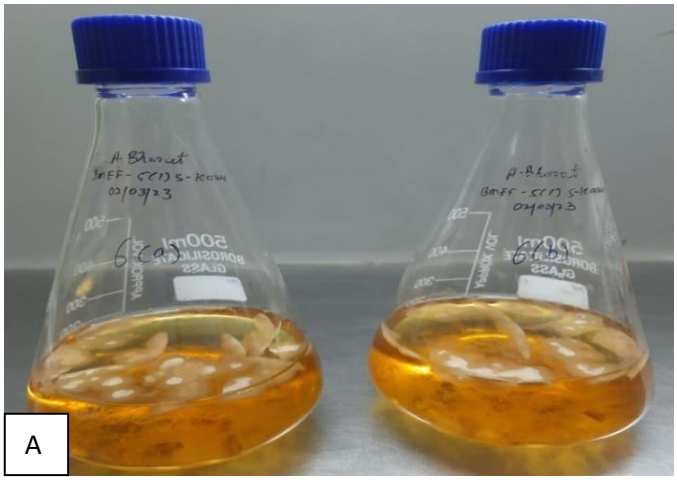
4.3 Production of bioactive compounds by fermentation

For fermentation, the purified endophytic fungal isolates obtained from *B. monnieri* were inoculated in potato dextrose broth (PDB) and incubated for 21 days at 25 ± 2°C under stationary conditions.

4.3.1 Extraction of the crude extract from the fermented broth

After 21 days of the fermentation process, the mycelia and broth were separated from each other with the help of muslin cloth through a funnel (Fig 9). Then the broth was collected in a

separate flask, and mycelia was dried in the oven and weighed. The initial wet weight and final dry weight of the mycelia were recorded (Table 6). To obtain the fungal metabolites, the culture filtrate was first reduced up to 50% by using a rotary evaporator, and then the filtrate was extracted with an equivalent amount of ethyl acetate (1:1) by solvent extraction (Fig 10). Then again, with the help of a rotary evaporator, the organic phase was reduced to obtain the crude extract. The crude extract was collected in sterilized glass vials and kept at 4°C for further bioactive studies.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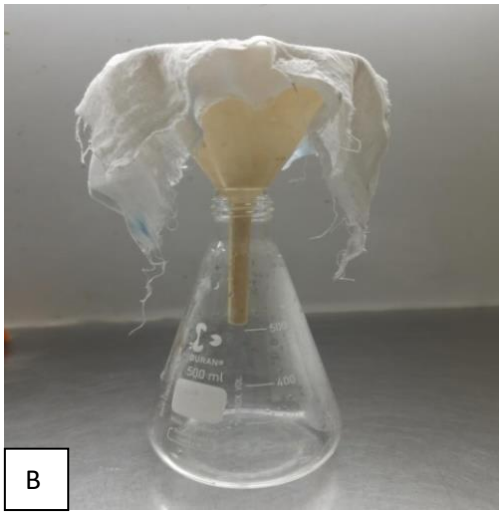
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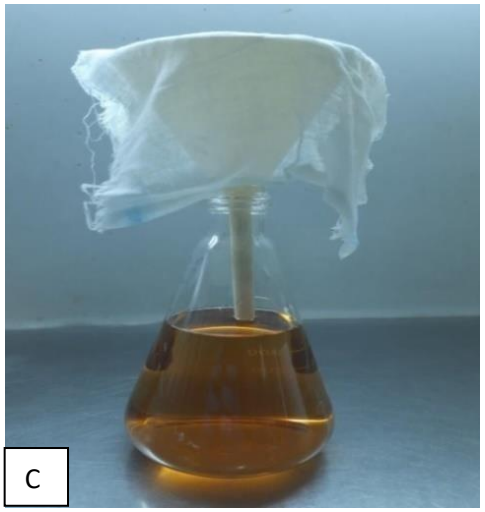
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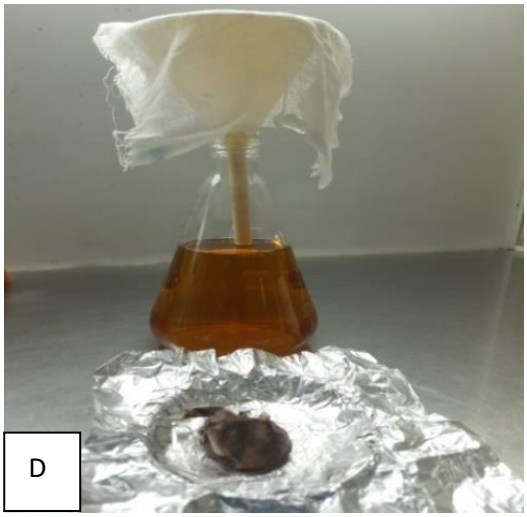


Fig 10: (A-E) Separation of fungal mycelia from the fermented broth

Fig 10 (F): Separating funnel

Table 6: The initial and final mycelial weight of fungal isolates

S.No.	Extract	Initial weight (wet) (in grams)	Final weight (dried) (in grams)
1.	BM 1	7.26	0.65
2.	BM 7	5.66	1.67
3.	BM 3	5.65	0.41
4.	BM 31	5.13	0.76
5.	BM 6	4.55	0.72
6.	BM 5	2.29	0.22
7.	BM 16	7.24	0.95

4.4 Preliminary tests

4.4.1 Antioxidant Potential of the Crude Fungal Extract (DPPH Assay)

The anti-oxidant effects of BM-1, BM-3, BM-5, BM-6, BM-7, BM-16, and BM-31 are shown in Fig 11. BM-5 showed maximum percentage scavenging activity of 33.40, 35.11, 75.51, and 71.64 at 62.5, 125, 250, and 500 µg/ml respectively.

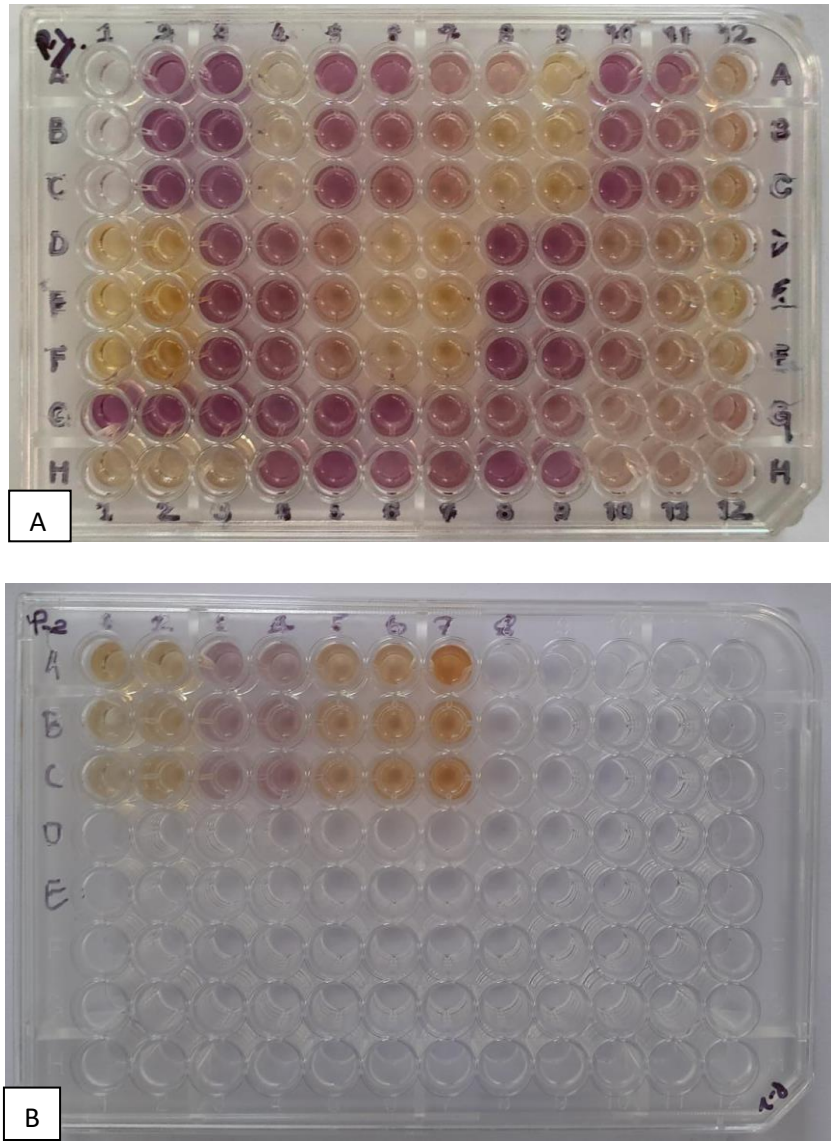
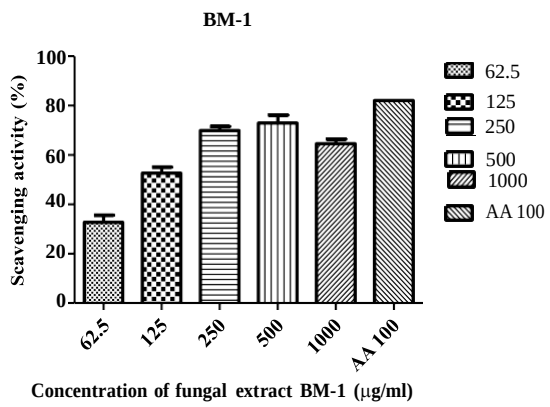
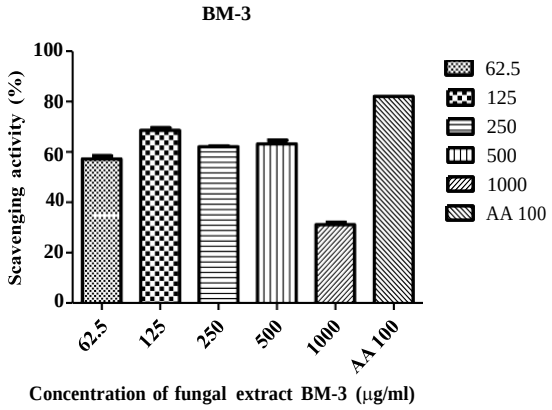


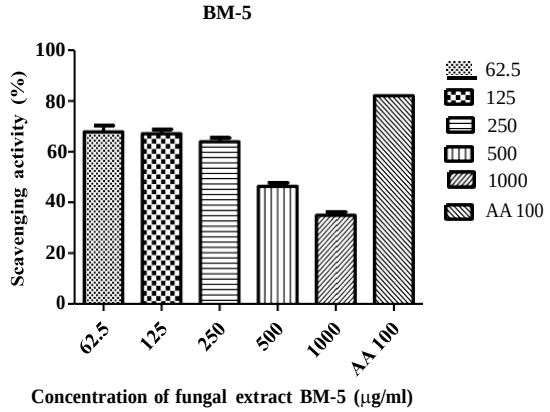
Fig 11: (A-B) Antioxidant (DPPH) Assay in 96 well plate

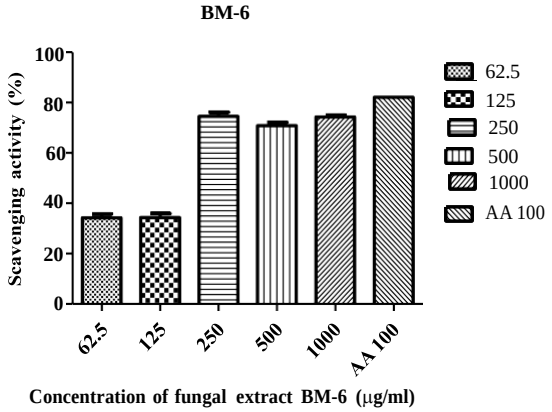
4.4.2 Assessment of anti-oxidant activity by fungal metabolites

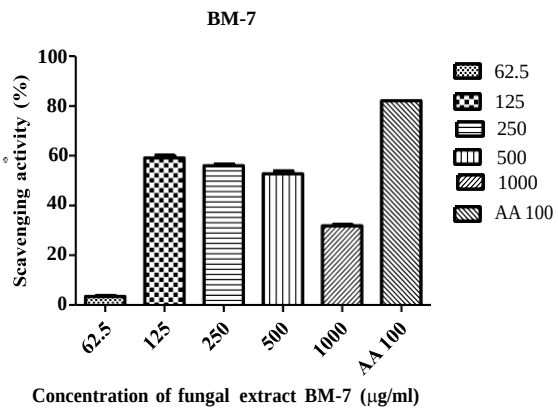
The anti-oxidant effects of BM-1, BM-3, BM-5, BM-6, BM-7, BM-16 and BM-31 are shown in Fig 12. BM-6 showed percentage scavenging activity of 35.11, 33.40, 75.50, 71.64 and 73.94 at 62.5, 125, 250, 500 and 1000 µg/ml respectively. BM-16 showed percentage scavenging activity of 22.25, 34.04, 58.29, 29.19 and 16.24 at 62.5, 125, 250, 500 and 1000 µg/ml respectively. Both the samples BM-6 and BM-16 were statistically different from each other in comparison to the positive control. Each fungal extract showed maximum anti-oxidant activity at 250 µg/ml which were comparable to that of AA at 100 µg/ml (82.1%).

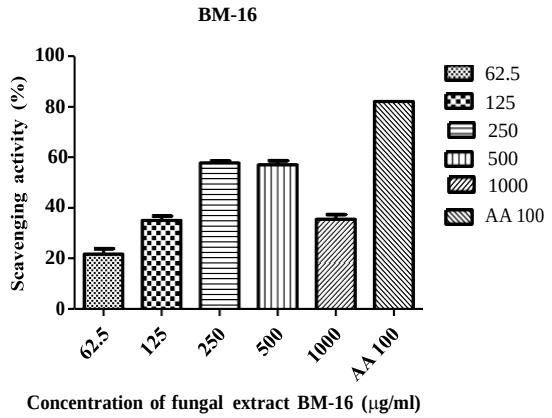

BM-1
Scavenging activity (%)
100
80
60
40
20
0
62.5
125
250
500
1000
AA 100
Concentration of fungal extract BM-1 (µg/ml)


BM-3
Scavenging activity (%)
100
80
60
40
20
0
62.5
125
250
500
1000
AA 100
Concentration of fungal extract BM-3 (µg/ml)


BM-5
Scavenging activity (%)
100
80
60
40
20
0
62.5
125
250
500
1000
AA 100
Concentration of fungal extract BM-5 (µg/ml)


BM-6
Scavenging activity (%)
100
80
60
40
20
0
62.5
125
250
500
1000
AA 100
Concentration of fungal extract BM-6 (µg/ml)


BM-7
Scavenging activity (%)
100
80
60
40
20
0
62.5
125
250
500
1000
AA 100
Concentration of fungal extract BM-7 (µg/ml)


BM-16
Scavenging activity (%)
100
80
60
40
20
0
62.5
125
250
500
1000
AA 100
Concentration of fungal extract BM-16 (µg/ml)

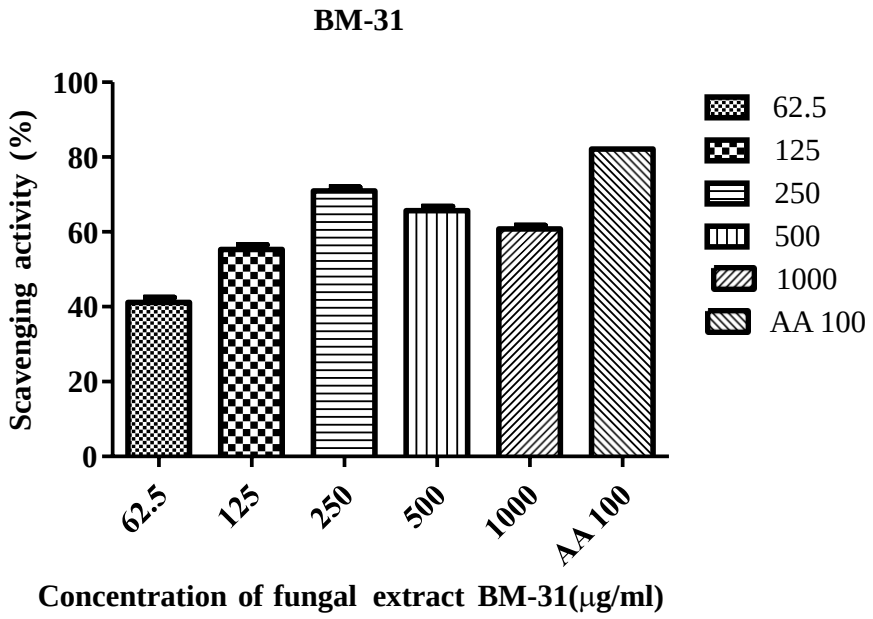


Fig 12: Radical Scavenging activities of the fungal extracts BM-1, BM-3, BM-5, BM-6, BM-7, BM-16 and BM-31 by DPPH assay. Ascorbic acid was used as positive control. Data are expressed as means ± SD (n = 3). All the samples were significantly different at a level of $p < 0.05$ (n = 3) by one-way ANOVA-Tukey's test.

4.4.3 Preliminary in-vitro screening for AChE inhibition

A variety of concentrations of each fungal extract (BM-1, BM-3, BM-5, BM-6, BM-7, BM-16, and BM-31) were produced by solubilizing the crude extracts in DMSO. As a reference compound 100 µg/ml of donepezil (DPZ) was employed. Acetylcholine is broken down by AChE into choline and acetic acid in this assay. A yellow colour is produced when choline binds to DTNB (Fig 13). Because choline does not bind to DTNB in the presence of an AChE inhibitor, a white colour develops. It was determined what percentage of AChE was inhibited by extracts (BM-1, BM-3, BM-5, BM-6, BM-7, BM-16, and BM-31). Out of seven crude fungal extracts, BM-6 and BM-16 showed maximum acetylcholinesterase inhibition with 57.10% and 64.10% at concentrations of 250 µg/ml respectively.

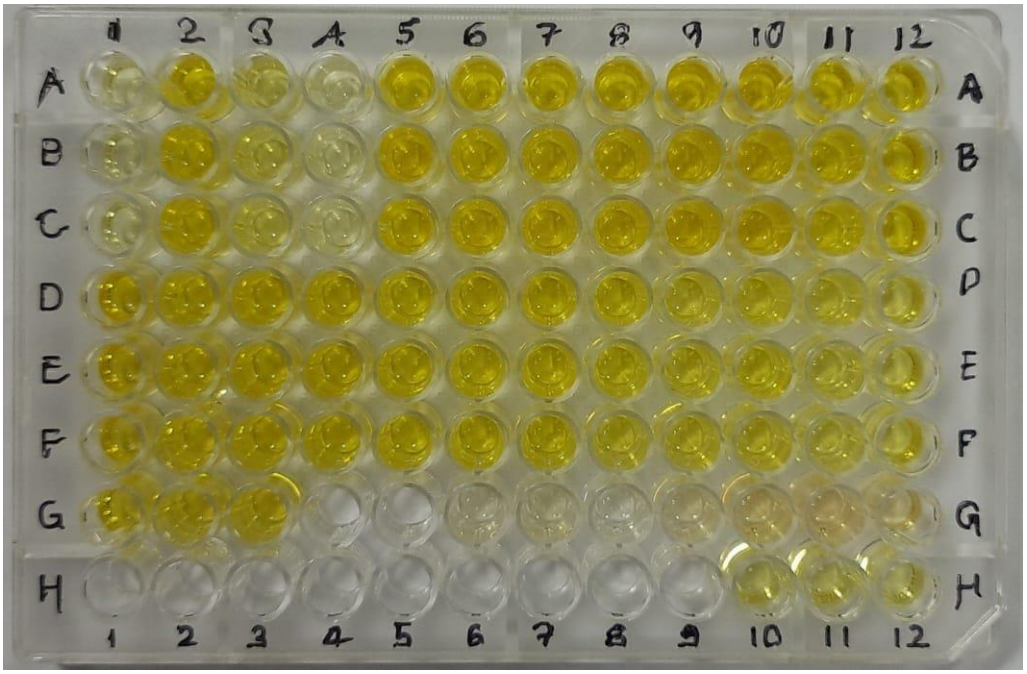
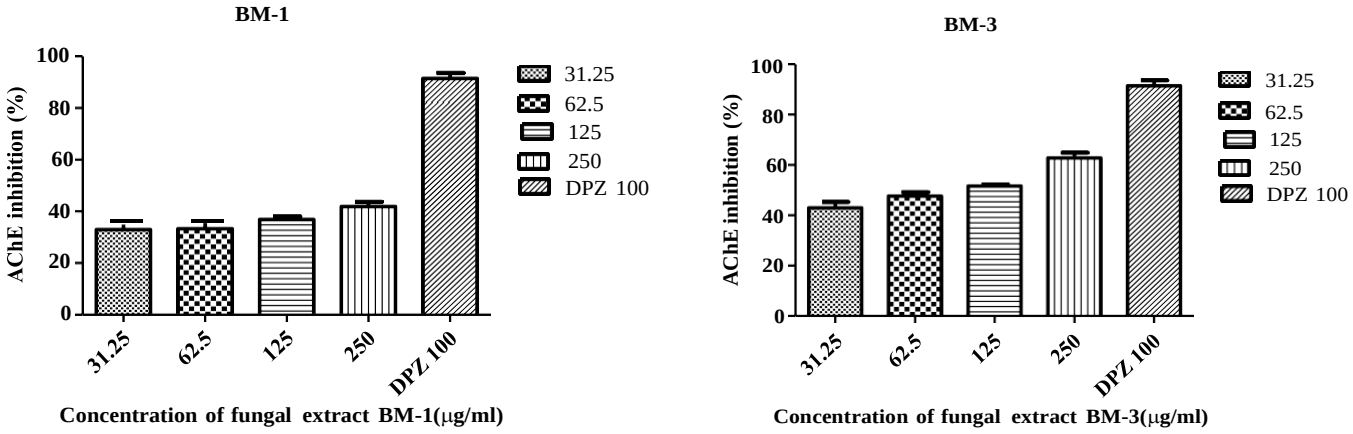


Fig 13: AChE inhibitory activity in 96 wells plate

4.4.4 Assessment of AChEI activity by fungal metabolites

The percentage AChEI by seven fungal extracts BM-1, BM-3, BM-5, BM-6, BM-7, BM-16 and BM-31 is shown in Fig 14. BM-6 showed percentage inhibition of 52.38, 55.22, 60 and 57.10 at 31.25, 62.50, 125 and 250 µg/ml respectively. BM-16 showed significant variation in percentage inhibition of 53.59, 58, 60.11 and 64.10 at 31.25, 62.50, 125 and 250 µg/ml, in comparison with DPZ at 100 µg/ml (92%).



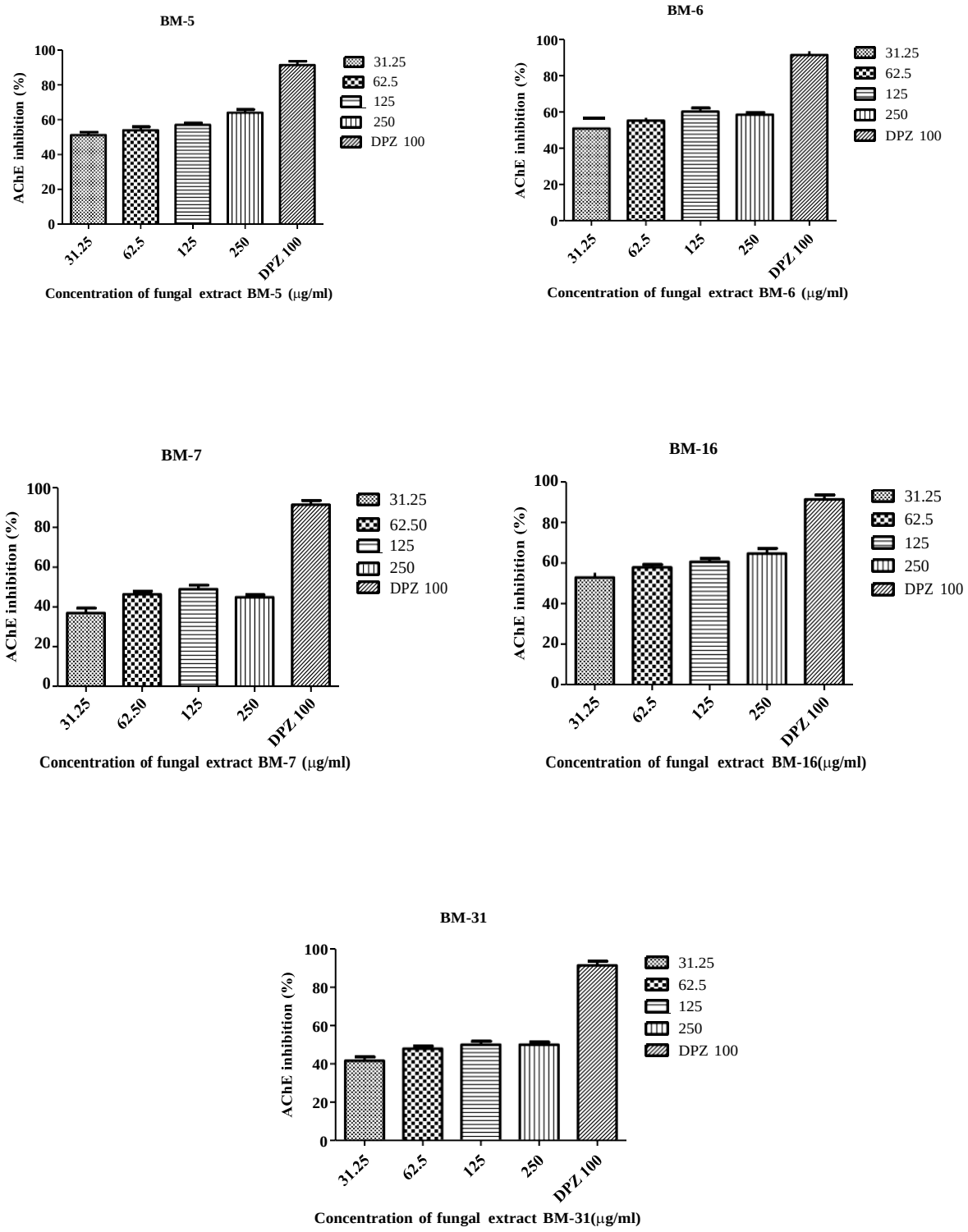


Fig 14: In vitro AChE inhibitory potential of BM-1, BM-3, BM-5, BM-6, BM-7, BM-16 and BM-31. Donepezil was used as positive control. Data are expressed as means ± SD (n = 3). All the samples were significantly different at a level of $p < 0.05$ (n = 3) by one-way ANOVA-Tukey'stest

4.5 Preliminary qualitative analysis of crude extracts BM-6 and BM-16

The fungal extract BM-6 shows the presence of condensed tannins, alkaloids, steroids, flavonoids, glycosides, and triterpenoids, whereas hydrosylate tannins and saponins were found absent (Fig 15) (Table 7). The fungal extract BM-16 shows the presence of alkaloids, steroids, glycosides, saponins, and triterpenoids, whereas hydrosylate tannins and condensed tannins, as well as flavonoids, were found absent. The formation of a reddish violet color confirms the presence of triterpenoids; the appearance of a greenish color shows the presence of condensed tannins; the appearance of reddish brown precipitates by Wagner's reagent test confirms the presence of alkaloids; and the formation of soapy bubbles confirms the presence of saponins through the saponification test (Fig 16) (Table 8).

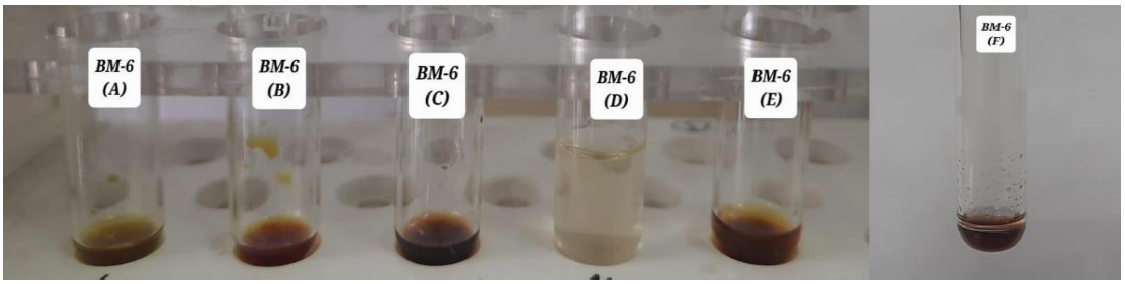


Fig 15: Phytochemical evaluation of different categories of chemical constituents in fungal extract BM-6. (F) The formation of reddish violet color confirms the presence of triterpenoids

Table 7: Qualitative analysis of phytochemicals for fungal extract BM-6

S.No.	Chemical constituents	Fungal extract BM-6
1.	Tannins	(++)
2.	Alkaloids	(++)
3.	Steroids	(+)
4.	Flavonoids	(+++)
5.	Glycosides	(-)
6.	Triterpenoids	(+++)
7.	Saponins	(-)

(+) Positive test, (-) Negative test

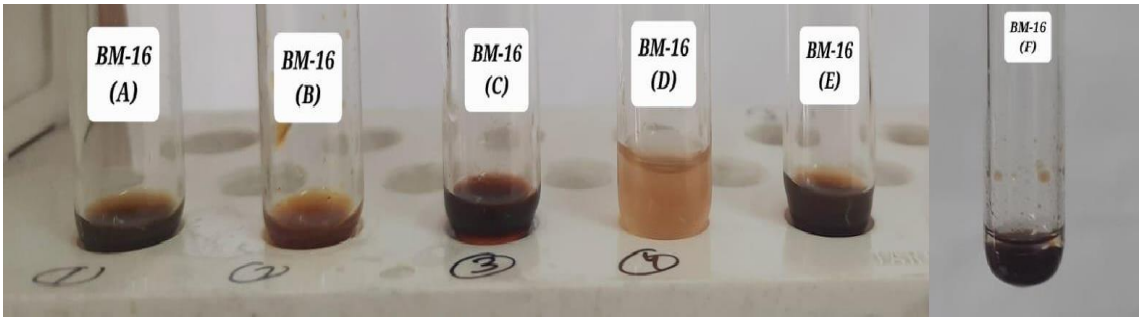


Fig 16: Phytochemical evaluation of different categories of chemical constituents in fungal extract BM-16. (F) The formation of reddish violet colour confirms the presence of triterpenoids

Table 8: Qualitative analysis of phytochemicals for fungal extract BM-16

S.No.	Chemical constituents	Fungal extract BM-16
1.	Tannins	(-)
2.	Alkaloids	(++)
3.	Steroids	(+)
4.	Flavonoids	(+++)
5.	Glycosides	(-)
6.	Triterpenoids	(+++)
7.	Saponins	(+)

(+) Positive test, (-) Negative test

4.6 Purification of Fungal Crude Extracts BM-6 and BM-16

Thin-layer chromatography was carried out using different solvent systems after optimization based on the polarity of the solvents. Silica plates were made, and the methanolic extracts of BM-6 and BM-16 were spotted on these plates for the separation of fractions and Rf values were calculated for both BM-6 (Table 9, 10) and BM-16 (Table 11, 12) at UV-254 and 366 respectively and bands were seen (Fig 17).

Table 9: Different solvent systems used for separation of compounds in fungal extract BM-6 at UV-254

S.No.	Solvent system	Ratio of solvents	No. of fractions	Rf values
1.	Toulene:ethylacetate:methanol: formic acid	3:3.5:2.5:1	3	0.54,0.61,0.87
2.	Ethylacetate:methanol:water	4:1:1	3	0.45,0.6, 0.65
3.	Toulene:ethylacetate:methanol: glacial acetic acid	3:4:3:1	3	0.72,0.66,0.45

Table 10: Different solvent systems used for separation of compounds in fungal extract BM-6 at UV-366

S.No.	Solvent system	Ratio of solvents	No. of fractions	Rf values
1.	Toulene:ethylacetate:methanol: formic acid	3:3.5:2.5:1	3	0.61,0.75,0.87
2.	Ethylacetate:methanol:water	4:1:1	4	0.64,0.69,0.73,0.84
3.	Toulene:ethylacetate:methanol: glacial acetic acid	3:4:3:1	3	0.72,0.64,0.37

Table 11: Different solvent systems used for separation of compounds in fungal extract BM-16 at UV-254

S.No.	Solvent system	Ratio of solvents	No. of fractions	Rf values
1.	Toulene:ethylacetate:methanol: formic acid	3:3.5:2.5:1	3	0.54,0.61,0.87
2.	Ethylacetate:methanol:water	4:1:1	1	0.61
3.	Toulene:ethylacetate:methanol: glacial acetic acid	3:4:3:1	2	0.72,0.64

Table 12: Different solvent systems used for separation of compounds in fungal extract BM-16 at UV-366

S.No.	Solvent system	Ratio of solvents	No. of fractions	Rf values
1.	Toulene:ethylacetate:methanol: formic acid	3:3.5:2.5:1	2	0.75,0.87
2.	Ethylacetate:methanol:water	4:1:1	5	0.62,0.68,0.73, 0.8,0.88
3.	Toulene:ethylacetate:methanol: glacial acetic acid	3:4:3:1	3	0.74,0.64 ,0.45

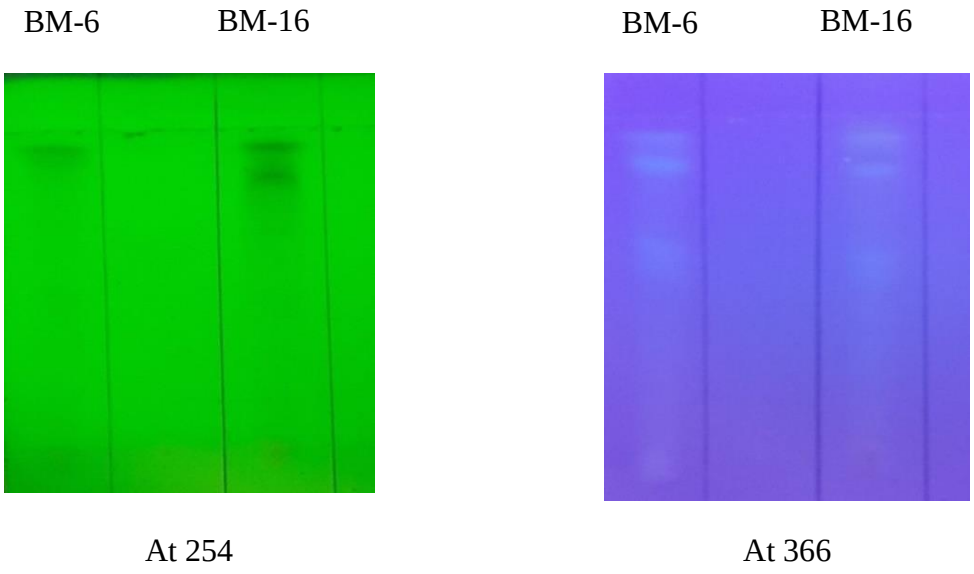


Fig 17: Thin layer chromatography results using solvent system Toulene:ethylacetate: methanol:glacial acetic acid (3:4:3:1) (A) BM-6 and BM-16 at UV 254; (B) BM-6 and BM-16 at UV 366 respectively.

4.6.1 Purification of crude extracts BM-6 and BM-16 through column chromatography

Silica gel G, 60–120 mesh size, was dissolved in 100% hexane and loaded onto the column. Twelve fractions were obtained in both cases, i.e., BM-6 and BM-16, with the following eluents: F1 in 100% hexane, F2 in hexane:chloroform (9:1), F3 in hexane:chloroform (7:3), F4 in hexane:chloroform (5:5), F5 in hexane:chloroform (3:7), F6 in hexane:chloroform (1:9), F7 in chloroform:methanol (9:1), F8 in chloroform:methanol (7:3), F9 in

chloroform:methanol (5:5), F10 in chloroform:methanol (3:7), F11 in chloroform:methanol (1:9), F12 in 100% methanol (Fig 18-19). These fractions were subjected to TLC, and based on the bands and RF values; different fractions were pooled together and evaporated to obtain dried fractions. Further, these pooled and dried fractions were subjected to AChE inhibitory activity.

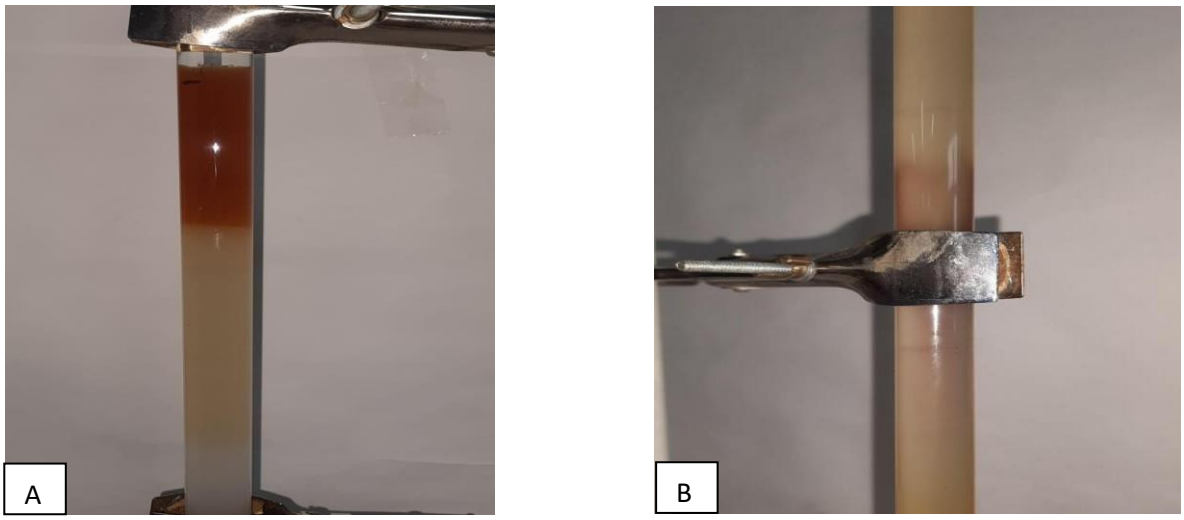


Fig 18: (A-B) Column chromatography of sample BM-16

Fig 19: Column chromatography of sample BM-6

4.7 Assessment/screening of bioactive fractions after purification of crude extracts BM-6 and BM-16 with column chromatography

The AChE inhibitory effect of fractions obtained from column chromatography of crude extract of BM-6 and BM-16 are shown in Fig 20. The concentration of each fraction was 160 µg/ml while that of reference compound DPZ was 100µg/ml. Out of both the samples fraction-7 of BM-16 was further analyzed for its inhibition activity at different concentrations. Fraction F7 showed the percentage inhibition of 2.25, 14.68, 45.19, 50.28, 53.67, 58.75, 60 and 65.53 at 2.5, 5, 10, 20, 40, 80, 160 and 320 µg/ml respectively (Fig 21), which were statistically showed variation in comparison to DPZ at 100 µg/ml (92%).

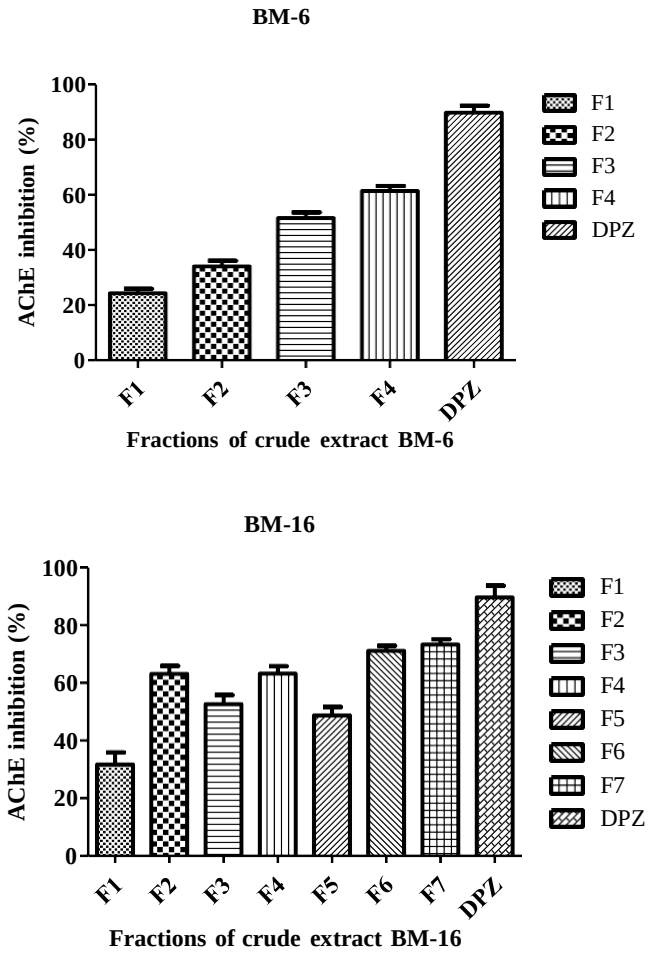


Fig 20: Effect of four fractions of BM-6 and seven fractions of BM-16 on percentage inhibition of acetylcholinesterase in vitro. Data is expressed as means ± SD (n = 3). All the samples were significantly different at a level of $p < 0.05$ (n = 3) by one-way ANOVA-Tukey's test.

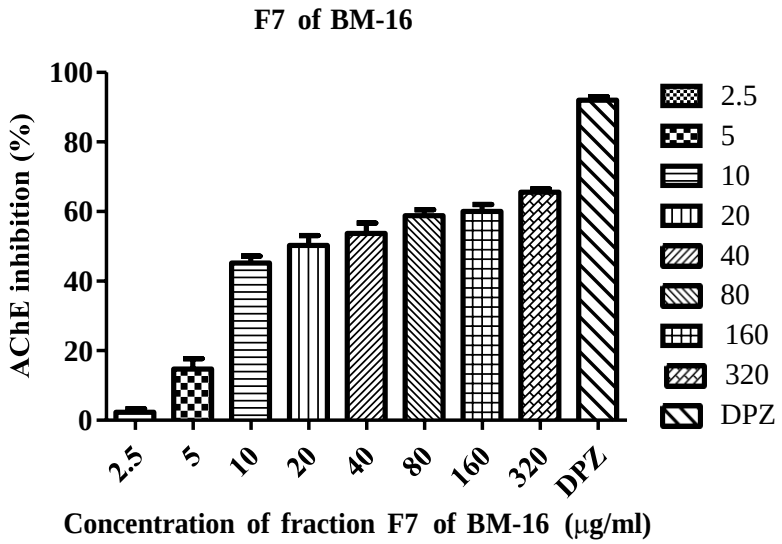


Fig 21: In vitro AChE inhibitory potential of fraction F7 of BM-16. Data is expressed as means ± SD (n = 3). All the samples were significantly different at a level of $p < 0.05$ (n = 3) by one-way ANOVA-Tukey's test

4.8 UV absorption spectrum of bioactive fraction

Sample was dissolved in 100 percent methanol. As shown in Fig 22 absorption was measured from 190 to 800 nm. Fraction F7 of BM-16 exhibited λ max at 204 nm and 282 nm.

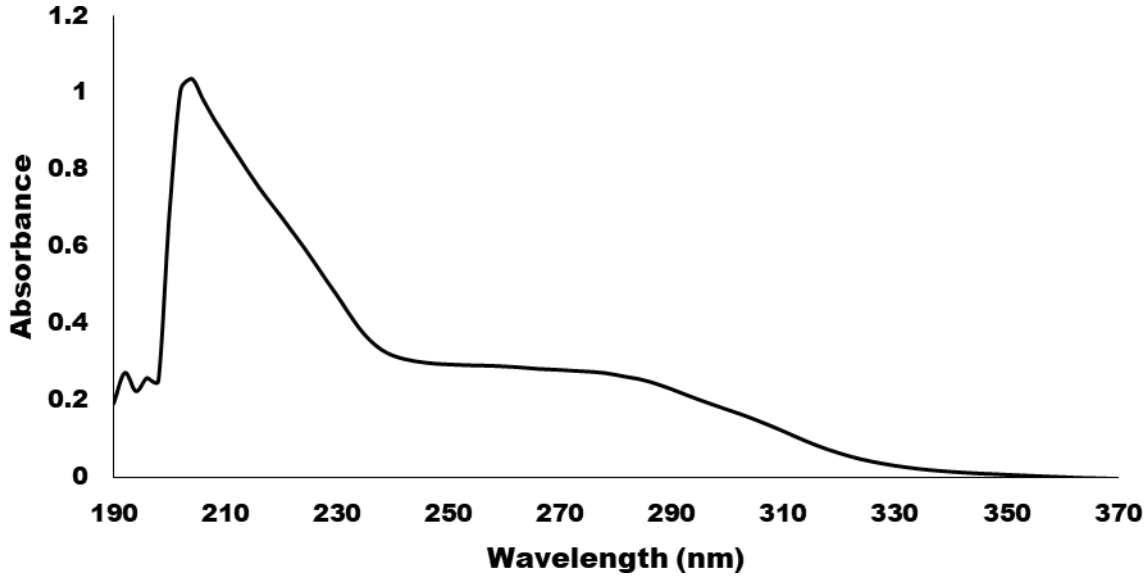


Fig 22: UV absorption spectra (190-400nm) of bioactive fraction F7 of BM-16

4.9 High resolution mass spectroscopy (HRMS)

The fraction F7 of BM-16 was analyzed using various mass libraries. The two intense peaks were observed that shows the presence of the two unknown compounds in abundance in the sample (Fig 23). According to NIST 20 database for compounds table 13 shows the list of some compounds which may be present due to their similar m/z values as compared to the results found in the HRMS. Further analyses need to be done in order to identify the particular compounds on the basis of observation with their theoretical and observed charge to mass ratio in positive ion mode in ESI as presented in Fig 23.

Table 13: Putative list of compounds which can be present in fraction 7 of BM-16

S.No.	Compound name	Molecular weight
1	Agnucastoside C	684.6
2	Monensinmethyl ester	684.9
3	Kijimicin	684.9
4	Podospermic acid	684.6
5	Linezolid	337.1437
6	Lobeline	337.5
7	Tenoicam	337.0910
8	Olopatadine	337.1677
9	Famotidine	337.0448
10	Danozol	337.0448
11	Decimemide	337.5

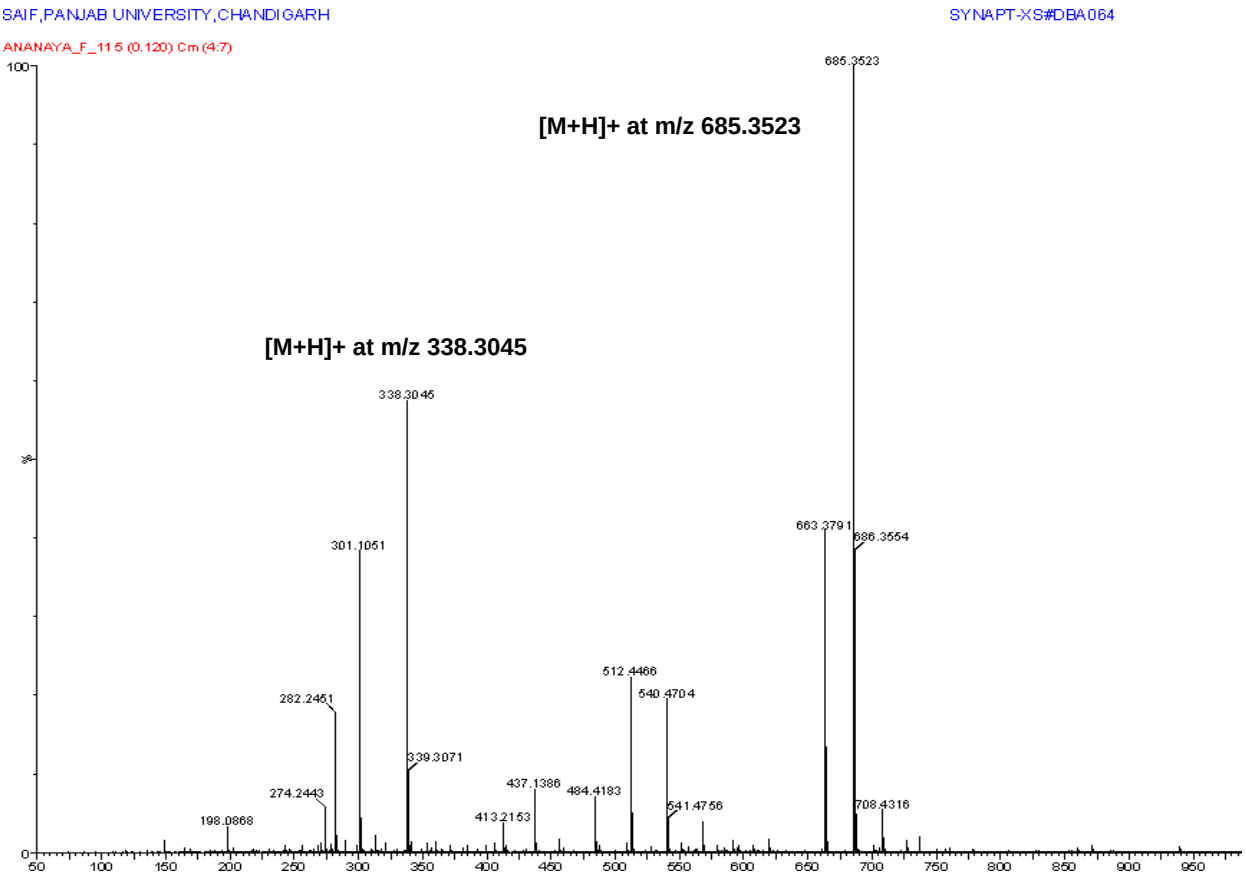


Fig 23: HRMS spectrum of bioactive fraction F7 of BM-16 showing m/z value of 685.3523 and 338.3045

4.10 Fourier-transform infrared spectroscopy (FTIR) of fraction 7 of BM-16

The range of FTIR spectrum was from 500 to 4000 cm-1. Analysis was done through IRT racer-100 (SHIMADZU). FTIR spectrum of bioactive fraction 7 of BM-16 was recorded (Fig 24). The FTIR spectrum of bioactive fraction showed a broad peak in the region of 3337.87 which was due to presence of hydroxyl group. The peak at 2973.32 region is due to the presence of C-H group. Other peak values of the spectrum are mentioned in the table 14.

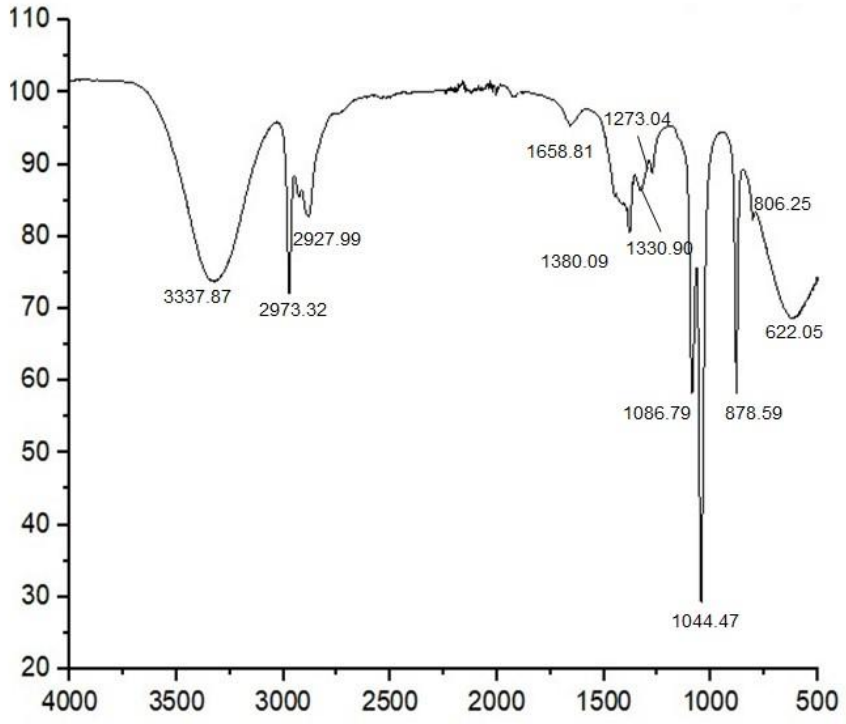


Fig 24: FTIR spectrum of fraction 7 of BM-16

Table 14: FTIR data of fraction 7 of crude extract BM-16

S.No.	Wave number	Functional group/characteristics
1	3337.87	O-H stretching
2	2973.32	C-H stretching
3	2927.99	C-H
4	1658.81	S=O stretching
5	1380.09	O-H bending
6	1273.04	C-N stretching
7	1086.79	C-N stretching
8	1044.47	S=O stretching
9	878.79	Si-OH
10	806.25	C-Cl stretching
11	622.05	Halo group

4.9 Identification of endophytic fungi BM-16 via morphology and molecular methods

4.9.1 Identification based on morphological characteristics

The endophytic fungus BM-16 grows on PDA plate, in the initial days (7 days), fungal growth observed with white margins. The overall growth during seven days was found to be evenly white, cottony, and somewhat protruding at the centre. After 7 days, the mycelia spreads out and the cotton growth is confined to the centre only. On the 15th day, the culture turned greyish without any visible spores on the plate (Fig 25). The Microscopic images of BM-16 represented the thin and long aseptate hyphae. This observation indicates that BM-16 is coenocytic in nature. Spores formed individually on swollen parts inside the hyphae. The spores are uniseptate with a slight mark of division and are mostly visible as conical (Fig 26). These characteristics were similar to *Alternaria sp.* The classification of *Alternaria tenuissima* is given in table 15.

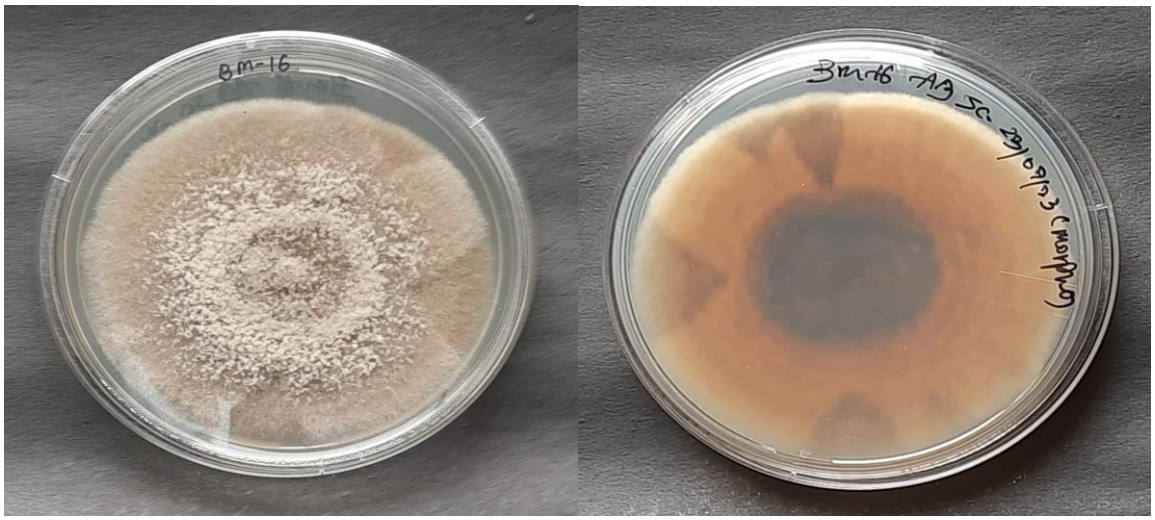


Fig 25: BM-16 on PDA plate-front and back-view

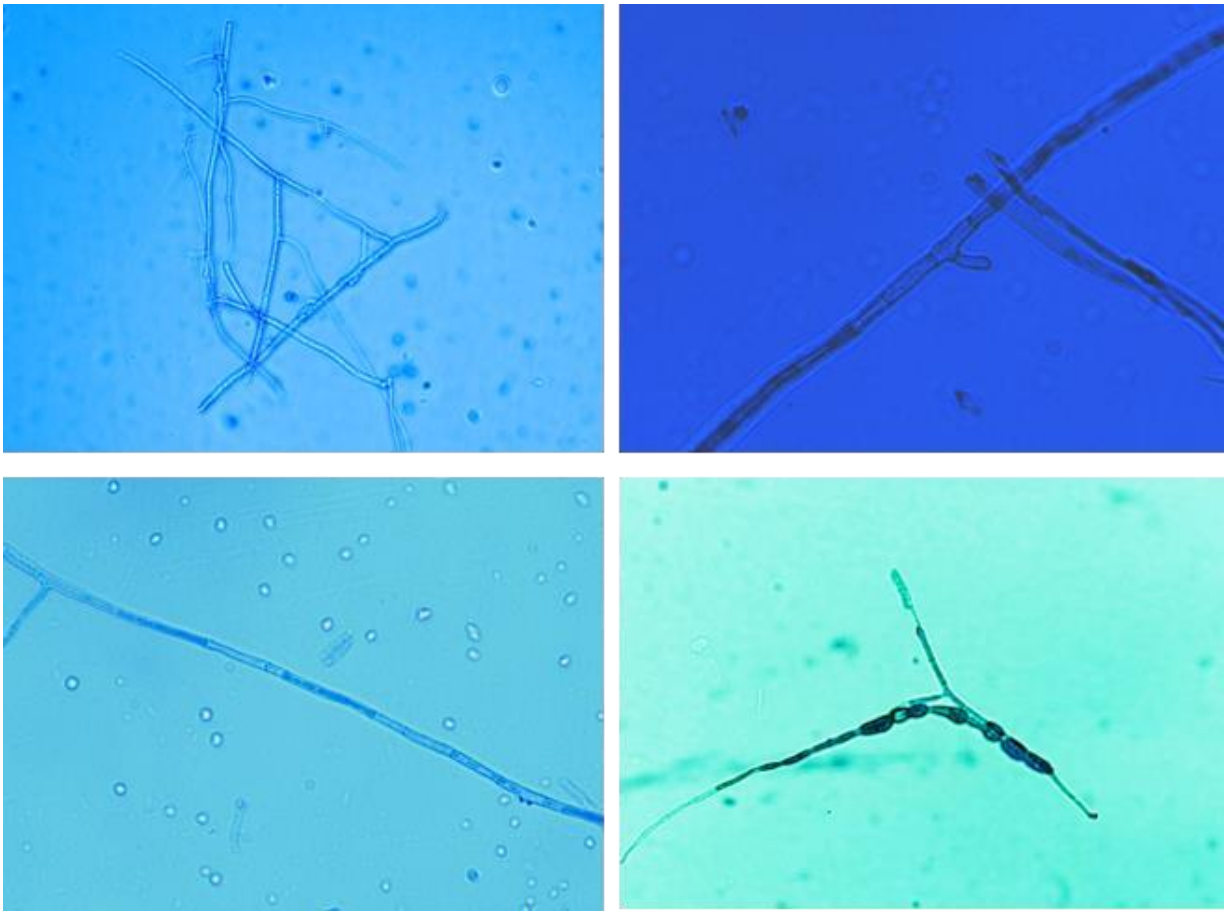


Fig 26: Microscopic analyses of BM-16

Table 15: Classification of *Alternaria tenuissima*

Classification of *Alternaria tenuissima*	
Kingdom	Fungi
Division	Ascomycota
Class	Dothideomycetes
Order	Pleosporales
Family	Pleosporaceae
Genus	*Alternaria*
Species	*Tenuissima*

4.10.2 Identification of endophytic fungi on molecular basis

Genomic DNA was isolated using CTAB method. The concentration of isolated DNA was found to be 378 ng/µl. ITS regions were PCR amplified using forward primer ITS1 and reverse primer ITS4. After purification, PCR product was evaluated by running on electrophoresis gel unit. 1 kb ladder was loaded in lane 1 while amplified PCR products were loaded in lane 2 and 3 (Fig 27). The size of amplicon was noted after the gel electrophoresis that came out to be 545 bp. The Sanger sequencing of the same has been done. The sequences were aligned and phylogenetic analysis was done using Mega11 software. Evolutionary relationships between homologous sequences were found by neighbor-joining method with a bootstrap value (1000) (Fig 28). The BM16 was identified as *Alternaria tenuissima*.

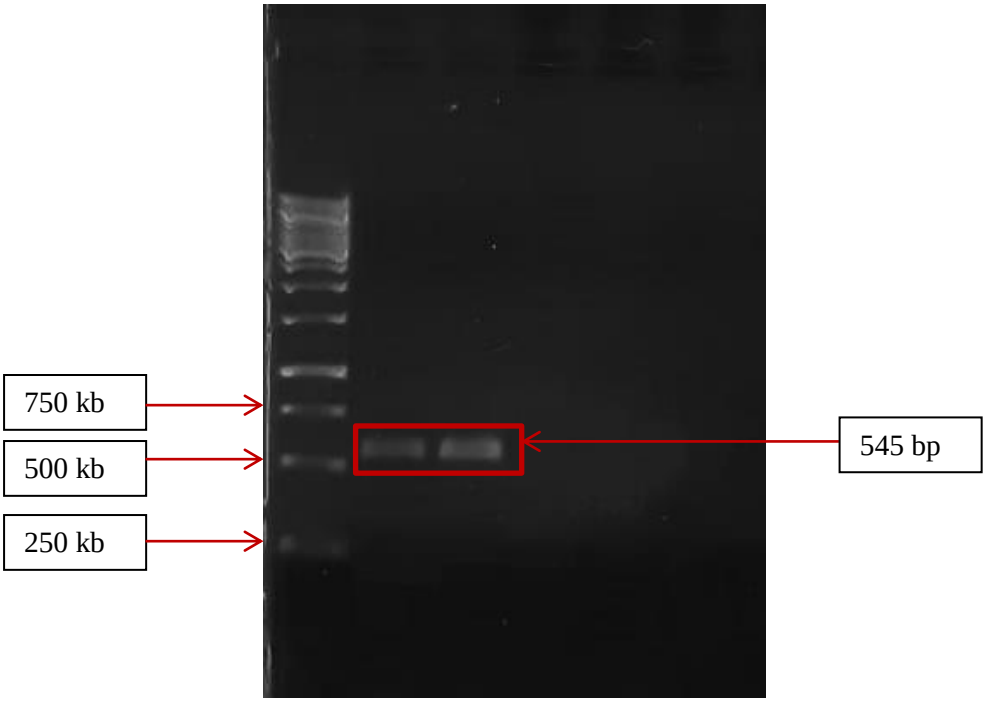


Fig 27: PCR amplified product of BM-16. Lane 1 - 1kb ladder, lane 2 and 3 - ITS amplified product

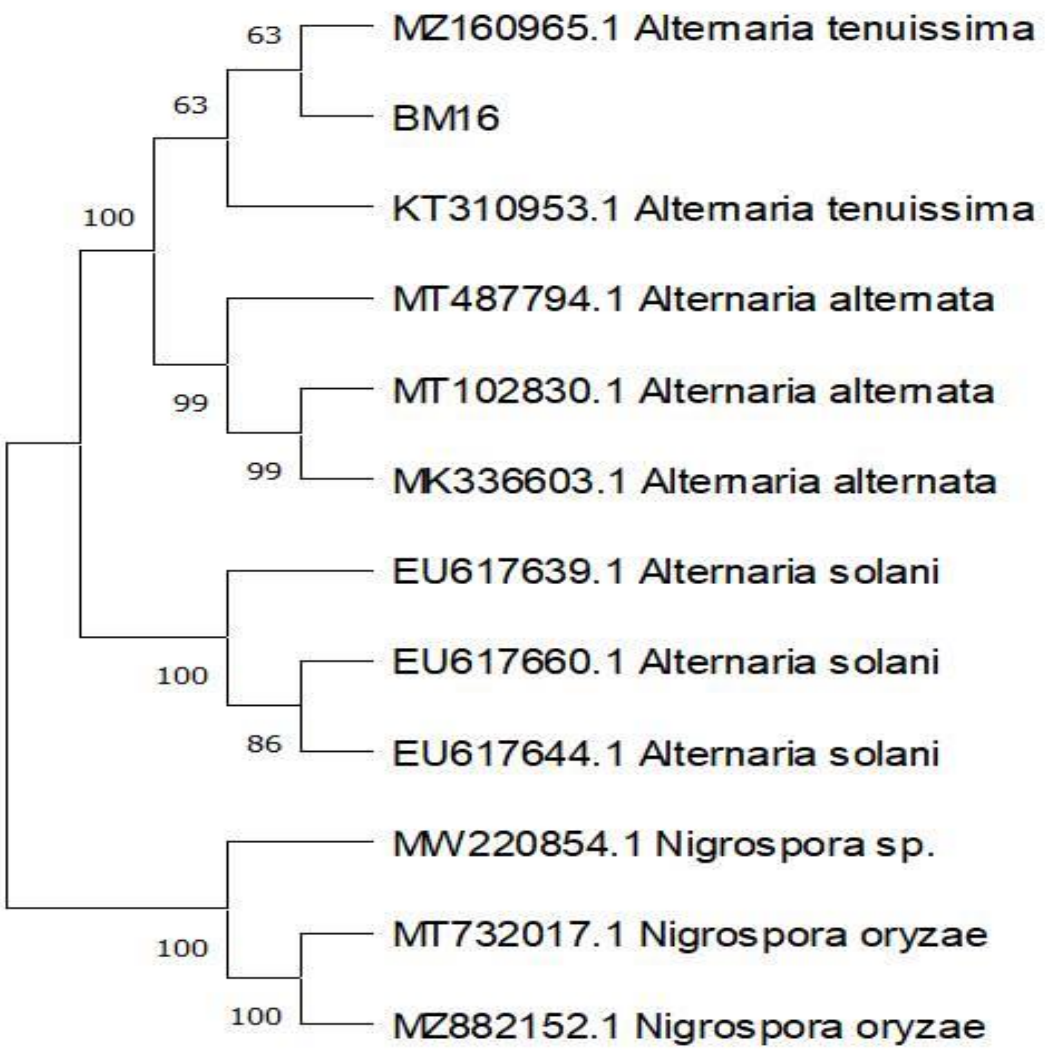


Fig 28: BM-16 was identified as *Alternaria tenuissima* after phylogenetic analysis via MEGA11 software (Neighbor Joining model with bootstrap value 1000).

Discussion

In the stems, leaves, and roots of many plants, there are tiny microorganisms called endophytic fungi. Endophytic fungi have mutually beneficial relationships with the plants which serve as their hosts. In exchange for the host plant's ability to benefit from their improved tolerance to biotic and abiotic stress, increased resilience to herbivores, diseases, etc., they obtain sustenance, shelter, and protection from the host plant. A wide variety of metabolites produced by endophytic fungi help plants flourish. Some fungal endophytes target woody plants and boost their growth and resilience, especially those that spread horizontally (Saikonnen *et al.,* 1998). Numerous elements, including the pattern of infection, environmental variables, the mode of transmission, and genetic makeup, may have an impact on the interactions between endophytes and host plants (Aly *et al.,* 2011). According to Qin *et al.,* (1998), medicinal plants have a distinct invasive microbiome that has the capacity to produce different and unique bioactive chemicals. According to Zhang *et al.,* (2006), the potential applications of secondary metabolites in the realms of medicine, agriculture, and industry are astounding.

In this study, the medicinal herb *B. monnieri* was selected for the isolation of endophytic fungi with acetylcholinesterase inhibitory potential. We chose this plant for the present study because of its neuroprotective effects against many neurological conditions like poor memory, anxiety, etc., and also because not much work has been done on the isolation of endophytic fungi and characterization of bioactive compounds for acetylcholinesterase inhibition on this plant. According to Jasim *et al.,* (2017), the valuable medicinal herb *B. monnieri* is used in traditional medicinal preparations to treat neurological conditions, improve memory, and stimulate brain processes. A total of seven endophytic fungi have been isolated from *B. monnieri.* For fermentation, the isolated fungal isolates were put in the PDB for a certain period of time. The endophytic crude fungal extract exhibiting acetylcholinesterase inhibitory potential was fractionated, purified, and characterized after preliminary tests for acetylcholinesterase (AChE) inhibitory and anti-oxidant activities (DPPH assay) on the crude extracts obtained from these isolates.

During preliminary in vitro screening of free radical scavenging activity BM-6 and BM-16 exhibited percentage free radical scavenging activity of 75.51 and 58.29% at 250 µg/ml respectively. Literature studies have revealed that the endophytic fungi *Arcopilus aureus* isolated from the grape plant *Vitis vinifera* showed significant antioxidant properties

(Dwibedi and Saxena 2019). In previous reports crude extract obtained from the endophytic fungus *Fusarium sp.* exhibited strong antioxidant property with IC50 values of 37.5 ± 3.5 µg/l (ABTS assay) and 191.3 ± 17.6 µg/ml (DPPH assay) respectively (Al-Qaralleh *et al.,* 2021). According to Chatterjee *et al.,* (2019) an endophytic fungus *Alternaria alternata* isolated from the medicinal plant, *Azadirachta indica* exhibited strong antioxidant activity with IC50 value of 38 ± 1.7 µg/mL. In vitro screening of AChE inhibition activity during preliminary tests showed that out of 7 fungal extracts BM-6 and BM-16 exhibited maximum percentage AChEI activity of 57.10% and 64.10% at 250 µg/ml respectively compared to that of the reference compound DPZ at 100 µg/ml (92%). In comparison to our findings, 5-methoxy-2-methyl-3-tricosyl-1, 4- benzoquinone and 1-O-methylemodin from the endophytic fungus *Colletotrichum* of *Huperzia serrata* possess AChE inhibitory activity (Li *et al.,* 2018). Long *et al.,* (2017) also reported that austin, dehydroaustinol, and preaustinoid isolated from *Aspergillus sp*. exhibited AChE Inhibitory activity with IC50 values of 2.50, 0.40, and 3.00 µM, respectively. Azaphilones, armochaetoglobins, and xanthenone from the endophytic fungus *Chaetomium sp.* indicated AChE inhibitory activity with IC50 values of 7.34, 5.19, and 4.23 µM, respectively (Xu et al., 2017). According to Bhagat *et al.,* (2016) a natural AChE inhibitor was reported from the endophytic fungus *Alternaria alternata* which was identified as 'altenuene' and exhibited AChE inhibitory activity of 78%. Vig *et al.,* (2021) also reported that quercetin showed significant AchE inhibitory activity produced by an endophytic fungus *Nigrospora oryzae* isolated from medicinal plant *Tinospora cordifolia.*

The UV absorbance spectrum of fraction F7 of BM-16 showed λ max at 204 nm and 282 nm. The FTIR spectrum of bioactive fraction showed a broad peak in the region of 3337.87 which was due to presence of hydroxyl group. The peak at 2973.32 region is due to the presence of C-H group. Other peak values of the spectrum are mentioned in the table 14. HRMS analyses of bioactive fraction F7 of BM-16 showed m/z values of 685.3523 and 338.3045. The peaks observed in UV spectroscopic analyses showed resemblance to certain compounds having similar or nearby m/z values (table 13).

According to Remya *et al.,* (2023) lobeline, a piperidine alkaloid, has been found to be a cholinesterase inhibitor. Due to the structural resemblances between lobeline and donepezil, a well-known acetylcholinesterase (AChE) inhibitor, it is hypothesized that lobeline may also possess AChE-inhibiting properties. In silico, in vitro, and biophysical investigations confirmed that lobeline may inhibit cholinesterase. According to the binding profiles, lobeline has a greater affinity for AChE. Molecular weight of the lobeline was found to be 337.5

which indicates the resemblance of m/z value of lobeline to that of the unknown compound whose m/z value observed in UV spectroscopic analyses, which was 338.3045 (Fig 23). As the results showed that fraction F7 of BM-16 exhibited a significant amount of AChE inhibitory activity, and HRMS peaks also show the resemblance of the m/z value to that of lobeline, preliminary phytochemical testing also indicated the presence of alkaloids in sample BM-16. So, it can be concluded that lobeline might be present in the fraction F7 of BM-16, which could be responsible for the AChE inhibitory activity of the fraction. Although further analyses need to be done to identify the particular compound showing the above-mentioned results.

Microscopic and molecular identification confirmed that the endophytic fungal isolate BM-16 was *Alternaria tenuissima.* Chatterjee *et al.,* (2022) isolated *Alternaria tenuissima* from the leaves of *Psidium guajava* L. producing neuroprotective, antimicrobial, antioxidant compounds like Cyclohexane, 1-hexyl-1-nitro, 2,3-Dihydrothiophene 1,1-dioxide etc. In another study by Wang *et al.,* (2014) an endophytic fungus Alternaria *tenuissima* SP-07 was isolated from the fresh root of Chinese herbal medicine Salvia *przewalskii* producing class of Solanapyrones which are potent antimicrobial compounds.

Conclusions

In the present study, medicinal herb *B. monnieri* was selected for the isolation and identification of endophytic fungi for acetylcholinesterase inhibitory potential. The above mentioned plant was selected as not much work has been done on the identification and characterization of bioactive compounds with AChE inhibition activity and moreover it has been used traditionally as a therapeutic tool since ages and have wide range of therapeutic applications in modern drug discovery as well as developmental processes and thus it could be explored for isolation of endophytic fungi and production of bioactive compounds.

In vitro studies on extract BM-16 obtained from endophytic fungus isolated from *B. monnieri* showed significant anti-oxidant and AChEI activities which were comparable to ascorbic acid and DPZ respectively. Various fractions were obtained after purification done via column chromatography and pooled to 7 fractions. The fraction 7 of BM-16 exhibited maximum AChEI activity of 65.53% at 320 µg/ml of concentration. UV spectroscopic analyses, HRMS and FTIR spectroscopic analyses of bioactive fraction F7 of BM-16 showed the presence of therapeutic compound which is AChE inhibitor. Further analyses need to be done in order to identify the particular compound. Microscopic and molecular identification confirmed that the endophytic fungal isolate BM-16 was *Alternaria tenuissima.*

So, it can be concluded that endophytic fungus *Alternaria tenuissima* is a hub of incredible compounds that have vast therapeutic potential. Such compounds needed to be elicited out and more research should be done. So that they can play a key role in the management of various neurodegenerative disorders like Alzheimer's disease.

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