

**Bioactive potential of endophytic fungi isolated from the medicinal plant
- *Terminalia arjuna***

A Dissertation submitted in partial fulfillment of the requirement for the award of
Degree of

Master of Technology

In

Biotechnology



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DECLARATION

I hereby declare that work presented in this thesis entitled "**Bioactive potential of endophytic fungi isolated from the medicinal plant-*Terminalia arjuna***" submitted under the supervision of Dr. M. Vasundhara in the partial fulfilment of the requirement for the award of the degree of Master of Technology in Biotechnology, Department of Biotechnology, TIET, Patiala, is my own original research work done during the period of July 2018 to July 2019. This report has not been submitted for the award of any other degree or certificate in this institute or any other institute.

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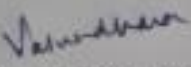
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CERTIFICATE

This is to certify that the work embodied in this thesis entitled "**Bioactive potential of endophytic fungi isolated from the medicinal plant-*Terminalia arjuna***" has been carried out by **Naina Saini**, Master of Technology (Biotechnology), under my supervision and guidance. She has fulfilled all the requirements for the award of the degree of Master of Technology in the subject of Biotechnology at Thapar Institute of Engineering and Technology, Patiala.

This is further certified that the work embodied in the thesis, is student's own work and has not been submitted for any other degree and unless otherwise stated, is all original.


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LIST OF ABBREVIATIONS

%	Percent
°C	Degree celcius
ml	Millilitre
µg	Microgram
µg/l	Microgram per litre
mg	Milligram
µl	Microlitre
m	Meter
nm	Nanometer
mm	Millimeter
g	Gram
mg/L	Milligram per litre
mg/ml	Milligram per millilitre
mg/kg	Milligram/kilogram
mg/dl	Milligrams per decilitre
w/v	Weight by volume
cm	Centimeter
IU/ml	International Units per Milliliter
rpm	Revolutions per minute
T2DM	Type-2 Diabetes mellitus
TA	<i>Terminalia arjuna</i>

MIC	Minimum Inhibitory Concentration
DPPH	2,2-Diphenyl-1-picrylhydrazyl
NO	Nitric Oxide
TLC	Thin layer chromatography
NaOCl	Sodium hypochlorite
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PBS	Phosphate buffer saline
DNS	Di-nitrosalicylic acid
Ac	Acarbose control
As	Acarbose standard
STZ	Streptozotocin
i.p	Intraperitoneally
GYP	Glucose Yeast and Peptone
DMSO	Dimethyl sulfoxide
MHA	Muller-hinton agar
MHB	Mueller-hinton broth
SEM	Standard error mean
MCD	Modified Czapek Dox
SD	Standard deviation
TB	<i>Terminalia</i> sp. Bark
AJ	<i>Arjuna</i>

Abstract

Terminalia arjuna is widespread in deciduous region of India. *Terminalia arjuna* exhibits anti-diabetic, anti-oxidant and anti-microbial activities. Endophytic fungi isolated from *Terminalia arjuna* possess antibacterial, anticancer and antioxidant properties. Therefore, the present study investigated the therapeutic effects of endophytic fungi obtained from medicinal plant, *Terminalia arjuna*. A total of 10 endophytic fungi were obtained from *Terminalia arjuna*. In *in vitro* alpha amylase inhibition assay TB3 showed significant percentage inhibition. Moreover, TB3 showed anti-diabetic effects in experimental rats. TB3 decreased the blood glucose level which was analogous to reference drug metformin. Fungal extracts TB1, TB3 and AJ showed free radical scavenging activity which was comparable to standard compound Ascorbic acid. All fungal extracts showed significant percentage inhibition against bacteria like *P. aeruginosa*, *S. Aureus* and *B. subtilis*. TB1, TB2, TB3 and TB4 showed amylolytic activities. Fungal cultures AJ, TB1 and TB3 showed the positive results in L-Asparaginase activity. Thus, it can be inferred that endophytic fungi obtained from *Terminalia arjuna* showed anti-diabetic, anti-oxidant, anti-bacterial, amylolytic and L-Asparaginase activities.

Endophytic fungi belong to a varied polyphyletic groups of organisms that dwell in coalition with their host plant mainly within leaves, stems, roots (Bacon and White 2000). They exhibit variety of relationships with their host plant ranging from mutualistic to antagonistic relation. Chemotaxis refers to movement of plant towards chemical secretion and it plays a key role in the colonization of fungal endophytes. At the same time, variety of defense secondary metabolites like oils and saponins are produced by the plants, as a impedance mechanism to the foreign plethora of microorganisms including endophytic fungi present around or within the plant. Therefore, these secondary metabolites create a problem in colonization of endophytic fungi. In response of metabolites fungal endophytes activates their self defense system by releasing detoxifying enzymes such as proteases, laccases, cellulases, lactases and xylanases for the detoxification of secondary metabolites to make their pathway easy for entering and colonizing inside the host plant.

Endophytic fungi are source of varied bioactive compounds. Sometimes endophytes produce similar novel secondary metabolites that are formed by their host plants (Tan and Zhou 2001). Discovery of *Taxomyces andreanae* an endophytic fungus from pacific yew (*Taxus brevifolia*) that can produce Taxol, a potent anti-cancer drug originally derived from its host plant, is an instance of production of novel compounds from the fungus (Stierle *et al.*, 1993). During evolution, endophytic fungi might have undergone genetic recombination by inheriting the ability to synthesize the metabolites similar to host plant. The bioactive compounds obtained from endophytic fungi could be categorized as alkaloids, steroids, tannins, terpenoids, quinines, lignans, phenols and lactones. In literature many treasured secondary metabolites have been tapped from fungal metabolites having potential to use them as antimicrobial, herbicidal, antioxidant, insecticidal, cytotoxic and anticancer compounds (Vasundhara *et al.*, 2016).

Medicinal plants having a distinctive microbiome that have potential to produce unique and divergent bioactive compounds (Qin *et al.*, 2012). These bioactive metabolites have importance in pharmaceutical industry (Strobel *et al.*, 2004; Wiyakrutta *et al.*, 2004; Kumar *et al.*, 2005; Tejasvi *et al.*, 2007). Secondary metabolites obtained from endophytic fungi possess immense bioactivities in the field

of medicine, agriculture and industries (Tan and Zho, 2001; Zhang *et al.*, 2006). In the present study, *Terminalia arjuna* was selected for study as it has medicinal properties. Since ancient times ayurvedic formulations of *Terminalia arjuna* have shown therapeutic effects against various diseases.

WHO reported that approximations 80 % of the world's population together with India still depends on the medicinal plant products. The bulk of novel drugs (70 %) which are accessible in the market or still under clinical trials pioneered in the whole world are derivatives of natural products, primarily plants. These medicinal plant products are extensively used as curative tool for averting the growth of micro-organisms. These plant products include diverse group of metabolites (primary and secondary) and illustrated a range of medicinal activities e.g. anti-inflammatory, anti-viral, anti-diabetic etc. (Gupta *et al.*, 2015).

Vernacular name of *Terminalia arjuna* (family combretaceae) is arjuna. This plant is found in many parts of India. Arjuna is a common tree and used in ayurvedic medicines since 1900's. Different parts of *Terminalia arjuna* such as stem, bark, leaves and their fruits have shown remedial effects against various diseases. It has been demonstrated that bark of *Terminalia arjuna* exhibits cardioprotective, anti-ischemic, anti-oxidant, fungicidal, anti-bacterial, anti-nociceptive, anti-diabetic, anticancer and anti-inflammatory activities. It is useful in the treatment of obesity, hypertension, hyperglycemia. Furthermore, ethnopharmacological studies revealed that higher phenolic compounds and flavonoids play a chief role in the anti-oxidative potential of *Terminalia arjuna*. In addition to this, *Terminalia arjuna* is considered as cardiogenic agents. Thus, it is continuously used as a tonic for flourishing cardiovascular system (Kouipou *et al.*, 2019).

Recent report illustrated that taxol has been produced by endophytic fungus *Alternaria brassicicola* of *Terminalia arjuna* (Gill *et al.*, 2019). Similar to this, Gangadevi *et al.*, 2009 reported that endophytic fungus *Pestalotiopsis terminaliae* produced taxol as its secondary metabolite.

An endophytic fungus *Chaetomella raphigera* obtained from the medicinal plant *Terminalia arjuna* has shown taxol yield of 79.6 microg/L. Tejaswi *et al.*, 2008 have reported that endophytic fungus *Pestalotiopsis* isolated from *Terminalia arjuna* exhibits more than 75 percentage inhibition against five bacterial strains including

Bacillus subtilis, *Escherichia coli*, *Pseudomonas fluorescens*, *Xanthomonas axonopodis* pv. *malvacearum*, and *Staphylococcus aureus*. Bioactive metabolite from *Pestalotiopsis* showed anti-oxidant activity. Literature review suggests that inner bark of *Terminalia arjuna* attenuated cardiovascular abnormalities (Tejasvi *et al.*, 2008).

Therefore, in the present report, bioactive potential of endophytic fungi isolated from *Terminalia arjuna* were explored as the plant is known to produce a variety of bioactive compounds.

Objectives:

- Isolation of endophytic fungi from medicinal plant *Terminalia arjuna*
- To perform bioactivity studies on the isolated endophytic fungi

2.1 Endophytes

The term endophyte has been characterised by different authors in numerous ways. Initially, De Bary (1866) named the term ‘endophyte’ for the fungi that stay within the host plants and distinguish them from epiphytes. Carroll (1986) explained these as the organisms that live in plant tissues without showing asymptomatic infection to the host plant except mycorrhizal and pathogenic fungi.

Afterwards, Petrini (1991) elaborated Carroll’s definition as all the organisms that colonize within the plant tissue without causing any negative effects to the host at any stage of their life cycle. Wilson (1995) classified endophytes into two type’s fungi and bacteria, which dwell in plants during their life cycle and does not cause any infections. This term was further explained by Bacon and White (2000) by defining endophytes as the microbes that colonize the plant without causing immediate harmful effect to plants. Some endophytes mediate interaction between plants and their competitors.

2.2 Classification and transmission of endophytic fungi

Endophytes transmit through two modes of transmission, one is horizontal and another is vertical. Horizontal mode of transmission is followed by majority of endophytes to reach their host plants via airborne spores. Other endophytes may follow vertical mode of transmission through seeds. The endophytes which never appear outside their host and are considered as true endophytes and these are vertically transmitted to the host (Hartley and Gange 2009). Primarily based as the variations in the host plant, taxonomy, ecological features, and evolutionary relatedness, Rodriguez (2009) placed endophytic fungi into two groups (Clavicipitaceous endophytes and non-clavicipitaceous endophytes).

Class 1 Clavicipitaceous endophytes: Endophytes of this group infects grasses, sedges and rushes. These invade as endophytes, saprophytes and pathogens believed to produce some bioactive compounds.

Class 2 Non-clavicipitaceous endophytes: Majority of them belongs to ascomycetes and minority to basidiomycetes. They inhabit inside the stems, roots and leaves of host plants (Steiner *et al.*, 2006).

2.3 Fungal endophytes isolated from medicinal plants

Since times, large number of medicinal plants have been explored to cure various diseases. In ancient records such as Vedas and Bible it is seen that herbal remedies and their preparation are useful in treating various ailments, and these are found to be helpful in curing disease (Kaul *et al.*, 2012). Diverse microbiome resides within medicinal plants that produce novel and varied secondary metabolites (Qi *et al.*, 2012). The microbiome that resides on plants are called epiphytes and the other class is classified as “endophytes”. These are the microorganisms that resides inside the host tissue by following distinct metabolic pathway. The metabolic pathway of host and endophytes is little similar, that increases the probability of production of secondary metabolites similar to the host plant. Basically production of such compounds increases under stress conditions.

Researchers are exploring new medicinal plants for the isolation of novel compounds from them and their residing endophytes. This would be beneficial for pharmaceutical industries to develop novel molecules to treat diseases. Endophytes are reported to mimic their host and produce similar kind of bioactive compounds. Therefore, it is significant to explore fungal endophytes. Isolation of endophytes will result in ecofriendly, inexhaustible and inexpensive production of interested compounds and therefore help in conservation of biodiversity. These also provide chance to isolate the bioactive compounds from the endemic plants. These compounds can be produced on large scale by fermentation. Their capability to synthesize bioactive metabolites has created the enthusiasm on bioprospecting them from medicinal plants (Kusari *et al.*, 2012).

Table 1: Endophytic fungi isolated from different medicinal plants

S. No	Endophytic fungi	Medicinal plants	Activity	References
1	<i>Rhizoctonia spp.</i>	<i>Annona crassiflora</i>	Antimicrobial	(De Mendonca <i>et al.</i> , 2015)
2	<i>Aspergillus sp.</i>	<i>Limonia acidissima</i>	Cytotoxic	(Siriwardane <i>et al.</i> , 2015)
3	<i>Penicillium funiculosum</i> <i>Alternaria solani</i>	<i>Gloriosa superba</i>	Antimicrobial	(Devi <i>et al.</i> , 2014)
4	<i>Alternaria alternata</i>	<i>Tabebula argentea</i>	Cytotoxic	(Guevara <i>et al.</i> , 2019)
5	<i>Fusarium oxysporum</i>	<i>Zingiber zerumbet</i>	Antioxidant	(Keerthi <i>et al.</i> , 2016)
6	<i>Chaetomium globosum</i>	<i>Withania somnifera</i>	Antifungal	(Kumar <i>et al.</i> , 2013)
7	<i>Periconia spp.</i>	<i>Piper longum</i>	Antimycobacterial	(Verma <i>et al.</i> , 2011)
8	<i>Phyllosticta sp.</i>	<i>Cuazuma tomentosa</i>	Antioxidant	(Srinivasan <i>et al.</i> , 2010)
9	<i>Trichoderma sp.</i>	<i>Centaurea stobe</i>	Antifungal, Cytotoxic	(Li <i>et al.</i> , 2016)
11.	<i>Fusarium sp.</i>	<i>Dysosma versipellis</i>	Antimicrobial, Anticancer	(Xiaoming <i>et al.</i> , 2018)
12	<i>Botryosphaeria fusarium</i>	<i>Camptotheca acuminata</i>	Antimicrobial	(Ding <i>et al.</i> , 2009)

2.4. *Terminalia arjuna*

Arjuna is located in deciduous region of India. It is a large tree with the height of 20-25 metres. It forms spreading crown with wide canopy with drooping branches. Leaves are elliptical having green upperside having a pinch of brown color. Flowers appear between March and June and are glabrous. The bark of *Terminalia arjuna* has unique features. Bark of *Terminalia arjuna* has smooth-appearance and is pinkish grey from periphery (Manu *et al.*, 2019).

Table 2: Classification of *Terminalia arjuna*

S.No	Classification	<i>Terminalia arjuna</i>
1	Class	Magnoliopsida
2	Division	Magnoliophyta
3	Family	Combretaceae
4	Genus	Terminalia
5	Kingdom	Plantae
6	Order	Myrtales
7	Species	Arjuna

(Paarakh 2010)

2.4.1 Endophytic fungi isolated from *Terminalia arjuna*

Researchers have been working since many years for exploring endophytic fungi from *Terminalia arjuna*, a number of fungal isolates have been isolated from *Terminalia arjuna* (Table 3).

Table 3: List of Endophytic fungi from *Terminalia arjuna*

S.No	Endophytic fungi	Medicinal plant	References
1	<i>Aspergillus flavus</i> <i>Diaporthe arengae</i>	<i>Terminalia arjuna</i>	(Maheshwari <i>et al.</i> , 2014)
2	<i>Pestalotiopsis clavispora</i> <i>Pestalotiopsis theae</i> <i>Pestalotiopsis microspora</i>	<i>Terminalia arjuna</i>	(Tejesvi <i>et al.</i> , 2019)
3	<i>Chaetomella raphigera</i>	<i>Terminalia arjuna</i>	(Gangadevi <i>et al.</i> , 2009)
4	<i>Pestalotiopsis terminaliae</i>	<i>Terminalia arjuna</i>	(Muthumary <i>et al.</i> , 2009)
5	<i>Hyphomyces</i> <i>Alternaria alternata</i> <i>Nigrospora oryzae</i> <i>Cocblonema spp.</i> <i>Penicillium spp.</i> <i>Monocillium spp.</i> <i>Peslalotiopsis spp.</i> <i>Botryodiplodia theobromae</i> <i>Epicoccum nigrum</i> <i>Alternaria spp.</i> <i>Cladosporium elatum</i> <i>Gliocladium penicilloides</i>	<i>Terminalia arjuna</i>	(Tejesvi <i>et al.</i> , 2005)

2.5 Anti-diabetic activity

Diabetes mellitus is the rapid growing metabolic endocrine disease worldwide. Diabetic patients are increasing speedily worldwide with a projected assessment of about 439 million diabetic patient globally by 2030. Majority of people suffers from

type-2 class of diabetes mellitus (T2DM). Numerous biological and behavioral parameters play a pivotal role in the devolution of T2DM (Garabadu *et al.*, 2014).

2.6 Assessment of bioactive compounds of *T. arjuna* for lesion therapeutic activity in rats.

The effect of topical use of phytoconstituents isolated from a hydroalcohol concentrate of the bark of *Terminalia arjuna* tree, was analysed for the healing of rat dermal injuries using *in-vivo* models. However, topical treatment with fraction 1 possesses tannins and topical treatment of fraction 1 increased the tensile strength of wounds. Moreover, fraction 1 showed anti-microbial activity against various microorganisms like *Escherichia coli*. Fraction 1 has also shown astringent effects of tannins (Chaudhari *et al.*, 2006).

2.7 Antimicrobial potential

It has been seen that quite ailments are recovering from *Terminalia arjuna* and *Camellia sinensis*. *Camellia sinensis* and *Terminalia arjuna* showed anti-cancer effects in addition to curing of cardiovascular ailments. Ethylacetate extracts of above mentioned plants showed anti-microbial effects against bacteria like *S. aureus* and fungus *C.albicans*. Range of inhibition zone was from 9 to 18 mm. The minimum inhibitory concentration (MIC) of TA and CS extracts ranged from 6.25 to 12.5 mg/ml. The minimum bactericidal concentration (MBC) of *T. arjuna* was observed to be 25 mg/ml. (Gupta *et al.*, 2016).

In this study, unorganized cultures were extracted from medical specialty tree *Terminalia arjuna* was implanted on MS medium with suitable concentrations of growth regulators and used seeds as explant. The callus extract were used to analyse the antimicrobial activity. There were many tests used for qualitative calculation of flavonoids and quantitative calculation of flavonoids such as TLC, infrared spectral and spectrophotometry studies. It also confirmed that Luteolin, Kaempferol were present in callus with higher amount as compared to all plant parts. The flavonoid extracts and callus extract were used to analyse the antimicrobial activity against bacteria's (For example: Gram-positive bacteria *B. subtilis*, *S. aureus*, Gram- negative bacteria *E. coli*, *K. pneumoniae* and *C.*

albicans). The extract have taken from bark of *T. arjuna* has also showed greater antimicrobial activity against microorganisms because of maximum concentrations of Flavonoids (Sharma et al., 2012).

2.8 Antioxidant activity

Ethanollic extracts of leaves and bark of *T. arjuna* were used to assess the antimicrobial and anti-oxidant properties. Disc diffusion method was used to evaluate the antimicrobial activity and DPPH, FRAP, NO assays were used to estimate the anti-oxidant activity. The n-butanolic fraction was shown more antimicrobial activity. The zone of inhibition of bark (15.0 ± 0.7 mm, 15.5 ± 0.7 mm, 15.0 ± 1.5 mm, 15.5 ± 0.7 mm, 15.0 ± 0.7 mm, 15.0 ± 0.7 mm) was seen against *B. subtilis*, *S. aureus*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. typhi* respectively. The n-butanol showed highest anti-oxidant activity as compared with ethanolic extract, ethyl acetate fraction, chloroform fraction, aqueous fraction and ascorbic acid. Overall, it can be clearly seen that the n-butanolic fraction of the parts of *Terminalia arjuna* showed more antioxidant and antimicrobial activity (Kumar et al, 2013).

2.8 Screening for L-asparaginase activity

It is examined that L-asparaginase isolated from different microbial sources (for example: bacteria produce glutaminase upto 10%). It can be isolated from plant tissues and animals, but generally glutaminase derived from microbial sources, for instance: bacteria, fungi and yeast. (Campbell and Mashburn, 1969; Sajitha et al., 2013). In this method, using glutamine was used instead of phenol red dye in MCD agar plates and then fungal extracts were screened for L-Asparaginase production (Thangavel et al., 2013). Few ascomycetes fungi showed L-asparaginase and L-glutaminase activity. Whereas, *Candida scottii* and *Cryptococcus albidus* showed only L-Asparaginase activity. The inhibitors were used for biosynthesis of glutamine and asparagine in combination with L-asparaginase leads to increase in the therapeutic efficiency L-asparaginase enzyme (Li et al., 2019).

Table 4: List of Bioactive compounds of *Terminalia arjuna*

Sr. No.	Bioactive compounds of <i>Terminalia arjuna</i>	References
1.	Alkaloids, carbohydrates, phytosterols, phenolics, tannins, flavonoids, triterpenoids	(Ramesh <i>et al.</i> , 2010)
2.	Coumarins, saponins, terpenes	(Kaul <i>et al.</i> , 2010)

3.1 Collection of plant material

The medicinal plant, *Terminalia arjuna* was selected for present study. Fresh aerial parts viz leaves and bark of *T. arjuna* were collected from TIET campus, Patiala (Punjab).

3.2 Surface sterilization of the collected samples

Samples collected were brought to TIFAC-CORE in a glass jar for further processing. Excised plant parts were stored in glass jars and covered with a mesh and were left under running water for a period of 10 minutes for the removal of debris. After this detergent was added and jars were shaken properly for proper cleaning of samples and were kept under running water for another 10 minutes for removal of traces of detergent. Cleaned samples were sterilized further in 70% ethanol for 1 min. Sodium hypochlorite (NaOCl) was added and left for 5 minutes for disinfection purpose and another washing was performed with 70% ethanol. Finally, three washings were given using deionised water in the laminar air flow (Wiyakrutta *et al.*, 2014).

3.3 Isolation of Endophytic Fungi

For isolation of endophytic fungi following steps were performed in the laminar air flow. Plant pieces which were previously surface sterilized were dried using autoclaved tissue paper and cut upto 1 cm in length with sterile blade (Kumaresan *et al.*, 2015). Samples were then inoculated on freshly made PDA plates supplemented with an antibiotic i.e streptomycin (200 mg/l). After inoculation the plates were incubated for 7 to 14 days at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and were checked regularly after every 2 to 3 days for the growth of endophytic fungi.

3.4 Purification of Fungal endophytes

Purification of fungal endophytes was done by transferring the hyphal growth from a mixed population of fungal endophytes onto the potato dextrose agar plates with the help of sterile blade. The plates were incubated at $25 \pm 2^{\circ}\text{C}$ for 7-14 days. Preservation of master culture obtained was done by storing at 4°C (Toghueo *et al.*, 2019).

3.5 Subculturing of the isolated endophytic fungi

For sub-culturing of the isolated fungi, 1 cm of discs of mycelia grown on PDA plate were excised and inoculated on freshly made PDA plates carefully in order to prevent dispersal of spores. Plates were then incubated for 7 days at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$. The pure cultures of isolated endophytic fungi were coded and preserved at 4°C . Subculturing of endophytic fungi was done periodically.

3.6 Fermentation of isolated endophytic fungi

To carry out fermentation, 250 ml of PDB was prepared in 500 ml of Erlenmeyer's flasks and was autoclaved. The discs were cut from the isolated fungal culture and added into PDB. Around two to three discs were inoculated into the medium. It was kept for incubation at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ under static condition for 21 days. The purpose of incubation at static condition is to allow adequate production of secondary metabolites (Tawfike *et al.*, 2019).

3.7 Extraction of crude extract from fungal broth

After 21 days of incubation, filtration was performed by using sterile muslin cloth and funnel. Filtrate was collected in sterile Erlenmeyer's flask and equal amount of ethyl acetate was added. Solvent extraction procedure was performed by taking filtrate and ethyl acetate in separating funnel and shaking it continuously for 15 to 20 minutes. Later, it was allowed to stand still until organic and inorganic phases were completely separated. Organic phase was accumulated in sterile flasks and sodium sulphate was added for the removal of water. Organic phases were further subjected to rotary-evaporation. Evaporation was done at 35°C under reduced pressure. The condensation temperature was 4°C . Dried crude extracts were collected in small glass vials and were stored for further testing of their biological activities. This extraction procedure was performed for all the filtered cultures (Tawfike *et al.*, 2019).

3.8 *In vitro* and *in vivo* anti-diabetic activity

3.8.1 *In vitro* α -amylase inhibition assay

Crude extract of *T. arjuna* was tested to evaluate α -amylase inhibition activity through the method documented in Popli *et al.*, 2018. Reaction mixture was composed of 50 μ l phosphate buffer saline (PBS) (100mM, PH-6.8), 10 μ l of alpha-amylase (2U/ml, 1mg= 2U and stock 1mg/ml) and 20 μ l (0.5 mg/ml) of extract. Incubation of reaction mixture was done at 37°C for 20 minutes.

After that 20 μ l of 1% soluble starch (100mmM phosphate buffer, pH-6.8) was added as a substrate and incubated further at 37°C for 30 min. After incubation 100 μ l of the DNS (di-nitrosalicylic acid) color reagent was added and boiled for 10 min. Acarbose with concentration of 100 μ g/ml was used as a standard. The color change was observed and absorbance was recorded at 540 nm in multiplate reader.

The results were expressed as percentage inhibition and calculate using the following formula:

$$\text{Inhibitory activity (\%)} = 1 - \text{Ac/As} \times 100$$

Acarbose stock: 1mg/ml

Negative control : 80 μ l buufer + 20 μ l soluble starch + 100 μ l DNS

Positive control: 50 μ l buffer + 10 μ l alpha-amylase + 20 μ l acarbose + 20 μ l soluble starch + 100 μ l DNS

Main reaction (MR): 70 μ l buffer + 10 μ l alpha-amylase + 20 μ l soluble starch + 100 μ l DNS

Samples (S): 50 μ l buffer + 10 μ l alpha-amylase + 20 μ l extract + 20 μ l soluble starch + 100 μ l DNS.

3.8.2 *In vivo* - Selection of animal models

The crude extract of TB1, TB2 and TB3 were sent to IIT, BHU for *in vivo* studies. The T2DM was induced in overnight fasted rats by a single injection of streptozotocin (45 mg/kg, i.p.), 15 min after nicotinamide (110 mg/kg, i.p.) administration (Garabadu *et al.*, 2014).

Mice were randomly divided into six groups with six animals namely control, STZ, TB1, TB2, TB3 and Metformin. TB1 (10mg/kg), TB2 (10mg/kg) and TB3 (10mg/kg) were administered intraperitoneally to TB1, TB2 and TB3 groups respectively. The metformin (10mg/kg) was also administered i.p. to Met group. Vehicle was administered to control and STZ groups. Rats were fasted overnight and T2DM was induced by a single injection of streptozotocin (45 mg/kg; i.p.), 15 min after nicotinamide (110 mg/kg; i.p.). Streptozotocin was dissolved in 0.1 M citrate buffer (pH 4.5) and nicotinamide was dissolved in physiological saline. After the administration of streptozotocin and nicotinamide 1 ml of blood was accumulated through retroorbital puncture and centrifuged at 3000 g for 5 min at 4°C. to obtain the plasma for measuring blood glucose level (GOD PAP kit) (Garabadu *et al.*, 2017)

3.9 Antioxidant activity

Antioxidant was done in 96-well microtitre plate. (2,2-Diphenyl-1-picrylhydrazyl) DPPH is a commercially available nitrogen containing radical. It is extensively used to measure the intensity of compounds, which are radical scavengers. DPPH gives violet color when in methanol. Once encountered by an antioxidant it gets stabilised by electron donation resulting in the change of colour from violet to yellow due to production of reduced product i.e diphenylpicrylhydrazine.

Fungal extracts were dissolved in methanol to make concentrations ranging from 100 µg/ml to 1000 µg/ml and further mixed with methanolic solution of DPPH. Methanol was used as blank. Ascorbic acid (1mg/ml) was used as standard antioxidant agent (Zhang *et al.*, 2019).

A template was designed at the start of the assay of 96 well plates for the performance of the experiment at different concentrations:

M: Methanol i.e (200 µl)

MD: Methanol (50 µl) + DPPH (150 µl)

AAS (Ascorbic acid standard): Ascorbic acid (20 µl) + DPPH (150 µl) + Methanol (30 µl)

AAC (Ascorbic acid control): Distilled water (20 µl) + DPPH (150 µl) + Methanol (30 µl)

Sample: Extract + Methanol (50 µl) + DPPH (150 µl)

Once the experiment was conducted in the 96 well plate it was kept in the dark for incubation i.e 45 minutes. On completion of incubation, reduction of DPPH radicals was observed by measuring absorbance in microplate reader at a wavelength of 517 nm.

$$\text{Percentage scavenging activity} = \frac{(\text{Control's Absorbance} - \text{Extract's Absorbance}) \times 100}{\text{Control's Absorbance}}$$

Table 5: Experimental design for DPPH assay

Sample concentrations (µg/ml)	Total volume (µl)	Fungal extracts (µl)	Methanol (µl)	DPPH (µl)
100	200	4	46	150
250	200	10	40	150
500	200	20	30	150
1000	200	40	10	150

3.10 Antibacterial-activity

3.10.1 Agar well diffusion antimicrobial assay of crude extract

Cultures used for antibacterial testing

Test bacterial cultures

- ✓ Gram positive bacteria: *Bacillus subtilis*, *Staphylococcus aureus*
- ✓ Gram negative bacteria: *Pseudomonas aeruginosa*, *Escherichia coli*

Procedure: Agar well diffusion assay was performed to evaluate the antibacterial (bacterial) potential of crude extracts against the above test cultures.

Preparation of McFarland Standard

The approximate number of bacteria in liquid suspension was standardized by using McFarland standard. This was used by comparing the turbidity of McFarland standard with the turbidity of test organism's suspension. The standard was prepared by addition of barium chloride into the sulfuric acid for the precipitation barium sulfate. By altering the volume of these two reagents standard of different degree of the turbidity can be prepared to represent the many different concentrations of bacteria. Most commonly used standard in clinical microbiology laboratory for antimicrobial testing is 0.5 which represents 1.5×10^8 (these range from 1.0×10^8 to 2.0×10^8 bacteria/ml).

1. 85 ml of 1% H₂SO₄ was added to a 100 ml volumetric flask.
2. 0.5 ml of 1.175% BaCl₂.H₂O was added drop wise with auto-pipette to H₂SO₄ by constantly swirling the flask.
3. Final volume of 100ml was made with 1% H₂SO₄.
4. The flask was kept on magnetic stirrer for 4-5 min. And the OD was recorded at 600 nm.

Well grown colonies of the bacteria on the Mueller-hinton agar plate was sub cultured on the MHA plates and incubated overnight at 37°C. From the MHA plates colony was picked with a sterile loop and transferred to MHB. This was then placed on shaker at 37°C for 1-2 hours, so that the O.D reaches 0.5 McFarland. 100 µl of the test organism was then spread over the MHA plates.

Then 6 mm bore was made with the help of cork borer and 50 µl of the crude extract was loaded into the wells and then incubated at 37°C for 24 hours. Then the diameter of zone of inhibition was measured that formed around the wells (Burhamzah *et al.*, 2018).

Ampicillin was used as antibacterial control (Hadacek and Greger, 2000).

3.11 Screening for fungal extracellular enzymes

Exploring large number of microorganism for production of useful enzymes provides an idea that filamentous fungi are most effective amongst all other microorganism. Easy recovering and high production of extracellular enzymes by filamentous fungi gain interest of many researchers (Bhagobaty and Joshi 2012) to obtain enzymes from these species. These enzymes are more thermo tolerant than the enzymes obtained from other sources, because of that they gain interest in many fields such as human health, agriculture and industry. Endophytic fungi are known to produce many novel molecules and represent an interesting option to be explored for the production of enzymes. Varied extracellular enzymes such as lipase, pectinase, laccases, cellulases, chitinases etc. are produced by fungal endophytes. These have the potential to use them in various industrial processes such as textile, leather, pulp, paper, food, beverages. The extracellular enzyme production varies among the endophytes.

3.11.1 Amylolytic activity

Amylase activity of TB1, TB2, TB3, TB4 and AJ was determined by growing the fungi on Glucose Yeast Extract Peptone Agar (GYP) medium and supplemented with 0.2% soluble starch and incubated for 7 days. After the incubation iodine treatment was given with 1% iodine and 2% potassium iodide. Thereafter, a visible zone of starch degradation on the plate indicated the presence of amylase (Popli *et al.*, 2018).

3.11.2 L-Asparaginase activity

L-Asparaginase production screening was done for five endophytic fungi according to (Li *et al.*, 2019) with slight modifications. Modified Czapek Dox (MCD) media was used for screening of endophytic fungi. Phenol red and L-Asparaginase were added to MCD media and stirred properly. Plates were kept in incubator at 28°C for 12 hrs. The mycelium plugs of 7 days old culture were inoculated on MCD agar plates and plates were incubated at 28°C. Plates were observed regularly for formation of pink zone for seven days.

3.9.2.1 Qualitative test

Agar well diffusion assay is performed according to Heatley 1994. Filtrates of fungal isolates were screened for L-Asparaginase production. Agar plates containing phenol

red and L-Asparaginase were prepared and 5 mm wells were made using sterilized cork borer. Culture broth was suspended in wells and plates were incubated at 37°C for two days. Plates were observed regularly for the formation of pink zone (Chou *et al.*, 2015)

In this present study, medicinal plant *Terminalia arjuna* has been selected for the isolation of endophytic fungi. The above medicinal plant was chosen for study as not much work has been done on the isolation of endophytic fungi and bioactivity of these fungi. *Terminalia arjuna* has therapeutic potential, hence it could be explored for the presence of endophytic fungi having capability to produce metabolites having bioactivity.

4.1 Collection of samples from *Terminalia arjuna*

The samples were collected from the campus of TIET, Patiala. The bark and the leaves of the plant *Terminalia arjuna* were collected from G block area (Fig 1). Samples were then transferred into sterile bottles and brought to TIFAC-CORE laboratory for further studies.



Fig. 1 Tree of *Terminalia arjuna*

4.2 Surface sterilization and isolation of endophytic fungi from *Terminalia arjuna*

The tissues of the plant were surface disinfected and transferred to the PDA plates which were then incubated at $25 \pm 2^\circ\text{C}$ for 7-14 days. The plates were observed regularly to check for the growth of endophytes. A total of 10 endophytic fungi were

obtained from *Terminalia arjuna* during a period of 7-14 days. Endophytic fungi obtained from *Terminalia arjuna* and named as TB1-TB10 (*Terminalia* sp. Bark). Pre-isolated culture AJ (Arjuna) from *Terminalia arjuna* was also taken for studies.

The purification of the fungal endophytes from a mixed population of fungal endophytes was done so as to get the pure strains. It was done by using sterile blade and inoculum was taken from plate and placed onto the potato dextrose agar medium. The plates were incubated for 3-7 days at $25 \pm 2^\circ\text{C}$. After the growth of the isolated endophytic fungi, the master cultures were given respective codes and preserved at 4°C for further studies (Fig. 2).

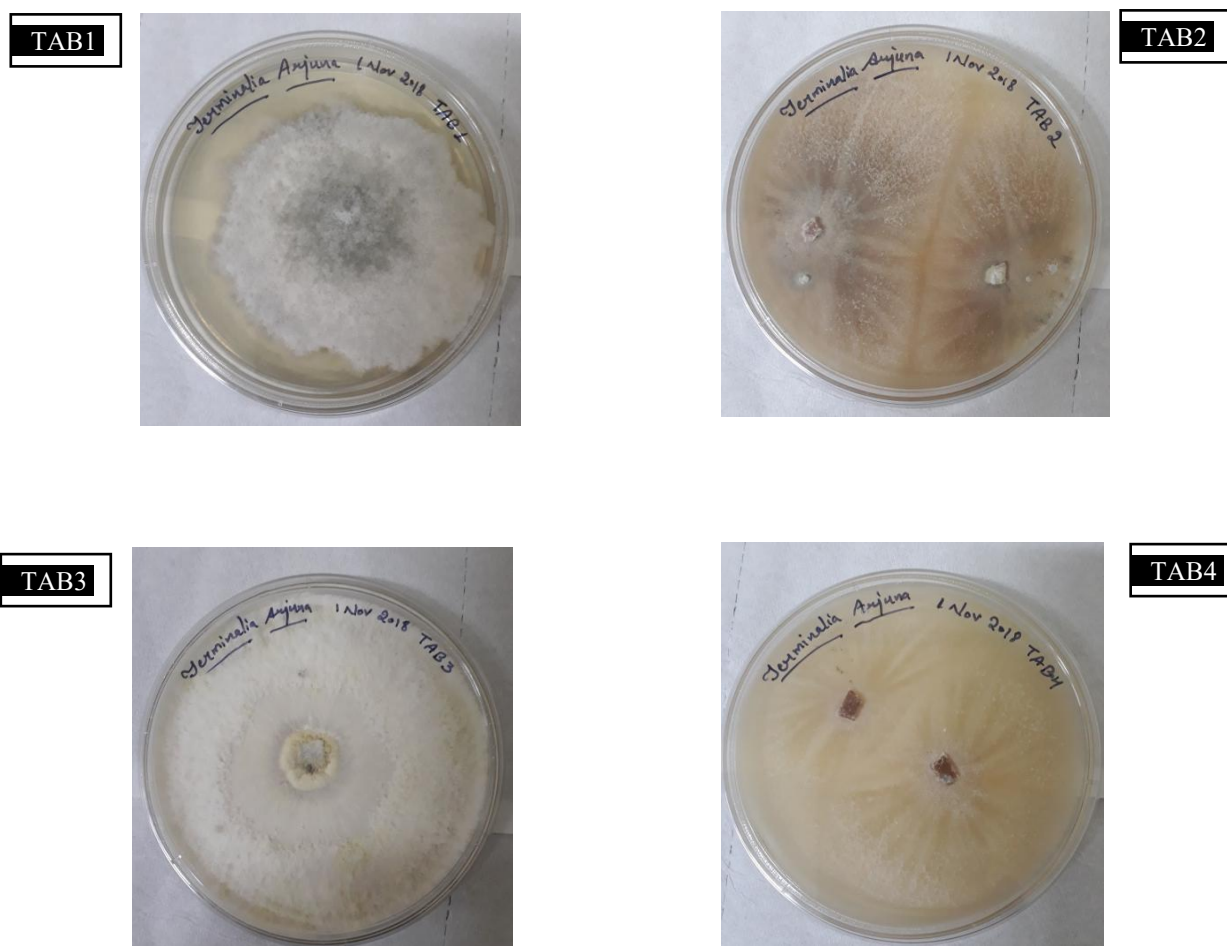


Fig. 2 Endophytic fungi obtained from *Terminalia arjuna*

4.3 Fermentation of endophytic fungi

Isolated fungal endophytes were sub-cultured to get pure fungal cultures and 1 cm² fungal discs were cut and inoculated in potato dextrose broth (PDB), which was kept for 21 days incubation at 25°C ± 2°C under stationary conditions. After the incubation period, the broth was filtered from the mycelia followed by ethyl acetate extraction of the broth (Fig. 3). The organic layer of ethyl acetate containing metabolites was evaporated using rotary evaporator. The residues obtained was collected in glass vials and stored for further experiments (Fig. 4).

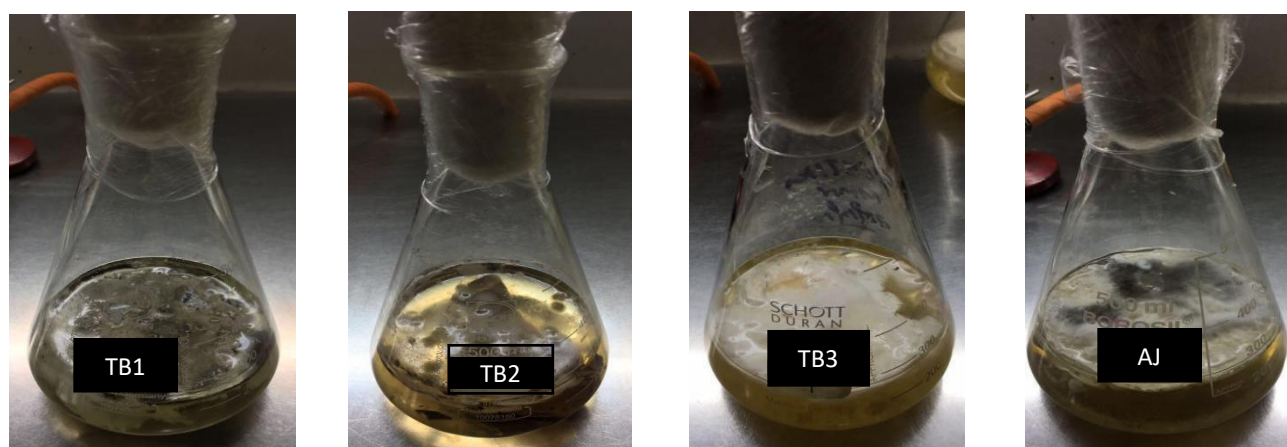


Fig. 3 Fermentation broth of the isolated endophytic fungi

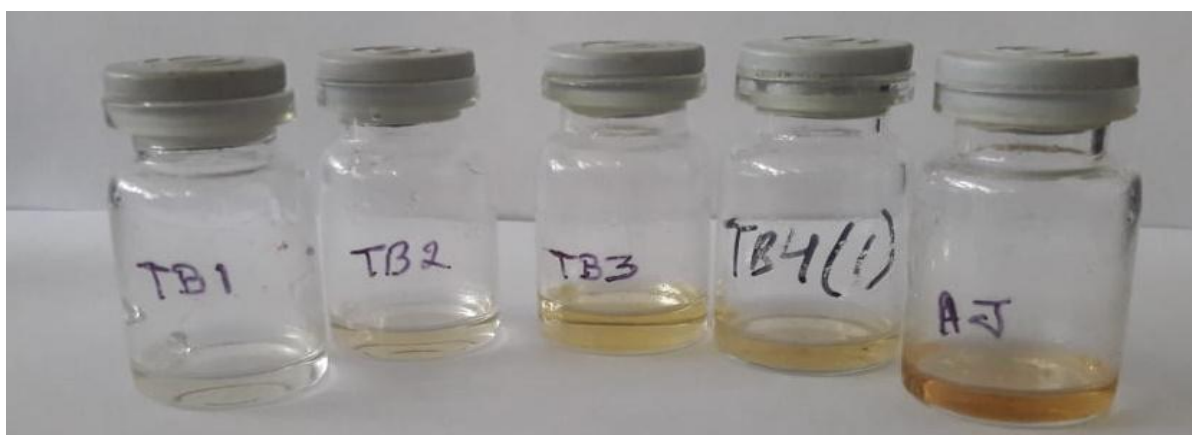


Fig. 4 Crude extracts of isolated endophytic fungi

4.4 Dry weight of mycelia

The discs of fresh mycelia from PDA plates were transferred to 500 ml Erlenmeyer flask containing 250 ml of the PDB. The cultured flasks were incubated for 21 days at $25 \pm 2^\circ\text{C}$ under stationary conditions. After the incubation period of 21 days, the culture was filtered with sterile muslin cloth to separate the mycelia from the broth. The sterile muslin cloth containing mycelia was dried in an oven till constant weight was achieved. Dry weight of mycelia was determined of all the isolated fungi.

Table 7: The dried mycelial weight of isolated fungi

S.No.	Extracts	Dried mycelial weight (mg)
1	TB1	200
2	TB2	180
3	TB3	210
4	TB4	190
5	AJ	208

4.5 Bioactivities of crude extracts (*In vitro* and *in vivo* anti-diabetic activity)

4.5.1 *In vitro* alpha-amylase inhibition assay

This assay involves the conversion of amylose into glucose. Four different concentrations of 100, 250, 500 and 1000 $\mu\text{g/ml}$ fungal extracts (TB1, TB2, TB3, TB4 and AJ). Since TB3 showed 43% inhibition at 1000 $\mu\text{g/ml}$, the concentration of TB3 was further increased to 200-1600 $\mu\text{g/ml}$ (Fig. 7). The concentration of reference compound acarbose was 100 $\mu\text{g/ml}$. Only TB3 showed significant percentage inhibition of alpha-amylase at 1000 $\mu\text{g/ml}$. The color change of yellow to brown was observed in TB3.

Statistical analysis revealed that there were significant differences in alpha amylase inhibition by TB3 [F (4, 10) = 1126] among groups. TB3 at concentration of 200, 400,

800 and 1600 $\mu\text{g/ml}$ showed the percentage inhibition of 2.87, 32.3, 40.2 and 49.71 respectively. TB1, TB2, TB4 and AJ did not show significant alpha-amylase percentage inhibition (Fig. 5, 6, 7, 8 and 9). TB3 showed maximum percentage inhibition of 49.7% of alpha amylase at 1600 $\mu\text{g/ml}$ which was comparable to that of standard compound acarbose. Acarbose at 100 $\mu\text{g/ml}$ show alpha-amylase inhibition of 76.5%.

Bioactive metabolites from endophytic fungus *Alternaria longipes* strain VITN14G of mangrove plant *Avicennia officinalis* showed 70 percent inhibition of alpha amylase (Ranganathan et al., 2019). Endophyte fungi, *Cladosporium sp.*-mediated synthesis of silver nanoparticles showed 56.19 percent alpha amylase inhibition (Popli et al., 2018).

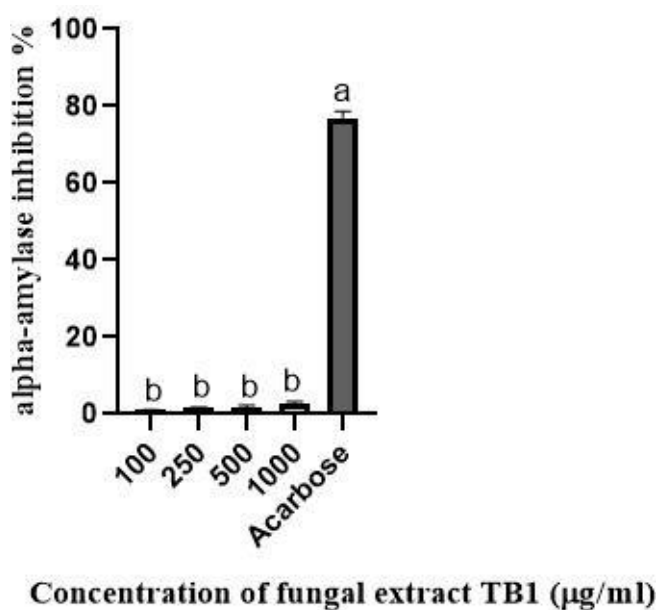


Fig. 5 Alpha-amylase inhibition activity of fungal extract TB1. Bars with the similar alphabets did not show significant difference at $P < 0.05$.

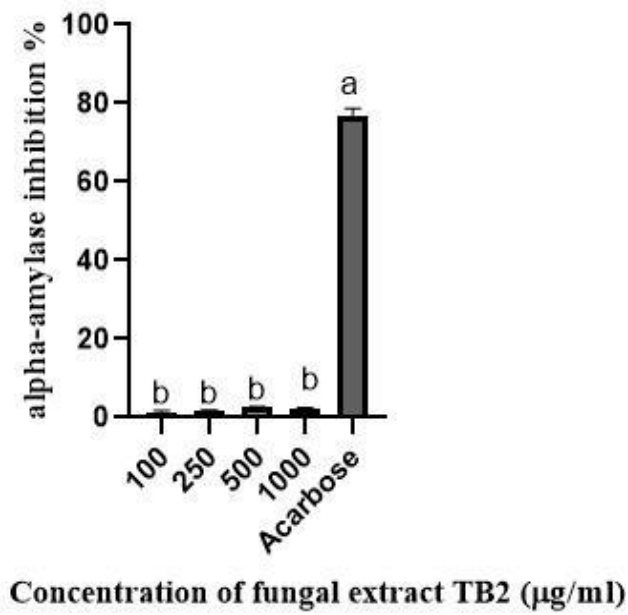


Fig.6 Alpha -amylase inhibition activity of fungal extract TB2. Bars with the similar alphabets did not show significant difference at $P < 0.05$.

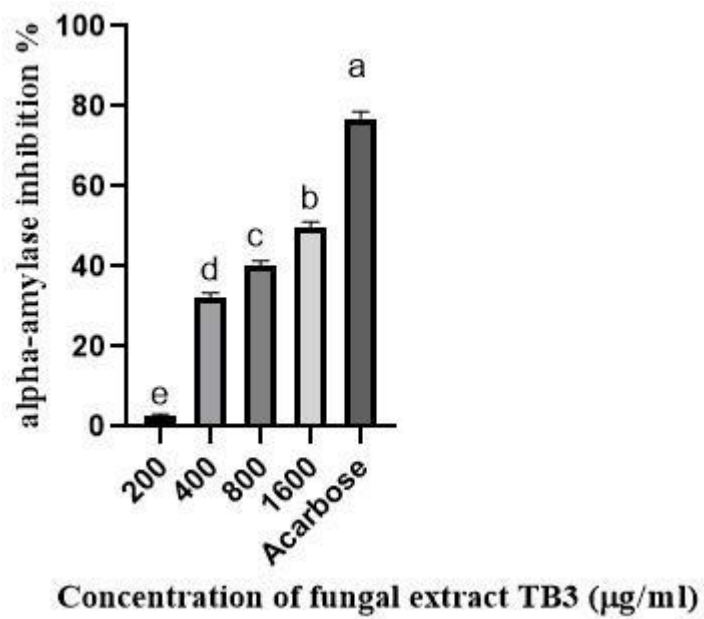


Fig. 7 Alpha-amylase inhibition activity of fungal extract TB3. Bars with the similar alphabets did not show significant difference at $P < 0.05$.

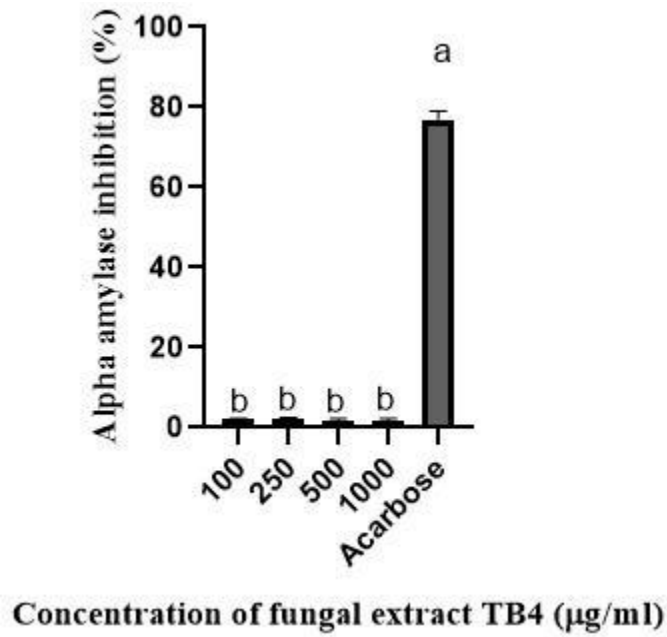


Fig. 8 Alpha-amylase inhibition activity of TB4. Bars with the similar alphabets did not show significant difference at $P < 0.05$.

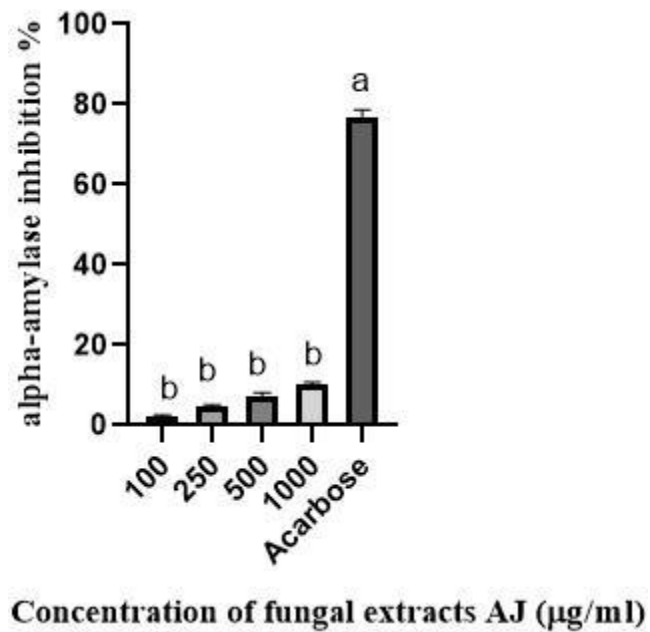


Fig.9 Alpha-amylase inhibition activity of extract AJ. Bars with the similar alphabets did not show significant difference at $P < 0.05$.

4.5.2 *In-vivo* antidiabetic activity

TB1-TB3 were subjected to *in vivo* anti-diabetic activity. Mice were randomly divided into six groups with six animals namely control, STZ, TB1, TB2, TB3 and Metformin. TB1 (10mg/kg), TB2 (10mg/kg) and TB3 (10mg/kg) were administered intraperitoneally to TB1, TB2 and TB3 groups respectively. The metformin (10mg/kg) was also administered i.p. to Met group. Vehicle was administered to control and STZ groups. Rats were fasted overnight and T2DM was induced with a single injection of streptozotocin (45 mg/kg; i.p.), 15 min after nicotinamide (110 mg/kg; i.p.). Streptozotocin was dissolved in 0.1 M citrate buffer (pH 4.5) and nicotinamide was dissolved in physiological saline. After the administration of streptozotocin and nicotinamide 1 ml of blood was collected through retroorbital puncture and centrifuged at 3000 g for 5 min at 4°C. to obtain the plasma for measuring blood glucose level (GOD PAP kit).

Statistical analysis revealed that there were significant differences in blood glucose level ($F(5, 30) = 98.72; P < 0.05$) among groups. Post hoc test revealed that streptozotocin increased the blood glucose level. TB3 and Met attenuated streptozotocin induced increase in level of blood glucose. TB1 and TB2 did not exert any anti-diabetic effects. Met (10 mg/kg) decreased blood glucose level to 80 mg/dl. TB3 (10 mg/kg) decreased blood glucose level to 92 mg/dl. TB3 showed significant decrease in the level of blood glucose as compared to standard metformin (Fig. 10).

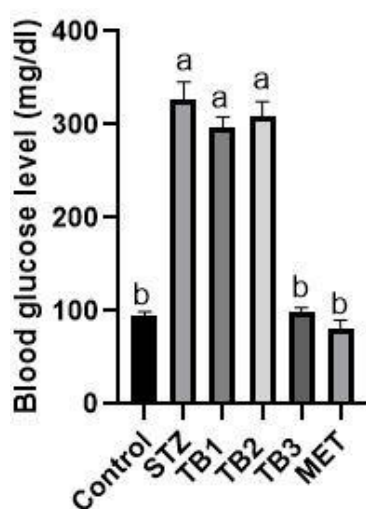


Fig. 10 Effect of fungal extracts on blood glucose level in experimental rats. Bars showing a common letter within the treatments are not significant at $P < 0.05$.

4.6 Antioxidant assay

Four different concentrations of 100, 250, 500 and 1000 µg/ml of fungal extracts TB1-TB4 and AJ were used to evaluate the free radical scavenging activity. Reference compound was ascorbic acid (AA) and its concentration was 100 µg/ml. Different concentration of fungal extracts were mixed with 150 µl of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and methanol into 96 wells micro titer plate. DPPH is violet in color but if the scavenging of free radical occurs then yellow color appears which implies that the extract has shown antioxidant activity.

The antioxidant effects of TB1, TB2, TB3, TB4 and AJ are shown in (Fig. 11, 12, 13, 14 and 15). One-way ANOVA showed that there were significant variations in antioxidant activity among groups in TB1 [F (4, 10) = 855.8], TB3[F (4, 10) = 416.2], AJ[F (4, 10) = 715.0] and TB4 [F (4, 10) = 1361]. TB1 showed percentage scavenging activity of 7.19, 22.2, 40.2 and 40.46 at 100, 250, 500, 1000 µg/ml respectively. TB3 showed percentage scavenging activity of 7.29, 40.46, 54.88 and 59.62 at 100, 250, 500 and 1000 µg/ml respectively. TB2 did not show significant anti-oxidant activity. TB4 showed scavenging activity of 30% at 1000 µg/ml. Ascorbic acid (100 µg/ml) showed antioxidant activity of 79.5%.

Past reports suggest that, endophytic fungus *Cladosporium. velox* obtained from *Tinospora cordifolia* has shown anti-oxidant activity with IC₅₀ value of 22.5µg/mL which was comparable to ascorbic acid (Singh et al., 2016). *Alternaria alternata* an endophytic fungus from medicinal plant, *Azadirachta indica* has shown free radical scavenging activity with IC₅₀ value of 38 ± 1.7 µg/mL (Chatterjee et al., 2019).

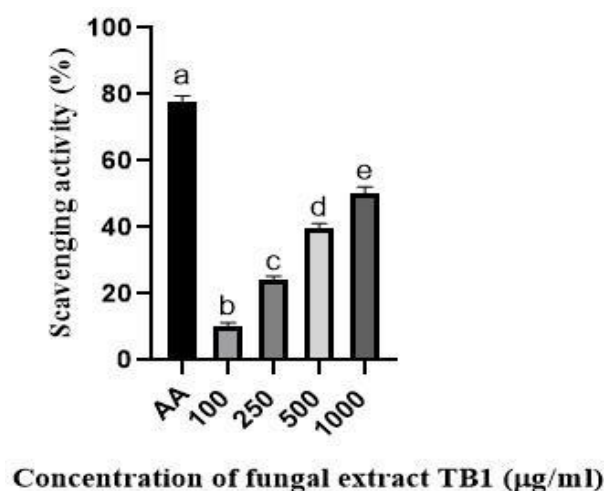


Fig. 11 *In-vitro* free radical scavenging activity of fungal extracts TB1. Bars with the similar alphabets did not show significant difference at P<0.05.

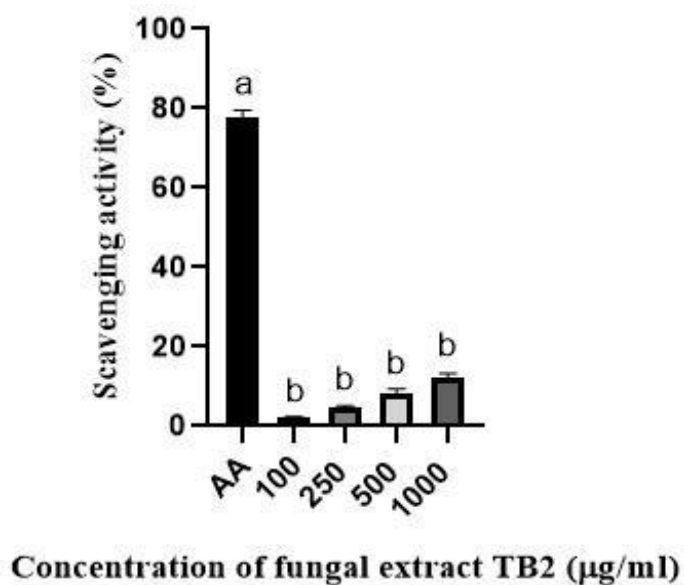


Fig. 12 *In-vitro* free radical scavenging activity of fungal extracts TB2. Bars with the similar alphabets did not show significant difference at $P < 0.05$.

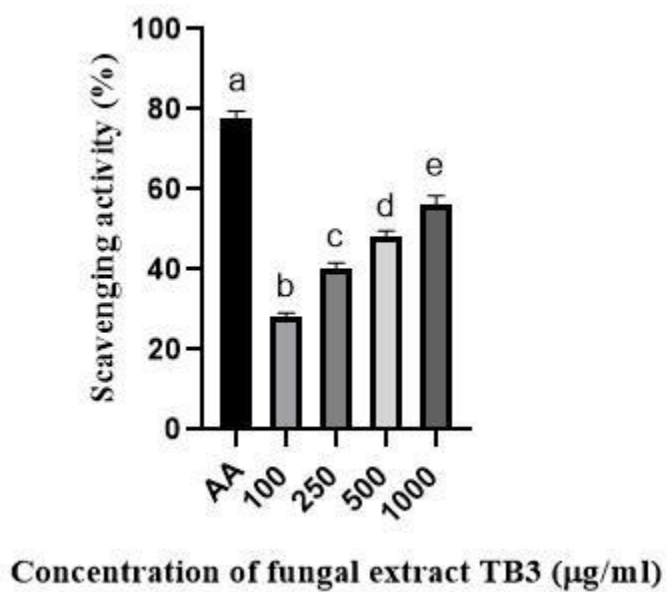


Fig. 13 *In-vitro* free radical scavenging activity of fungal extracts TB3. Bars with the similar alphabets did not show significant difference at $P < 0.05$.

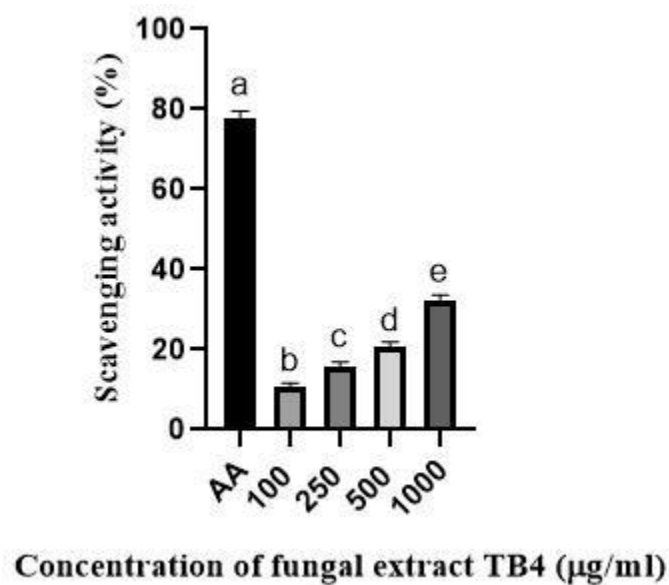


Fig. 14 *In-vitro* free radical scavenging activity of fungal extracts TB4. Bars with the similar alphabets did not show significant difference at $P < 0.05$.

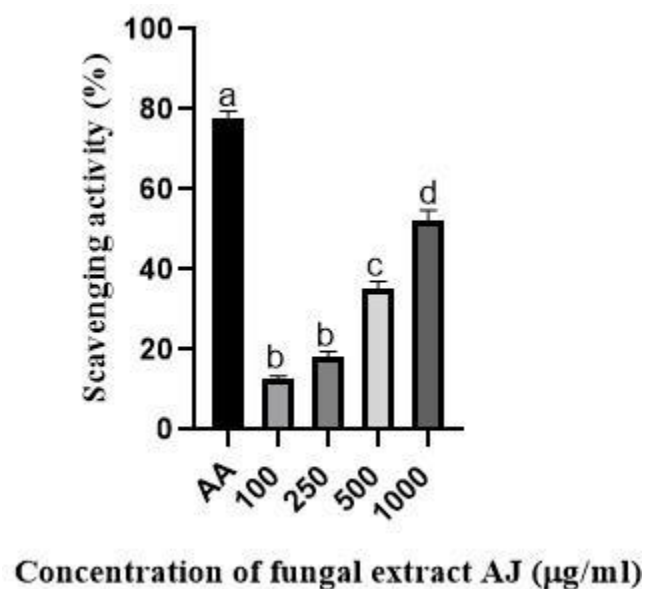


Fig. 15 *In-vitro* free radical scavenging activity of fungal extracts AJ. Bars with the similar alphabets did not show significant difference at $P < 0.05$.

4.7 Antimicrobial activity of the crude fungal extracts

The activities of the crude fungal extracts were tested on microorganisms (bacterial and fungal) by agar well diffusion method. The zone of inhibition (clear zone where bacteria did not grow) formed was measured in millimeter (mm). Fungal extracts of *Terminalia arjuna* were tested for preliminary antimicrobial activity with the bacterial test cultures (*E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis*).

Agar well diffusion method

The activities of the crude fungal extracts were tested on bacteria by agar well diffusion method. The zone (clear zone where bacteria did not grow) formed was measured in millimeter (mm). Four crude extracts of *T. arjuna* were tested for antimicrobial activity with the bacterial test cultures (Table 11).

Table 8: Antimicrobial activity of screened crude fungal extract with their zone diameter

Crude extracts	<i>E.coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>B. subtilis</i>
TB1	0	0	13	8
TB2	0	0	8.2	11.1
TB3	0	12	10	14
TB4	0	8	0	0
AJ	0	6	0	8

Crude extracts of TB1, TB2, TB3, TB4 and AJ from bark of *Terminalia arjuna*, displayed inhibitory activity on MHA medium, while the same extracts showed no activity against *E. coli*, that illustrates the extracts do not move into the cell membranes. The isolated crude extract TB3 showed zone of inhibition ranging from 10-14 mm against *S. aureus*, *B. subtilis*, *P. aeruginosa*. TB3 was the only extract which showed 14 mm zone of inhibition against *B. subtilis*. The isolated endophytic crude extract TB1 showed zone of inhibition range 8 and 13 mm against *S. aureus*, *B. Subtilis* respectively. Fungal crude extract AJ showed zone of inhibition ranging from

6-8 mm against *B. subtilis*, *P. aeruginosa* respectively and the isolated crude extract TB4 showed zone of inhibition 8 mm only against *P. aeruginosa* (Table 11). All the antibacterial activities of the extracts are shown in (Fig. 16, 17 and 18).

Past reports suggest that, the flavonoid extracts and callus extract were used to analyse the anti-microbial activity against bacteria's (For example: Gram-positive bacteria *B. subtilis*, *S. aureus*, Gram- negative bacteria *E. coli*, *K. pneumoniae* and *C. albicans*). The extract have taken from bark of *T. arjuna* has also showed greater antimicrobial activity against microorganisms because of maximum concentrations of Flavonoids (Sharma et al., 2012).

4.7.1.1 Antimicrobial activity against *Staphylococcus auerus*

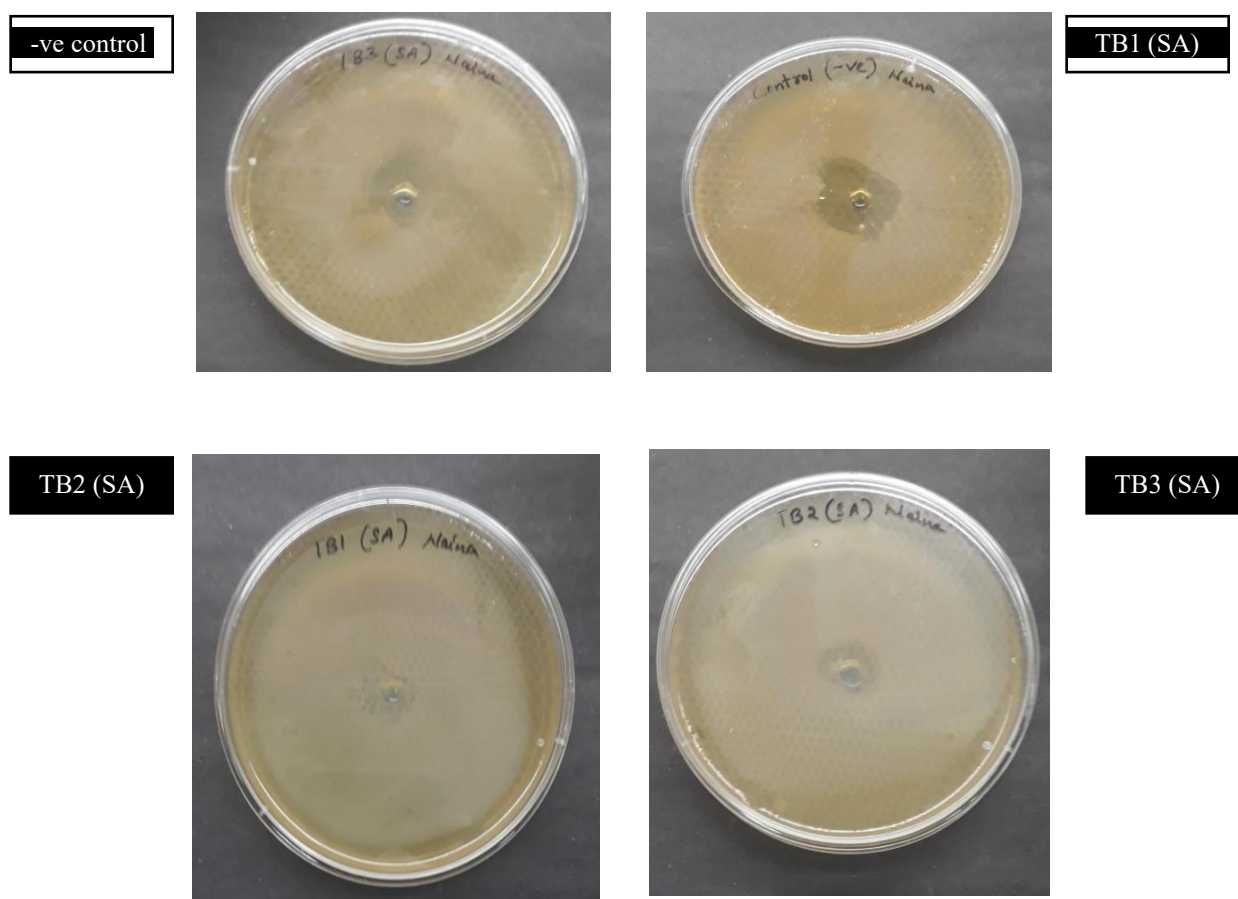


Fig 16. Antibacterial activity of fungal crude extracts against *S. aureus*. The positive control is ampicillin and negative control is methanol.

4.7.1.2 Antibacterial activity against *Bacillus subtilis*

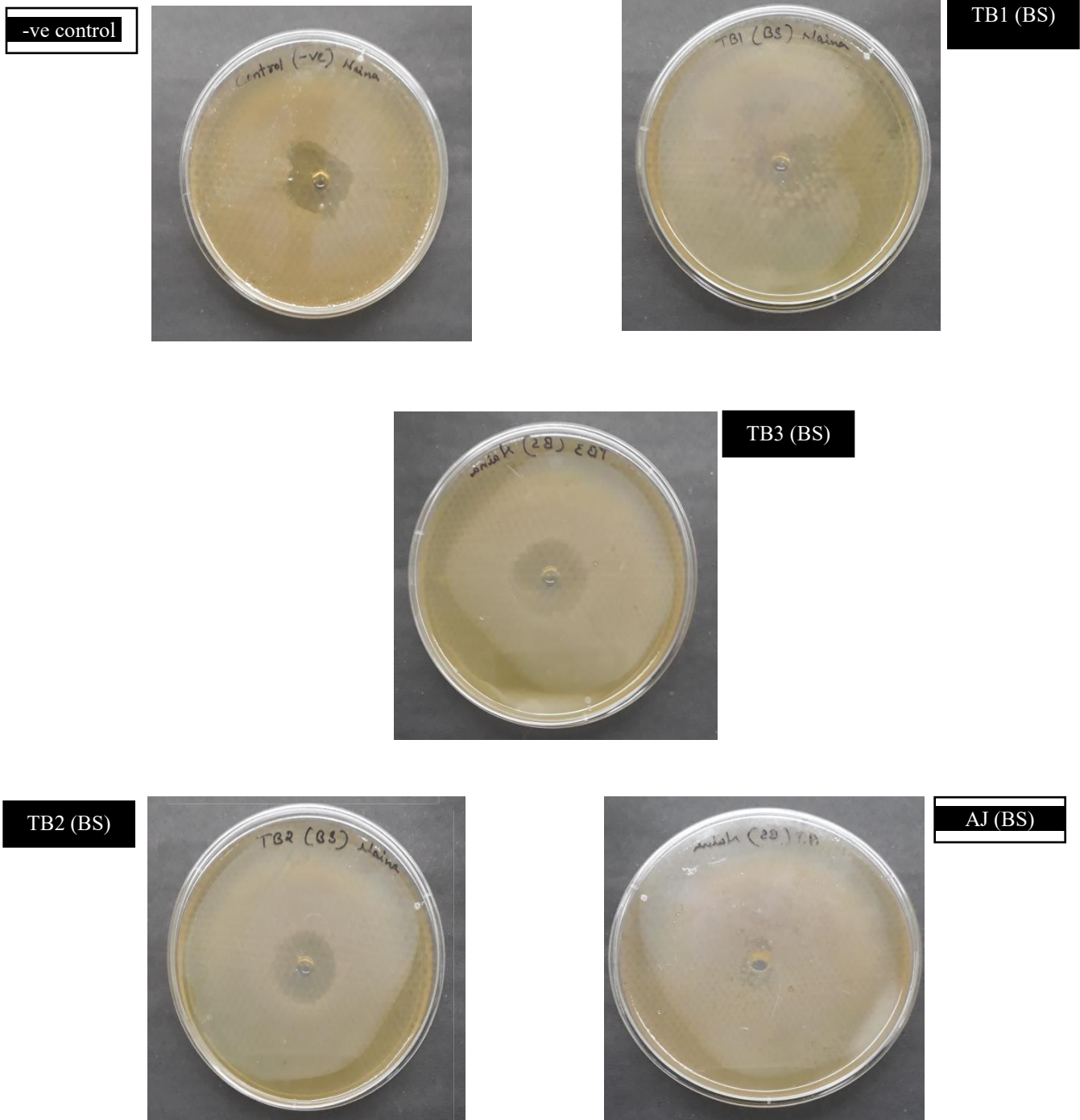


Fig 17. Antibacterial activity of fungal crude extracts against *Bacillus subtilis*.

4.7.1.3 Antibacterial activity against *Pseudomonas aeruginosa*

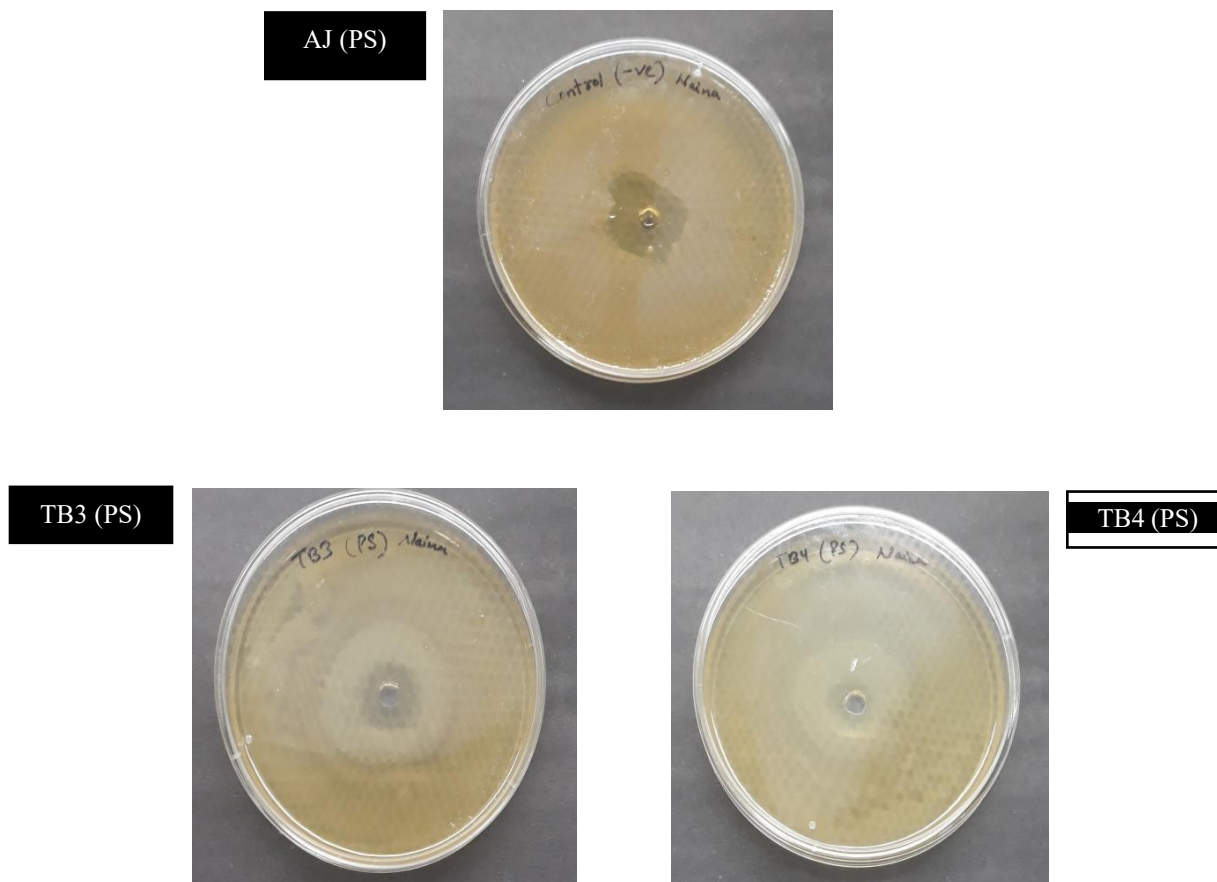


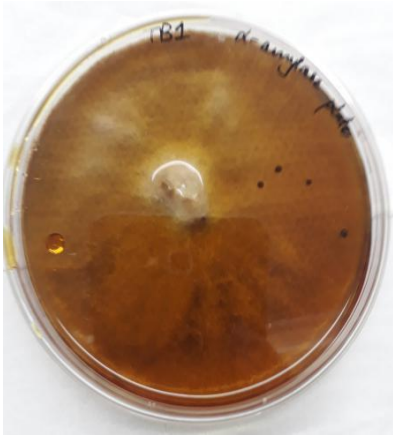
Fig. 18 Antibacterial activity of fungal crude extracts against *P. aeruginosa*.

4.8 Screening for fungal extracellular enzymes

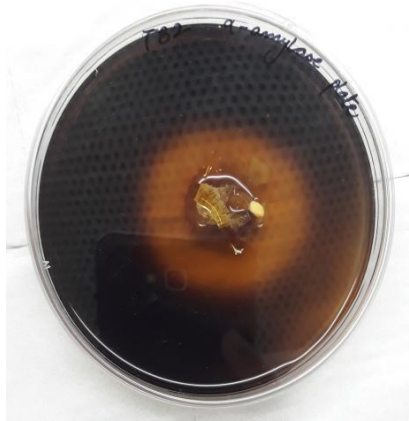
4.8.1 Amylolytic activity

It is the conversion of starch into sugar by the function of acids or enzymes. This method is used to identify the amylolytic activity of the fungal extracts TB1, TB2, TB3, TB4 and AJ. GYP medium was prepared with 0.2% soluble starch. Then mycelial discs of endophytic fungi were cut into small pieces and inoculated onto agar plates and incubated for 7 days. Plates were filled with iodine solution after 7 days and observed for yellow colony around the fungus. Out of the above five cultures TB1, TB2, TB3 and TB4 showed yellow color and indicated the amylolytic activity (Fig. 19). Zone diameter in mm is shown in table 9.

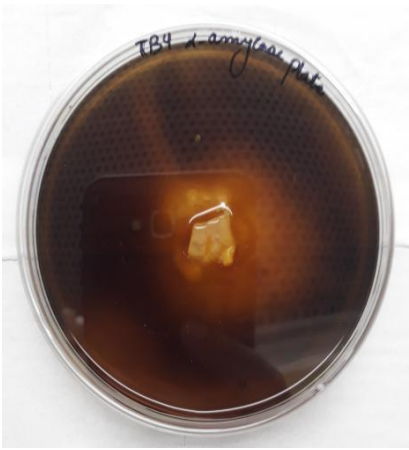
TB1



TB2



TB3



TB4

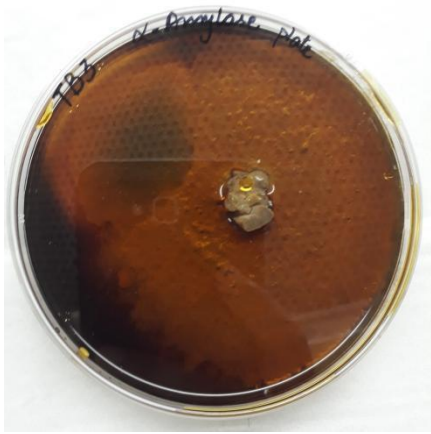


Fig. 19 Amylolytic activity of endophytic fungi TB1, TB2, TB3 and TB4.

Table 9: Zone diameter in amyolytic activity

Culture	Zone diameter (mm)
TB1	55 ± 0.04
TB2	48 ± 0.07
TB3	42 ± 0.02
TB4	38 ± 0.04

4.8.2 L-Asparaginase activity

L-asparaginase activity is indicated by the formation of pink zone around fungal mycelium. Endophytic cultures TB1, TB2, TB3, TB4, and AJ were used for screening, in order to check for their potential to utilize L-asparagine as nitrogen source. Only three cultures AJ, TB1 and TB3 showed the positive results. When the cultures were cut into small discs and inoculated onto modified Czapek Dox agar medium, addition of phenol red dye, which was used as an indicator, so, it has the ability to convert color of plates (pink zone) under alkaline conditions. Color change occurred due to the accumulation of ammonia which represents the conversion of L-asparagine into L-aspartic acid (Fig. 20). A zone diameter is shown in table 10.

This study seeks endophytes that produce the anticancer enzyme l-asparaginase, to harness their potential for mass production. Four plants with anticancer properties; *Cymbopogon citratus*, *Murraya koenigii*, *Oldenlandia diffusa* and *Pereskia bleo*, were selected as host plants. l-Asparaginase-producing endophytes were detected by the formation of pink zones on agar, a result of hydrolyzes of asparagine into aspartic acid and ammonia that converts the phenol red dye indicator from yellow to pink. The anticancer enzyme asparaginase was further quantified via Nesslerization. Results revealed that a total of 89 morphotypes were isolated, only 25 of these morphotypes

produced L-asparaginase, and their asparaginase activities were between 0.0069 and 0.025 $\mu\text{M mL}^{-1} \text{min}^{-1}$.

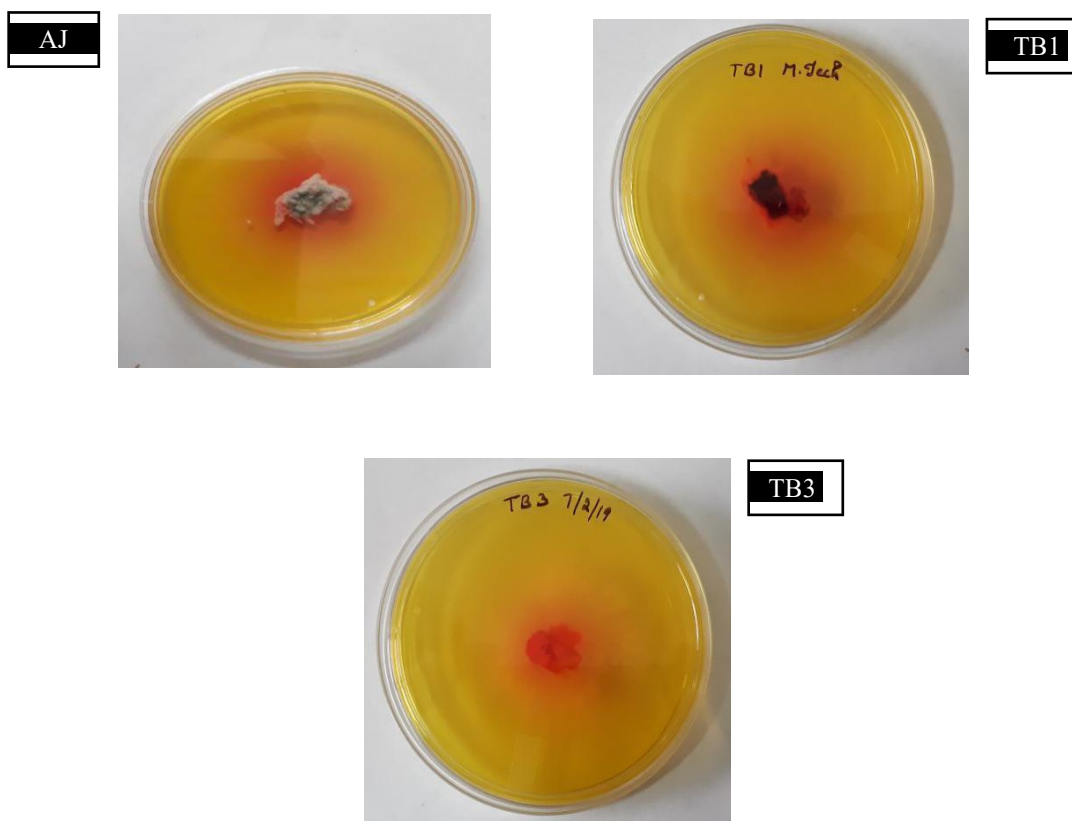


Fig. 20 L-Asparaginase activity of TB1, TB3 and AJ.

Table 10:. Zone diameter after 48 hours of cultures in L-Asparaginase activity

Culture	Zone diameter (mm), Mean \pm SD
AJ	51 \pm 0.02
TB2	49 \pm 0.03
TB3	47.5 \pm 0.72

In the present study, medicinal plant *Terminalia arjuna* has been selected for the isolation of endophytic fungi. The crude extract from isolated endophytic fungi from *T. arjuna* were subjected to anti-diabetic, antioxidant, antibacterial and extracellular enzyme activity. Five extracts TB1-TB4 and AJ were studied for the above activities. In alpha-amylase activity, TB3 showed significant percentage inhibition of alpha-amylase at 1000 µg/ml. TB3 at concentration of 200, 400, 800 and 1600 µg/ml showed the percentage inhibition of 2.87, 32.3, 40.2 and 49.71 respectively. TB3 showed maximum percentage inhibition of 49.7% of alpha amylase at 1600 µg/ml which was comparable to that of standard compound acarbose. TB3 (10 mg/kg) decreased blood glucose level to 92 mg/dl in experimental mice during *in vivo* study. TB3 showed significant decrease in the level of blood glucose as compared to the standard drug metformin. In antioxidant activity, TB1 showed percentage scavenging activity of 7.19, 22.2, 40.2 and 40.46 at 100, 250, 500, 1000 µg/ml respectively. TB3 showed percentage scavenging activity of 7.29, 40.46, 54.88 and 59.62 at 100, 250, 500 and 1000 µg/ml respectively. TB2 did not show significant anti-oxidant activity. TB4 showed scavenging activity of 30% at 1000 µg/ml. Ascorbic acid (100 µg/ml) showed antioxidant activity of 79.5% which was used as standard.

In antibacterial activity, the isolated crude extract TB3 showed zone of inhibition ranging from 10-14 mm against *S. aureus*, *B. subtilis*, *P. aeruginosa*. TB3 was the only extract which showed 14 mm zone of inhibition against *B. subtilis*. In amylolytic activity, out of the above five cultures TB1, TB2, TB3 and TB4 showed yellow color and indicated the amylolytic activity. In L-Asparaginase activity, only three cultures AJ, TB1 and TB3 showed the positive results.

Hence it may be concluded that TB3, an endophytic fungi isolated from *Terminalia arjuna* can be a potential source of bioactive compounds as it exhibits significant bioactivities. Further these therapeutic compounds can be purified and characterized and can play an important role in the treatment of diseases like diabetes, cancer, infection and other inflammable conditions.

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APPENDIX

Table 12: Compostion of modified media

Chemicals	Amount for 1000ml
Disodium hydrogen phosphate.2H ₂ O	6.0g
Potassium di hydrogen phosphate	3.0g
Sodium chloride	0.5g
L-asparagine	5.0ml
Magnesium sulphate (1mole/litre)	2.0ml
Calcium chloride (0.1mole/litre)	1.0ml
20% glucose stock solution	10.0ml
Agar	20.0ml
Distilled water	1000ml

Table 13: Compostion of GYP medium

Chemicals	Amount for 1000ml
Glucose	1g
Yeast Extract	0.1g
Peptone	0.5g
Agar	16g
Distilled water	1000ml

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