INTRODUCTION

As the population of the world is increased, it is suffering from various health problems caused by certain drug resistance bacteria, parasite protozoans, and fungi. Therefore, an intensive search for the development and invention of new effective agents to deal with these problems is now underway [1]. Since ancient times, people had exploited the nature, particularly, plants in search of new drugs. This has resulted in use of a large number of medicinal plants with curative properties which help in the treatment of various diseases caused by microorganisms [2]. A number of research observed that there are a number of endophytic fungi present inside the plants and protect their host from insects and other herbivores. The term endophyte refers to the group of microorganisms which is part of its life cycle, invades the tissue of living plants, and causes asymptomatic infection [3]. Endophytic fungi have been reported to produce similar compounds to the group of microorganisms which is part of its life cycle, invades the number of endophytic fungi present inside the plants and protect their host from parasites protozoans, and fungi. Therefore, in the present investigation, the endophytic fungi were isolated from the medicinal plant Tridax procumbens and evaluated their antibacterial activity against the pathogenic bacteria. The morphological identification of all the isolated endophytic fungi was observed on the basis of their macroscopic and microscopic characteristics, and the molecular identification of the potent fungal strain was observed through 18s rRNA sequence analysis. Using solvent-solvent extraction technique, different solvent residues of the potent fungal metabolite were extracted in benzene, n-butanol and toluene. The n-butanol solvent extract exhibited a maximum zone of inhibition against the test bacterial strains.

RESULTS

Five endophytic fungi were isolated from medicinal plant T. procumbens and screened for their antibacterial activity against E. coli (22.60±0.32 mm), S. typhimurium (19.26±0.23 mm), S. pyogenes (16.36±0.18 mm), K. pneumoniae (14.26±0.54 mm), and B. subtilis (14.43±0.27 mm) bacterial strain. The endophytic fungal strain A. japonicus isolated from T. procumbens was shown the significant antibacterial activity against the pathogenic bacteria. The morphological identification of all the isolated endophytic fungi was observed on the basis of their macroscopic and microscopic characteristics, and the molecular identification of the potent fungal strain was observed through 18s rRNA sequence analysis. Using solvent-solvent extraction technique, different solvent residues of the potent fungal metabolite were extracted in benzene, n-butanol and toluene. The n-butanol solvent extract exhibited a maximum zone of inhibition against the test bacterial strains.

CONCLUSION

The present study reveals that the endophytic fungi serve as a potential source for the production of effective bioactive compounds.
in fungal incubator at 26±1°C for 5–7 days. The pure colonies of the endophytic fungi were further transferred on the PDA plates at 26±1°C for 5–7 days. The pure cultures of the isolated fungi were preserved on PDA slant without antibiotic at 4°C.

Phenotypic identification of isolated endophytic fungi

The morphological identification of the isolated fungi was done by slide culture technique given by Aggarwal and Hasija, 1980 [14], and Domsch et al. 2007 [15]. The endophytic fungi were characterized on the basis of their color, size and shape of spores, hyphae characters, and reproductive structures [16].

Source of test bacterial strain

The pathogenic bacteria used in the study such as Salmonella typhimurium microbial type culture collection (MTCC) 733, E. coli MTCC 1679, Staphylococcus aureus MTCC 96, B. subtilis MTCC 441, and K. pneumoniae MTCC 4032 cultures were obtained from MTCC and GeniBank, Institute of Microbial Technology (IMTECH), Chandigarh, India. The organisms were preserved at 4°C in the presence of glycerol (15% v/v) for longer periods.

Screening of isolated endophytic fungi

The isolated endophytic fungi were subcultured in Petri plates containing PDA and incubated in a fungal incubator for 6–7 days at 26±1°C. Sterile cork borer of 8 mm was used to cut the portion of mycelia mat and transferred to 100 mL pre-sterile PDB and incubated at 26±1°C in a fungal incubator for 7th, 14th, and 21st day [17]. Under aseptic conditions, the metabolized broths were filtered with Whatman filter paper no.1 and centrifuged at 8000 rpm for 8 min. The pellet was discarded and the supernatant refiltered to get cell-free culture filtrate (CFCF) for the estimation of antibacterial activity.

Molecular identification of potent fungi

After screening, the molecular identification of potent fungal strain was performed by 18S rRNA sequence analysis. For the molecular identification, the DNA of potent fungal was isolated by LETS method [18]. In this method, loop full conidia were inoculated to the 10 mL media and that was incubated for 16–30 h. After that, the mycelia was harvested, washed with distilled water, and lyophilized by liquid nitrogen and crushed in a motor-pastel by adding 0.7 mL extraction LETS buffer (0.1 M LiCl, 10 mm EDTA, 10 mm HCL [pH 8], and 0.5% SDS). After that, crushed mycelia was poured in the centrifuge tubes and vortex for few minutes. After that, 1 mL phenol chloroform-isoamyl alcohol (25:2:1) were added and vortexed for 1 min at medium speed and centrifuged at 3000 rpm for 5 min. There were two layers formed in the tube as aqueous layer and pellet. The pellet was discarded and the aqueous layers were transferred in the other sterilized tube and add 1 mL absolute chilled ethanol in the aqueous layer and placed the tube on dry ice for 15 min. After that spun for 15 min in a microcentrifuge at 10,000 rpm for 10 min. The supernatant was removed and the pellet dissolved in TE buffer for further use.

Quantification and amplification of fungal DNA

The quantity of the isolated DNA was checked in ultraviolet (UV)-visible spectrophotometer. From the stock, 1 µL DNA was mixed with 49 µL sterile distilled water to get 50 times dilution. The A260/280 visible spectrophotometer. From the stock, 1 µL DNA was mixed with 49 µL sterile distilled water to get 50 times dilution. The A260/280 ratio was measured using HiMedia antibiotic zone scale and compared with the control. All experiments were performed in triplicate.

In vitro antibacterial activity by agar well diffusion method

For antibacterial activity, agar well diffusion method was performed given by Newyork, 1972 [19]. Nutrient agar medium (NAM) plates were prepared and 25 µL of bacterial suspension was spread out with the help of glass s preader on the NAM plates to make bacterial lawn and allowed to dry for 10 min. The agar wells were prepared by scooping out the medium with a sterile cork borer which had 8 mm diameter. The wells were then filled with 75 µL of the fungal crude extract and incubated at 37°C for 24 h. After the incubation period, the zone of inhibition was measured using HiMedia antibiotic zone scale and compared with the control. All experiments were performed in triplicate.

Statistical analysis

In the present study, antibacterial activity was conducted in triplicate and the results were calculated as mean (±) standard deviation. Data were analyzed with one-way ANOVA and significant differences between mean values at p<0.05 were determined using SPSS program (16.0 versions).

RESULTS

Isolation and phenotypic identification of isolated endophytic fungi

In the present investigation, five endophytic fungi were isolated from leaves of T. procumbens collected from Majholi, Jabalpur (M.P.), India, as shown in Table 1. Identification of fungal strains was done using standard protocol of Aggarwal and Hasija, 1980 [14], and Domsch et al. 2007 [15]. On the basis of their cultural and microscopic properties, these fungi show different characteristics and successfully identified as Aspergillus japonicus (SG1), Aspergillus niger (SG2), Alternaria sp. (SG3), Fusarium sp. (SG4), and Penicillium sp. (SG5) as shown in Fig. 1.

Screening of isolated endophytic fungi from the plant

For the screening of the antibacterial activity of isolated endophytic fungi from the leaves of plant, all fungal isolates were inoculated in PDB for 7th, 14th, and 21st day and incubated at 26±1°C in a fungal incubator. During the screening of endophytic fungi, CFCF of 7th, 14th, and 21st day were examined for their antibacterial activity against the test bacterial strain as shown in Fig 2 and depicted in Table 2.
After screening the antibacterial activity of the endophytic fungi, the potent fungal strain *A. japonicus* was showed and the maximum antibacterial activity against the test bacteria was further identified by molecular sequencing using 18S rRNA sequence analysis. In the molecular study, two primers, namely, ITS 4 (TCC TCC GCT TAT TGA TAT G) and ITS6 (GAA GGT GAA GTC GTA ACA AGG) were used for molecular identification. Based on BLAST search of rRNA gene sequence, the endophytic fungi were found to be closest homology to *A. japonicus* and submitted to the NCBI GenBank with accession no. KY218732 (Fig. 3). Phylogenetic tree was constructed based on the closest relationship with consensus sequences and shown in Fig. 4.

### Table 1: Phenotypic characteristics of isolated endophytic fungi from leaves of *T. procumbens*

| Fungal isolate | Macroscopic characteristic | Microscopic characteristic | Probable genera/species |
|----------------|----------------------------|----------------------------|-------------------------|
| SG1            | Colony wrinkled, near mummy brown, mycelium white, reverse uncolored | Conidial heads radiate, hyaline to pale brown, smooth, vesicles subglobose to globose, conidia spherical or ellipsoidal | *Aspergillus japonicus* |
| SG2            | Colonies were powdery in texture and black in color with conidial production, reversed plate showed pale yellow colored due to pigmentation | Hyphae septate hyaline dichotomously branched vesicle, round, radiate head, black conidia, spores erect, simple and thick walled | *Aspergillus niger* |
| SG3            | Woolly colonies greenish-black or olive brown with a light border | Single conidia, smooth-wall, dark in color, septate hyphae | *Alternaria* sp. |
| SG4            | White pink color, smooth swarming and raised colony | Multi-celled spores, conidia are oval shaped and attached to conidiophores arising from a septate mycelium | *Fusarium* sp. |
| SG5            | White and cottony colonies | Single-celled spores, conidia in chains develop at the end of the sterigma from the medulla of the conidiophores arise from a septate mycelium | *Penicillium* sp. |

### Table 2: Screening of isolated endophytic fungi against five pathogenic bacteria

| Name of fungi | Day of incubation period | Zone of inhibition (in mm) | B. subtilis | S. pyogenes | E. coli | S. typhimurium | K. pneumoniae |
|---------------|--------------------------|-----------------------------|------------|------------|--------|----------------|---------------|
| *A. japonicus* | 07 | 00.00±0.00 | 00.00±0.00 | 06.11±0.54 | 00.00±0.00 | 00.00±0.00 | 00.00±0.00 |
| | 14 | 14.43±0.27 | 16.36±0.18 | 22.60±0.32 | 19.26±0.23 | 14.26±0.54 | 14.26±0.54 |
| | 21 | 08.31±0.54 | 00.00±0.00 | 08.16±0.47 | 00.00±0.00 | 00.00±0.00 | 00.00±0.00 |
| *Fusarium* sp. | 07 | 05.05±0.39 | 00.00±0.00 | 07.23±0.58 | 00.00±0.00 | 00.00±0.00 | 01.45±0.00 |
| | 14 | 17.46±0.55 | 00.00±0.00 | 16.33±0.35 | 13.56±0.20 | 16.53±0.33 | 16.53±0.33 |
| *Alternaria* sp. | 07 | 09.10±0.47 | 00.00±0.00 | 11.23±0.52 | 00.00±0.00 | 00.00±0.00 | 00.00±0.00 |
| | 14 | 09.56±0.45 | 09.65±0.45 | 10.53±0.25 | 00.00±0.00 | 09.56±0.36 | 09.56±0.36 |
| | 21 | 11.04±0.19 | 09.22±0.42 | 13.23±0.36 | 08.45±0.72 | 06.16±0.14 | 06.16±0.14 |
| *A. niger* | 07 | 13.71±0.28 | 09.22±0.42 | 17.23±0.25 | 00.00±0.00 | 12.24±0.58 | 12.24±0.58 |
| | 14 | 08.50±0.22 | 06.05±0.47 | 12.23±0.54 | 10.66±0.46 | 00.00±0.00 | 00.00±0.00 |
| | 21 | 08.32±0.54 | 06.05±0.47 | 12.23±0.54 | 10.66±0.46 | 00.00±0.00 | 00.00±0.00 |
| *Penicillium* sp. | 07 | 00.00±0.00 | 12.04±0.56 | 07.00±0.38 | 00.00±0.00 | 00.00±0.00 | 00.00±0.00 |
| | 14 | 11.30±0.09 | 08.40±0.23 | 16.36±0.47 | 10.02±0.00 | 00.00±0.00 | 00.00±0.00 |
| | 21 | 00.00±0.00 | 14.16±0.32 | 12.20±0.17 | 00.00±0.00 | 09.50±0.41 | 09.50±0.41 |

### Molecular identification of potent fungus

After screening the antibacterial activity of the endophytic fungi, the potent fungal strain *A. japonicus* was showed and the maximum antibacterial activity against the test bacteria was further identified by molecular sequencing using 18S rRNA sequence analysis. In the molecular study, two primers, namely, ITS 4 (TCC TCC GCT TAT TGA TAT G) and ITS6 (GAA GGT GAA GTC GTA ACA AGG) were used for molecular identification. Based on BLAST search of rRNA gene sequence, the endophytic fungi were found to be closest homology to *A. japonicus* and submitted to the NCBI GenBank with accession no. KY218732 (Fig. 3). Phylogenetic tree was constructed based on the closest relationship with consensus sequences and shown in Fig. 4.

### In vitro antibacterial activity of potent fungi

In the present investigation, metabolites of endophytic fungi *A. japonicus* that showed the maximum zone of inhibition against the test bacterial strain were extracted with a number of organic solvent such as benzene, toluene, and n-butanol (1:1 v/v) and concentrated in rotary vacuum evaporator. After evaporation of solvent, the metabolites were mixed...
with sterilized distilled water and observed their antibacterial activity by agar well diffusion method, and the fraction of n-butanol extract was showed maximum antibacterial activity against B. subtilis, E. coli, MTCC1679, K. pneumoniae MTCC4032, S. typhimurium MTCC733, and Streptococcus pyogenes MTCC96 as shown in Table 3. The optimum inhibitory concentration of n-butanol fungal extract was also observed at different concentrations from a range of 25–800 µL. The antibacterial activity of the n-butanol extract was increased as the concentration was augmented, and after 800 µL concentration, the antibacterial activity of the solvent extracted metabolites was fairly stable as depicted in Table 4.

**DISCUSSION**

Endophytic fungi are the most promising bioagent that has a huge source of various bioactive compounds. Mainly, these compounds could be classified as alkaloids, terpenoids, quinones, steroids, isocoumarins, saponins, lactones, phenylpropanoids, and phenols [9,20]. Many of these compounds are being used for the treatment of a number of diseases [7]. Thus, in the present work, endophytic fungi were isolated from the medicinal plant T. procumbens L. and observed their antibacterial against the bacterial strain. Sandhu et al. 2016 [21] reported seven endophytic fungi such as Aspergillus niger, Penicillium citrinum, Cladosporium sp., Curvularia lunata, Aspergillus sp., Alternaria sp., and Aspergillus fumigates isolated from Bauwolffia serpentina and the morphological identification was completed based on mycelia type, aerial color, shape and kind of spores, presence of foot cells, conidiofomes, and the characteristics of spores. In another study, Tran et al. 2010 [22] isolated six endophytic fungi from phylodes of Acacia species and molecular identification was done through 18S rRNA gene sequencing. In the present study, five endophytic fungal strains were isolated from T. procumbens and identified as Aspergillus japonicus, Fusarium sp., Aspergillus niger, Alternaria sp., and Penicillium sp. using phenotypic characteristics, and the potent fungal strain was identified on the basis of 18S rRNA sequencing using ITS 4 and ITS6 primers. Parenicova et al. 2001 [23] used DNA sequence of the ITS1 and ITS2 region to make sure the close relationship between A. japonicus and A. aculeatus. In the present study, phylogenetic tree was constructed and that showed, 99% similarity with A. japonicus. During the screening of endophytic fungi, CPF of 7th, 14th, and 21st day was examined for their antibacterial activity. A. japonicus showed the maximum inhibitory effect against selected pathogenic bacteria. The optimum inhibitory concentration of n-butanol fungal extract was also observed at different concentrations that the antibacterial activity was elevated as the concentration of n-butanol extracted metabolites was increased. Similarly, Meenupriya and Thangaraj, in 2011 [24], also observed the inhibitory concentration of ethyl acetate extract of the Aspergillus ochraceus against the four pathogenic bacteria from a range of 25–1000 µL and the maximum antibacterial activity was examined as the concentration of the solvent extract was increased.

**CONCLUSION**

Endophytic fungi are those microorganisms that survive interior of plants an especially leaf, stems, and roots without any apparent harm to host. Endophytes are rich sources of bioactive metabolites, which can be potentially used in the field of medicine, agriculture, and industries. In the present investigation, fungal endophyte A. japonicus isolated from T. procumbens showed the maximum antibacterial activity against the test bacterial strain. Further study can be performed to identify the bioactive compounds present in the extract. A. japonicus exhibited the most significant inhibitory activity against five pathogenic bacteria. The isolation of these antibacterial compounds from the endophytic fungi and identification of bioactive compounds can be a crucial approach to search of novel natural products.

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Table 4: Well-diffusion method standardization (n-butanol fungal extract low and high concentration)

| Concentration (µL) | Zone of inhibition (in mm) |
|--------------------|---------------------------|
|                    | B. subtilis | S. pyogenes | E. coli | S. typhimurium | K. pneumoniae |
| Low concentration   |            |            |        |              |              |
| 25                 | 0.74±0.21  | 0.10±0.26  | 11.37±0.52 | 05.20±0.12 | 00.00±0.00   |
| 50                 | 12.20±0.34 | 14.2±0.53  | 17.34±0.36 | 13.2±0.74 | 08.16±0.52   |
| 75                 | 15.13±0.71 | 17.30±0.45 | 25.3±0.78  | 21.4±1.05 | 14.2±0.94    |
| 100                | 15.52±0.45 | 19.11±0.34 | 27.2±0.57  | 24.3±0.65 | 16.0±0.32    |
| High concentration  |            |            |        |              |              |
| 200                | 17.30±0.62 | 22.42±0.57 | 29.05±1.12 | 26.18±0.38 | 19.21±0.57   |
| 400                | 19.50±0.44 | 23.14±0.49 | 30.23±0.84 | 27.42±0.05 | 21.14±0.25   |
| 600                | 20.83±0.37 | 24.46±0.87 | 31.12±0.92 | 28.13±0.76 | 22.40±0.54   |
| 800                | 20.30±0.85 | 25.19±0.68 | 31.60±0.30 | 29.06±0.43 | 23.27±0.35   |

B. subtilis: Bacillus subtilis, S. pyogenes: Streptococcus pyogenes, E. coli: Escherichia coli, K. pneumoniae: Klebsiella pneumoniae, S. typhimurium: Salmonella typhimurium

Fig. 3: 1% Agarose Gel data showing the band of Amplified DNA (700bp).

Fig. 4: Phylogenetic analysis of the obtained sequence of ITS Region with Aspergillus japonicas sequences from Genbank

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